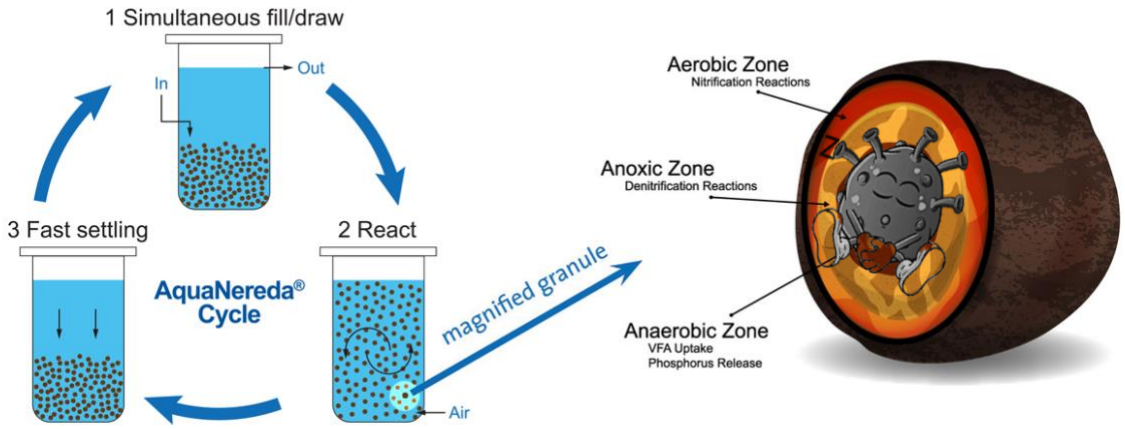
A study of starvation and dormancy of phosphate accumulating organisms and its effect on the Nereda® wastewater treatment

Sarah Jacob



TU Delft Delft University of Technology

Phosphate Accumulating Organisms in Aerobic Granular Sludge

A study of starvation and dormancy of phosphate accumulating organisms and its effect on the Nereda® wastewater treatment

by
Sarah Jacob – 5026547

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Committee members:

Prof. Dr. Ir. Merle de Kreuk,
Dr. Ing. Mario Pronk,
Ir. Lenno van den Berg

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ABSTRACT

Dormancy is a topic that is researched extensively for various microorganisms, especially in the field of medicine. However, not many studies have been conducted for the dormancy of microorganisms in the wastewater treatment sector. Treatment processes like the NEREDA® technology use Phosphate Accumulating Organisms (PAO) to remove phosphate from the wastewater. If dormancy affects these bacteria, it can cause the wastewater treatment plants to function at a lower capacity. Reviving dormant PAOs can increase treatment capacity and treat the wastewater during high influent loading without effluent deterioration. Studying the optimal conditions for granule storage without inducing dormancy can be advantageous during granule transport for inoculation of reactors during their start-up phase. This study presents the first independent assessment of the possible dormancy of PAOs in Aerobic Granular Sludge (AGS).

In this research thesis, dormancy is a survival strategy opted by PAOs to persist substrate limitation. There are two different pathways to dormancy that has been explored here. *Responsive switching* and *starvation* are studied as they express fast and slow transition respectively into dormancy. Lag faced during resuscitation of dormant cells is considered proportional to the length of dormancy in responsive switching.

The experimental phase of the research can be interpreted in three parts: first through method development, second through method testing and third through the application of the developed method. The method was developed to understand dormancy in PAOs by observing differences in their maintenance and activity rates. This method was then tested on granules where dormancy was induced by lowering the temperature to 4ºC and anaerobically storing without feed. This method was then applied to newly sampled granules from a full-scale reactor to study dormancy in the PAOs. The developed method increased the activity of the former granules by 60% but did not raise the activity of the latter. Maintenance rates increased by 167% and 68%, respectively, in the granules. Although dormancy could not be sufficiently proved by the method, starvation of PAOs in Utrecht NEREDA® reactors was possible.

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Nomenclature

AGS	Aerobic granular sludge
ALHs	Acyl homoserine lactones
AOB	Ammonia oxidizing bacteria
AS	Activated sludge
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DPAO	Denitrifying phosphate accumulating organisms
EBPR	Enhanced biological phosphate removal
GAO	Glycogen accumulating organisms
NOB	Nitrite oxidizing bacteria
PAO	Phosphate accumulating bacteria
PG	Peptidoglycan
PHA	Polyhydroxyalkanoates
PHB	Poly-β-Hydroxybutyrate
PHV	Polyhydroxyvalerate
qPCR	Quantitative polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
SIR	Substrate induced respiration
TSS	Total suspended solids
SND	Simultaneous Nitrification and Denitrification
VBNC	Viable But Non-culturable
VFA	Volatile Fatty Acids
VSS	Volatile suspended solids

1. INTRODUCTION

Phosphorus is an essential nutrient that must be removed before its release into water bodies. This is because phosphorus-rich industrial wastewater or sewage runoff can cause eutrophication of the aquatic ecosystems (Graham, 2018). This causes the algae to increase rapidly and decreases Dissolved Oxygen (DO) and transparency of the water body. Increased phosphorus content has caused environmental problems, ecological imbalance, a threat to water resources and much more (Li et al., 2021). The removal of P from wastewater is primarily by two methods; biological treatments and physio-chemical methods (Graham, 2018). Here we consider biological treatment, in which Phosphate Accumulating Organisms (PAO) play a significant role in the uptake of the P from the wastewater by biological processes.

PAOs are very important during Enhanced Biological Phosphate Removal (EBPR) processes like Activated Sludge (AS) and Aerobic Granular Sludge (AGS). The alternating aerobic and anaerobic conditions during the treatment processes aids the survival of PAOs. Their unique ability to take up Volatile Fatty Acids (VFA) anaerobically allows them to outgrow the other heterotrophs. This is facilitated by creating storage polymers like Poly-P and Polyhydroxyalkanoates (PHA), which is used by the PAOs, to survive aerobic conditions without any substrate (Filipe et al., 2001).

AGS reactors, a superior substitute for conventional Activated Sludge reactors, use granules of sizes varying from 100-2000μm (van Dijk et al., 2018). These granules have an aerobic, anoxic and anaerobic zone in the granule's outer, middle and inner layers, respectively (de Kreuk et al., 2005). This morphology of the AGS eliminates the need to build separate aerobic and anoxic tanks used in AS process, thereby saving cost and space. The differentiation of the zones in the granules helps with the Simultaneous Nitrification and Denitrification (SND) of the wastewater. The thickness of each layer depends on the penetration depth of oxygen into the granules during its formation. The granules mainly consist of Ammonium Oxidizing Bacteria (AOB), Nitrite Oxidizing Bacteria (NOB), PAOs and Denitrifying PAOs (DPAO) in a matrix of extracellular polymeric substances (EPS) (Wilén et al., 2018). During a computational study into the AGS, it was observed that PAOs contribute towards the largest (~27%) bacterial population present (Xavier et al., 2007), and this population mainly consisted of the genus *Candidatus* Accumulibacter phosphatis (here referred to as "*Ca.* Accumulibacter phosphatis") (Dasgupta et al., 2020). PAOs are an important aspect of the high performance of the AGS system for the treatment of wastewater.

Although there has been tremendous progress in understanding the newly developed full-scale AGS reactors, much knowledge regarding their full-scale working is yet to be researched. One such area is decreased maintenance energy requirement of PAOs in the AGS reactor. During a review of the research paper de Kreuk et al. (2007), which described a mathematical model of an AGS reactor, it was observed that the substrate required for the maintenance of the PAOs were much higher than what was available in wastewater treatment plants. The maintenance terms considered for the model were from the work of Murnleitner (1997), who formulated an integrated metabolic model for biological phosphorus removal. The maintenance terms are as shown.

Anaerobic maintenance = 0.05 $gP/gCOD_{PAO}/d$
Aerobic maintenance = 0.06 $gO_2/gCOD_{PAO}/d$
Overall maintenance term = 0.053 $gCOD_{PHB}/gCOD_{PAO}/d$

The model considers highly enriched PAO biomass, but in reality, that might not be the case. The amount of PAO in AGS for a full-scale NEREDA® plant was observed to be about 25-50% of total biomass in a Fluorescence in-situ Hybridization (FISH) analysis (Pronk et al., 2015). A multi-scale individual-based model of microbial and bioconversion dynamics in AGS showed a live PAO percentage of 27% (Xavier et al., 2007). Considering 1/3rd of biomass as PAO (2.7 gPAO/L) is a reasonable assumption for calculating the acetate requirement for maintenance.

For this amount of biomass, the required substrate quantity for anaerobic maintenance alone is ~220 mgHAc/$L_{influent}$. This amount of substrate raises concern as the influent Volatile Fatty Acids (VFA) in wastewater treatment plants are typically in the range of 10 to 80 mgHac/$L_{influent}$ (Henze et al., 2008). This amount of substrate concentration cannot satisfy the maintenance requirement of the PAO cells as well as their growth. Therefore, the discrepancy in this calculation led to the formation of a theory that granules in the AGS reactor might have PAO that are functioning with lowered maintenance energy because of dormancy.

Academically, dormancy in different single-celled organisms has been studied and researched. Microbial community in soil that assists with plant growth survive harsh climates by being in a state of dormancy (Stenstrom et al., 2001). Bacteria such as *Escherichia coli* that infect the human body tend to be resistant to antibiotics due to their dormant nature (Cesar et al., 2020). In PAOs, one could hypothesise that dormancy can reduce the efficiency of the treatment process as part of the biomass in the treatment plant can be inactive and does not contribute to the treatment process.
Fluctuations in the influent concentration are one of the leading causes of decreased activity in AOBs (Geets et al., 2006b). Even though not experimentally proven that the AOBs are dormant in the paper written by Geets et al. (2006), the behaviour exhibited by the bacteria under the fluctuating conditions leading to low growth rate was observed to be that of dormant bacteria cells. During the pilot runs of the Epe wastewater treatment plant, it was noticed that the plant has large influent hydraulic and organic load fluctuations due to the mixing of industrial wastewater (Khan et al., 2015). It would be interesting to know if the fluctuations lead to substrate limiting conditions for the PAOs. If substrate limitation leads to dormancy in PAOs, it could explain a potential delay in phosphate removal after a period of low organic loading (Yu et al., 2021). If the cause of this delay is unknown, the plant operator might increase the biomass concentration in the reactor to increase the activity, thereby decreasing the oxygen transfer efficiency and increasing the airflow rate for aerobic mixing (Cecconi et al., 2020). If dormancy does affect PAOs in AGS, reviving them to perform at their optimal activity could help manage the peak loadings and flows without compromising the effluent quality. Dormancy could also play a role during AGS transportation to new wastewater treatment plants. Granules transported for a few days could show a delay in treatment activities due to dormancy. If, indeed, dormancy is the cause of this delay or loss in activity, it would be beneficial to understand their revival techniques.
Therefore, this research thesis looks at the possible dormancy of PAOs and their effect on the NEREDA® treatment.

According to the author's knowledge, the ability of Phosphate Accumulating Organisms (PAO) to enter a state of dormancy is a topic that has not been researched by the scientific communities. Hence this research study will be conducted with themes shared by most bacteria along with organisms that share a similar physiological state as the PAOs.

2. LITERATURE SURVEY

2.1 Survival strategies of bacteria

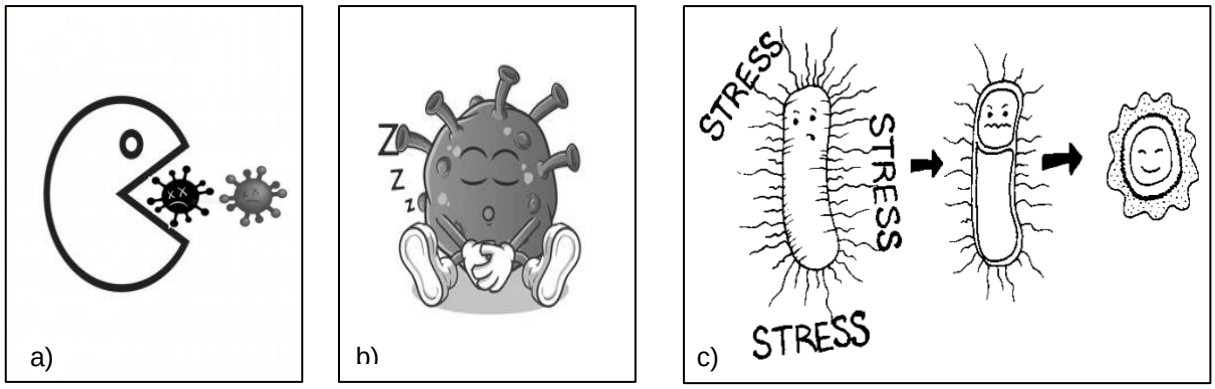


Figure 1: Bacterial survival strategies. a) cannibalistic behavior during which, the live bacteria consume the dead fraction b) Dormant stage - state of resting with lowered metabolism and no growth c) Sporulation or formation of metabolically inactive daughter cells

Bacteria's face many periodic stresses during their lifetime. Substrate limitation, temperature changes, altered pH and many more stresses force the bacteria to change their normal physiological functioning to suit the stressful climate. The different methods of survival followed by bacteria can be useful in narrowing down the research area for this thesis.
A study conducted by Steinhaus & Birkeland (1939) focuses on these different survival strategies of bacteria in stressful conditions. According to this study, three types of strategies help bacteria weather out harsh environmental conditions.

1. **Cannibalistic behaviour** is a strategy that is adopted by bacteria when nutrients have been depleted. In this approach, the energy for new growth is obtained by live cells feeding on the biodegradable part of the lysed cells. In simpler terms, Steinhaus & Birkeland (1939) referred to it as **cannibalistic behaviour** where live bacteria consume the dead bacterial portion for survival. It is pertinent for survival that the live bacterial population responds quickly to a substrate to out-compete other organisms for food.

2. In some cases, the microbes affected by the growth-limiting stress enter a resting stage. This stage is called **dormancy**. In this stage, the bacteria slow down or even entirely stop the growth function while limiting their metabolic activities (Rittershaus et al., 2013). This strategy does not cause apparent changes to the form of the bacteria. According to research, 60% of all the microbial populations in all ecosystems undergo dormancy due to stressful conditions (Cole, 1999).

3. **Sporulation** is a process where a growing cell is divided into two-cell-chamber sporangium in which the production of a spore takes place (Stragier & Losick, 1996). When the growth-limiting stress conditions disappear, the spores germinate, regrow, and establish their normal metabolic functions. In normal growth, there is a symmetrical division of the healthy cells, producing two identical daughter cells (Stragier & Losick, 1996). In spore formation, the division is asymmetrical where the mother cell has the more significant segment, and the forespore is in the smaller segment. With subsequent morphological changes and development, when the spore is matured, it is released by the mother cell (Stragier & Losick, 1996). The mother cell faces death as usual, but the

spore is resilient and reproduces when the right conditions arise. The spores are tolerant to most stresses like temperature even up to 150°C, chemical agents, substrate limitation and severe pH changes (Stragier & Losick, 1996).

The cannibalistic survival technique is not the interest of this research as the technique does not cause lower maintenance rates in individual cells but keep up their complete metabolism with growth by feeding on their fellow dead cells. Spore formation is typically used by gram-positive bacteria (Basta & Annamaraju, 2021). It is a multi-step process that sometimes requires external forces like heat shock to revive them (Luu et al., 2015). In both the above cases, either the cells cannot be revived, or it is not easy to break their survival state. Loss of activity during the NEREDA® wastewater treatment due to these survival techniques cannot be reversed (or need extreme external forces) and hence not important for this research thesis.

Dormancy is the focus of this research since it describes the condition where cells slow down their maintenance rate and entirely stop their cell growth in response to growth-limiting stresses. This strategy might best explain the difference in calculated and observed metabolic rates of the PAOs, and their cell revival can increase the treatment efficiency.

2.2How do we define dormancy in PAOs?

In the scientific community, the definition of dormancy is not fixed. Many different environmental stresses cause dormancy. There are infinite ways bacteria can react to these stresses and undergo many more changes that might not necessarily fit into the definition of dormancy provided.

Viable But Non-Culturable (VBNC) state is a dormant state in which cells live but become unculturable on routine growth media (Ayrapetyan et al., 2015; Colwell, 2000; Sachidanandham & Yew-Hoong Gin, 2009; Wagley et al., 2021).
Persister cells are also considered dormant cells, and they are defined as cells that receive tolerance to antibiotics after prolonged exposure to them (Dworkin & Shah, 2010; Lennon & Jones, 2011). In another research, continuous cultures at a slow growth rate create dormant cells with zero maintenance energy (Pirt, 1987).

The above papers show that dormancy in bacteria is not a specific topic and can have many definitions. It is hence imperative to define what properties of dormancy have been considered for this research thesis.

This thesis considers dormancy a survival strategy invoked by PAO cells because of substrate limitations. It is defined as "*a reversible state of lowered maintenance and activity rates with no growth".* For the strategy to be advantageous, it is pertinent that the dormant cells resume their normal growth and not die off after entering the state of dormancy (Lennon & Jones, 2011).

2.3General biological changes in cells during dormancy

There are some general themes of bacterial dormancy that can be an indicator towards dormancy in PAOs (Kadouri et al., 2005).

Like squirrels storing food for the winters, microbial organisms store their preferred substrate to stay in a state of dormancy. In *Vibrio cholerae,* Gram-negative gammaproteobacteria,

glycogen is collected and stored in the cells in response to the stress of available nutrients. The stored glycogen is immediately taken up for regrowth when the stressed environment ends (Bourassa & Camilli, 2009). In the same way, many microorganisms in various climates use Poly-β-Hydroxybutyrate (PHB) or other Polyhydroxyalkanoates (PHA) as carbon and energy source for survival during starved conditions (Kadouri et al., 2005). Organisms like *Legionella pneumophila* and *A. brasilense* are present in the soil and rhizosphere. They are constantly exposed to stresses like nutrient limitation or harmful physical or chemical factors and have higher survival rates due to their PHA producing capabilities (Kadouri et al., 2005). PHA also helps with spore formation and cyst encapsulation during the starved condition (Kadouri et al., 2005). Although it is not the only reason for the high survival rate, PHA producing organisms have an edge to outlive non-PHA accumulating organisms in stressed conditions.

Another theme of quiescent dormancy is the changes to the bacterial cell wall. The cell wall is composed of peptidoglycan (PG), a polymer made of sugars and amino acids and strands of polysaccharides, which are cross-linked with short peptide bonds (Vollmer et al., 2008). In organisms like *Staphylococcus aureus* and *Bacillus subtilis*, it is seen that the cross-linking peptide bonds become fewer in number but thicker during dormancy and some of the peptide's bonds are replaced by germination specific lytic enzymes (Rittershaus et al., 2013).

Cell wall thickening was first observed in mycobacteria, and then a computational model of *M. tuberculosis* predicted it in response to hypoxia (Rittershaus et al., 2013). The prediction was confirmed by electron microscopy that showed the thickening of capsule layers and the outer mycolic acid of the cell wall (Cunningham & Spreadbury, 1998). This thickening of the cell wall results in a decrease in cell wall permeability. In the case of bacteria like *M. tuberculosis*, this decreases the entry of administered antibiotics in the dormant cells (Rittershaus et al., 2013).
The signal to exit from the dormant state also can be brought about by the cell wall. In *B. subtilis*, the PrkC Ser/Thr kinase induces spore formation after recognising fragments of extracellular peptidoglycan when the cells start to grow again. In the same way, Ser/Thr kinase, PknB in *M. tuberculosis* might be a signal for the bacteria to exit the state of dormancy and might be a common trend in bacterial signalling to leave the state of dormancy (Rittershaus et al., 2013).

Protein production along with RNA development decreases when the cell is in a condition of dormancy, which seems logical, but this is not always the case (Rittershaus et al., 2013). When the cell enters and exits the state of dormancy, there seems to be an increase in proteins and RNA production, as seen in starved *E. coli* bacteria. When the cells are preparing for dormancy, protease is produced. Protease breaks the peptide bonds of the proteins and thus degrades proteins (López-Otín & Bond, 2008). Due to the presence of protease, the protein turnover during the transition increases five times the standard rate (Rittershaus et al., 2013).
In the same way, when cells exit dormancy, protein turnover is most likely required. Hence protein turnover can be an indicator of metabolic change required to shift between growing conditions. Once there is entry into dormancy, the RNA and protein production decreases up to 20 times the standard rates along with a five times dip in the transcription rate (Rittershaus et al., 2013). When the cells enter dormancy, the production rate is inversely proportional to the stability of the mRNA. It was noticed in many bacteria during conditions that induce dormancy or during the stationary phase of growth, the mRNA becomes stable (Rustad et al., 2013). This increased stability of mRNA provides the essential proteins for continual survival during the dormancy phase.

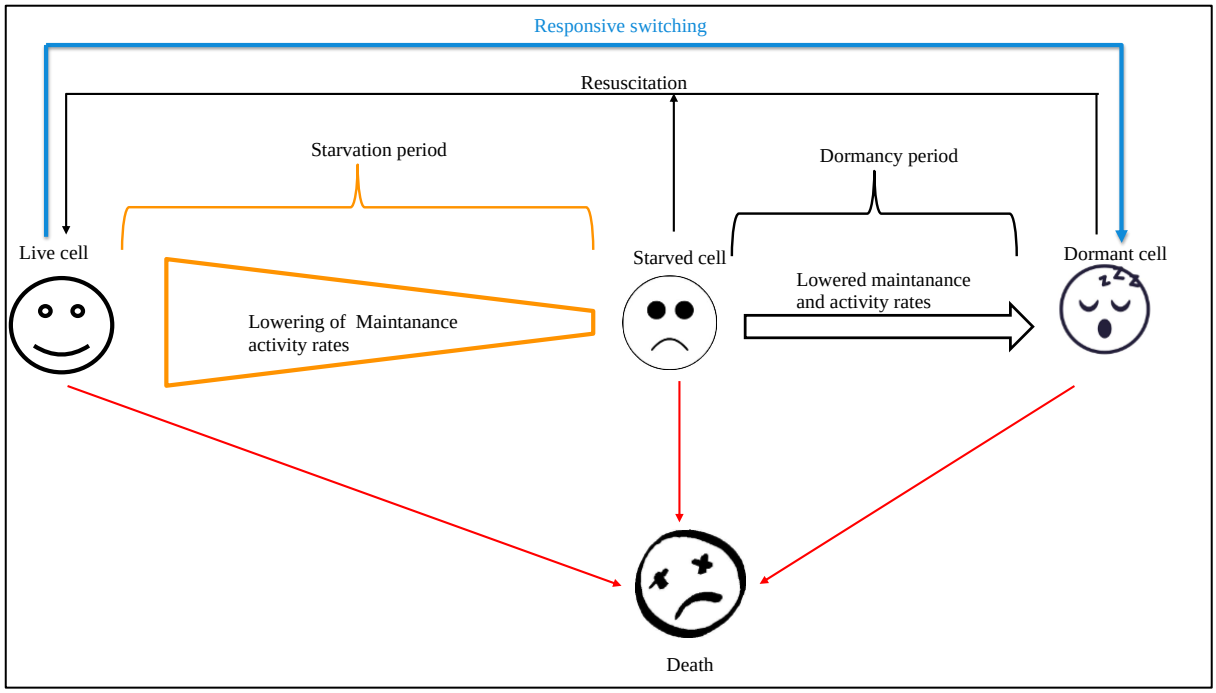


Figure 2: Survival of PAO during substrate limitation. Live PAO cells can become dormant through long term starvation (displayed in orange) where metabolic rates through time decreases. During the starvation period there is also decrease in growth rate. They have strong cell to cell communication for quick substrate uptake when they are abundant. They have stable RNA and enzymes in this stage. Live PAO cells can also become dormant through responsive switching (displayed in blue). Here the cells have good sensory machinery to keep track of the quick changing surroundings and help with live-dormant transition. Once in a state of dormancy the cells have constant lowered metabolic values, with no growth conditions. Here the cells display lowered RNA and proteins, with thickened cell wall making them resistance to outside stressors.

2.4Pathway towards dormancy in PAOs

Even though the survival strategy applied by PAOs can be dormancy, the path to dormancy might not be a straightforward one. Here we investigate two different ways PAOs can go into a state of dormancy.

2.4.1 Starvation leading to dormancy

Extended storage of *Micrococcus luteus*, an actinobacterium, in a spent growth medium was observed to essentially decrease the number of growing cells (Kaprelyants & Kell, 1993). The number of growing cells decreased to a constant value after 80 days of storage. During this time, there was also a significant decrease in the respiratory activity of the culture (Kaprelyants & Kell, 1993). During storage, the DNA content of the cells remained stable, but there was a decrease in the protein content of the cell (Kaprelyants & Kell, 1993). This could be a common effect of starvation in bacteria. After 80 days, penicillin was introduced to kill the live and growing cells while the dormant cells would remain unaffected (Kaprelyants & Kell, 1993). The dormant cells were separated from the dead fraction by flow cytometry and then introduced to a growth medium, allowing cell resuscitation (Kaprelyants & Kell, 1993). Cell growth was subsequently assessed by plate counts, which showed that 50% of the non-growing population in the spent medium were not dead but in a state of dormancy. This can be used to conclude that one of the pathways to dormancy is through the prolonged starved condition of the cells. In the research paper of Kaprelyants & Kell (1993), the cannibalistic behaviour of cells was disregarded as the survival strategy due to multiple reasons. Therefore, dormancy was considered a probable strategy used by the cells in this situation. The dormant cells were found

to have decreased protein content and cell size compared to values after resuscitation (Kaprelyants & Kell, 1993).

The study of starvation in *E. Coli*, a proteobacterium like PAO, has proved to be beneficial in stating that starvation can lead to dormancy (Cesar et al., 2020). When *E. Coli* was starved, three different fractions of cells were found. The first fraction immediately started growing in response to a freshly added substrate. The second fraction was a fraction of cells that showed delayed growth when introduced to a fresh nutrient medium. This fraction was the dormant cells. It must be noticed that the lag to re-grow the dormant fraction increased with a longer starvation period. The third fraction was a non-growing fraction of cells that could not be resuscitated. Fraction two and three increased with an increase in the stationary phase (Cesar et al., 2020).

Currently, there has not been any research into the topic of dormancy in PAOs for any stressors. Nevertheless, the research into the behaviour of PAOs during the starvation period has been looked into (Lopez et al., 2006; Vargas et al., 2013).
It was observed that during anaerobic starvation, PAOs preferred first to use polyP and then glycogen for their maintenance energy (Lopez et al., 2006; Vargas et al., 2013). This was not observed in Lu et al. (2007), where first glycogen was preferred and then polyP for energy purposes. The anaerobic utilization of glycogen resulted in the production of polyhydroxyvalerate (PHV), which decreased the phosphate uptake capacity of PAOs (Lopez et al., 2006). No significant decay of PAO was observed during the starvation period (Lopez et al., 2006).
PHA was used for energy during aerobic starvation, followed by glycogen and polyP (Lopez et al., 2006; Lu et al., 2007). There was a significant decrease in the active PAO fraction under aerobic starvation (Lopez et al., 2006; Lu et al., 2007).
Batch tests were conducted to observe the change in maintenance and activity rates during the starvation period. A decrease in maintenance and activity rates with an increase in starvation time was observed (Vargas et al., 2013). Starvation can therefore be a likely path taken by PAOs in AGS towards the path of dormancy.

2.4.2 Responsive switching to dormancy

In theory, when there are fluctuating environmental conditions like in water treatment plants, responsive switching can take place to transition live cells to dormant cells (Kussell, 2005). Responsive switching is the condition where cells can sense environmental fluctuations and enter and exit a state of dormancy as seen fit (Lennon & Jones, 2011). The greater the uncertainty of changing conditions, the faster the responsive switching takes place (Kussell, 2005). The cells need to have sensory machinery to constantly scan the environment for different stressors (Lennon & Jones, 2011).

The bacteria community in the soil is very vulnerable to dormancy due to ever-changing biologically, chemically, and physically stressful conditions imposed on them (Kadouri et al., 2005; Lennon & Jones, 2011). During a study to determine the soil microbial biomass dynamics, it was seen that the microbes do not follow a single dynamic for a long period (Stenstrom et al., 2001). Instead, they undergo responsive switching, which allows them to remain in alternating conditions by being in and out of dormancy (Lennon & Jones, 2011; Stenstrom et al., 2001). Stenstrom et al. (2001) focused on studying soil biomass as a whole instead of a particular microorganism, depending on their reaction to the addition of substrate. The Substrate Induced Respiration (SIR) method used in this experiment distinguished

between two respiration rates (Stenstrom et al., 2001). A higher rate of respiration was said to be from the growing community and a lowered rate of respiration from the non-growing community. The non-growing fraction of biomass with lowered respiration rate was in a state of dormancy, as defined in the scope of this research thesis. When the substrate was added, the non-growing organisms quickly transitioned to growing organisms. When the substrate was low, the growing organisms changed back to the non-growing state (Stenstrom et al., 2001).

The quick transition from active to dormant during substrate limitation and vice versa during high substrate availability points towards the responsive switching pathway. Hence in this research thesis, we consider responsive switching to dormancy as the second pathway to enter a state of dormancy.

2.5 *Review of dormancy study in PAO: Genus: Ca*. Accumulibacter phosphatis

In an attempt to study activated sludge treatment of wastewater with low C/N ratio (<4), the microbial community at a transcriptional level was analyzed and studied (Fan et al., 2020). This is as close as the scientific community has come to research regarding the dormancy of PAOs in EBPR. The main goal of the research was to maximize the treatment efficiency when there was low C/N ratio by utilizing their internal carbon source thereby minimizing the use of external carbon sources. The experimental plan was divided into three phases. In the first phase the activated sludge was being accustomed to the raw wastewater with C/N ratio less than 4 for 60 days (Fan et al., 2020). There was poor phosphate and total nitrogen removal from the system at this phase. In phase two, external carbon sources in the form of acetate and propionate were added to the system to raise the C/N ratio to 5 for 120 days (Fan et al., 2020). In phase three, which was for 154 days no more external carbon was provided instead it was observed that the internal carbon reserve was being utilized completely and the denitrifying phosphate removal increased to 92% in the anoxic zone (Fan et al., 2020).

During the three phases, transcriptional community study was conducted by DNA and cDNA high throughput sequencing at gene and transcription level. *Ca.* Accumulibacter phosphatis was found to be the effective and most abundant denitrifying phosphate removal bacteria in the system.

qPCR and RT-PCR method was used to calculate the live and dead/dormant portion of the different microbial community in all the three phases. Difference between the bacterial gene copy number and transcript copy number determined by qPCR and RT-PCR respectively gave the ratio of the dead or dormant portion (Fan et al., 2020).
During phase one the dead or dormant portion of *Ca.* Accumulibacter phosphatis was 70.65%, which decreased to 54.18% in phase two and in phase three there was the least dormant microbe of 45.49% in the system (Fan et al., 2020). The ANOVA results show that there is statistical significance among the results. In GAO, the dead or dormant portion was 78% in phase one, which can be indicative of GAOs being more affected by starvation than PAOs. The dead/dormant portion of GAO in phase three was 41% suggesting faster resuscitation compared to PAO, which could be due to their greater affinity to VFA.

Here it is unclear which strategy is used by the PAOs. It could be a state of dormancy, where the cells were resuscitated due to substrate addition. It could also be due to cannibalistic behavior of the cells, where the healthy cells eat the dead fraction. The decreased dead fraction along with new growth can decrease the dead/dormant fraction.

Even though the method might not point directly towards identifying dormancy, addition of substrate does increase the live population in the entire sludge. Substrate addition can perhaps increase the metabolic functions of the sludge as seen with the increased performance of the treatment plant.

2.6Entry and exit from dormancy

Research about ammonium-oxidizing bacteria, present in the AGS, led to a review of what happens during substrate limitation and starvation (Geets et al., 2006a). AOB are assumed to have a lower affinity towards ammonium and oxygen than other ammonium digesting heterotrophs at a high C/N ratio (Verhagen et al., 1992). This puts them at a greater risk for starvation and, therefore, towards a path to dormancy. The effect of oxygen and ammonium starvation on *Nitrosomonas europaea,* a betaproteobacteria like *Ca.* Accumulibacter phosphatis was studied by various researchers. However, these studies had different conclusions because one said that a *Nitrosomonas europaea* starved for months could rejuvenate within a matter of minutes in a retentostat reactor. At the same time, another concluded that just for 42 days of starvation, it required 154 hours before nitrite production resumed to its original value (Geets et al., 2006a). These different conclusions show that the resuscitation of *Nitrosomonas europaea* cells is intricate and depends on external factors.

The maintenance energy requirement was five times lower for *Nitrosomonas europaea* during ammonium limitation (Keen & Prosser, 1987). The author concluded that maintenance requirements are not constant but dependent on growth rate (Keen & Prosser, 1987). A lower growth rate can lead to lowered energy requirements for maintenance (Keen & Prosser, 1987). It was observed that at a lower growth rate, the cells were in a dormant state (Geets et al., 2006b).

The energy required by the bacteria during the dormant phase seems to be from storage polymers that are formed during the activated sludge process (Kadouri et al., 2005). It was reported that AOB cells store and increase their internal ammonium when they are in starved conditions. Although it was concluded that this internal ammonium did not serve as an internal substrate supply, it can be seen as a preparation towards dormancy or starvation in general (Schmidt et al., 2004).

For exit from dormancy, the theory formulated is that cell to cell communication using quorum sensing is used by the *Nitrosomonas europaea,* although no sensing regulation systems have been found (Chain et al., 2003; Chhabra et al., 2004). However, when gram-negative signalling molecule acyl homoserine lactones (AHLs) was added to starved cells, the lag to wake up from dormancy decreased almost five times (Batchelor et al., 1997). This could mean that cell to cell communication could be a preferred mechanism to wake up from dormancy.

Hence, we use the study of *Nitrosomonas europaea,* a betaproteobacteria, to design the experimental setup for this research. Here substrate limitation is identified as the cause/entry into dormancy. Therefore, substrate addition is chosen as the method to resuscitate the *Ca.* Accumulibacter phosphatis from dormancy. The change in maintenance energy is studied as the first step into understanding if dormancy does occur in the phosphate accumulating bacteria or not.

2.7Gaps in literature

Although a lot of research has been carried out on dormancy, most studies are focused on a select group of microorganisms. These microorganisms are generally well characterized and often relevant from a medical perspective. Organisms that are relevant for wastewater treatment have been studied much less with regards to dormancy. *Ca.* Accumilibacter phosphatis is an organism that is not well characterized yet. It has not been isolated in pure culture, and its complex and dynamic metabolism make it a difficult organism to study. As a result, there is almost no knowledge of how *Ca.* Accumilibacter phosphatis would respond to any kind of stress be it substrate limitation, temperature decrease etc. Only a handful of studies have investigated the effect of starvation on *Ca.* Accumilibacter phosphatis and they all have been studied for activated sludge. No research has been reported to study the effect of starvation in aerobic granular sludge. It is therefore difficult to predict if the granular morphology of the AGS and the EPS would influence the *Ca.* Accumilibacter phosphatis to act differently during starvation compared to the AS. The lack of research into different stresses also means that no research has been reported regarding dormancy in *Ca.* Accumilibacter phosphatis. It is not known yet if *Ca.* Accumilibacter phosphatis can go into a state of dormancy, and what would trigger them to go into and out of this state.

3. RESEARCH QUESTION

To better focus the thesis, research objective and questions are listed below.

The research objective of this thesis is to find out if there are PAOs that are dead or dormant in aerobic granular sludge by conducting activity and maintenance energy consumption tests.

The research questions that will be dealt with in this thesis are as follows:

1. Can there be dormancy in PAOs?
2. What causes the PAOs to go into a state of dormancy?
3. What are the different conditions that can be altered to change the status of the PAOs from dormant to active?
4. To what extent are the PAOs in the aerobic granular sludge dormant during the NEREDA® treatment?
5. How do the dormant PAOs affect the overall NEREDA® treatment?

This master thesis focuses on answering these research question with a three-part explanation. Part one will be to develop a methodology that can identify dormancy and revive the dormant PAOs. The second phase will be testing the developed method on granules where starvation/dormancy is induced. The third part will be extending the developed method to a full-scale reactor and understanding the behavior of the granules in a NEREDA® reactor from the point of view of starvation and dormancy.

4. METHODOLOGY

Chapter 4 describes the experimental phase of this master thesis. The experiments are based on the acclimatization of the granules in order to resuscitate any dormant PAOs in the granules. This acclimatization method, which is described in this chapter, was first developed and later applied. The method development is described in Chapter 5.
In this chapter, two main experiments were conducted. Experiment one was carried out to test the newly developed acclimatization method on AGS sludge where dormancy had been induced by conditions of low temperature (4ºC) and anaerobic storage without substrate.
Experiment two was carried out after the method was tested to observe the physiology of the newly sampled AGS granules with respect to starvation and dormancy.

4.1 AGS collection and preparations

The AGS used for experiment 1 was collected from the Utrecht NEREDA® plant on 25th May 2021 during the aeration phase. Experiments with the granules were conducted after 27 days of storage at a temperature of 4ºC.

The AGS for experiment 2 was collected on 28th July 2021 from reactor one during the aeration phase from the Utrecht NEREDA® plant.

The granules were first sieved through a 2.24mm mesh, and the granules collected on the sieve were used for further experiments. They were put into a plastic container and crushed with a PROLINE HB35 mixer till granules become a liquid consistency without any intact granules. Approximately 2/3rd of the crushed volume was transferred to the lab reactor for the acclimatization cycles.

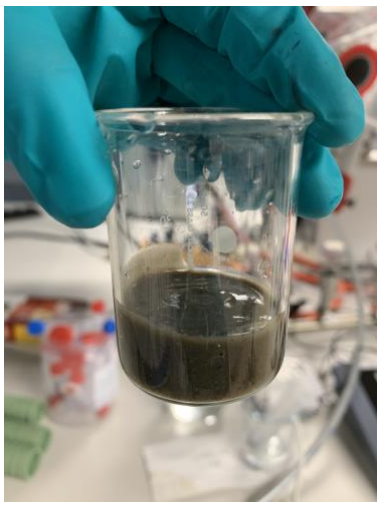


Figure 3: Crushed granules

4.2 Reactor-setup and operational conditions

The lab-scale reactor is a double-walled sequential batch reactor (SBR). The feed volume into the reactor for each cycle was 1.5 L, which brought the total volume (granules + water) during the aeration phase to be 2.9 L. The internal diameter of the reactor is 5.6 cm and has a height of 150 cm.

One entire cycle in the reactor consists of 60 minutes settling period, 10 minutes discharge time, 60 minutes feeding period and 110 minutes aerobic period. The aerobic period was adjusted from 110 minutes to 145 minutes if the acetate was wholly taken up in the aerobic period, but the phosphate was still left in the system.

The anaerobic feeding was conducted through a bed fed system. For the cycles in which concentration measurements were performed, the feed was added directly to the reactor as a short pulse. The pulse feed was followed by N_2 gas mixing for the entire feed duration at a flow of 2 L/min.
During the aerobic phase, the mixing was conducted by compressed air and nitrogen gas, which kept the DO in the system at 5 mg/L. Different mass flow controllers controlled both the gas flows. The off-gas recycle was adjusted to keep the total constant flow at 4 L/min.
The reactor was fitted with a DO probe for oxygen measurement, a pH probe to maintain the pH at 7±0.1 by introducing 1M HCl or 1M NaOH solution and a Cond 330i handheld conductivity probe.

The reactor was operated for four days, and the batch experiments were conducted on the 5th day.

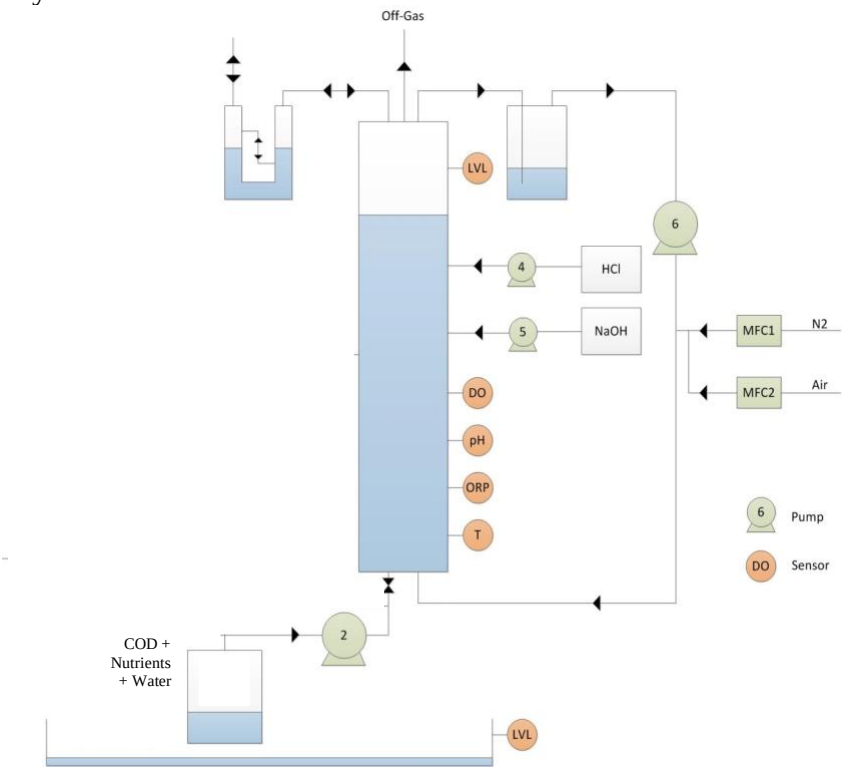


Figure 4: Reactor layout

Table 1 shows a sample COD and nutrient amount for the influent. The acetate concentration was calculated from equation 2, and the rest of the concentrations were calculated by their corresponding ratio. Nitrogen compounds were not added in the influent to prevent biomass growth.

Table 1: Sample influent concentrations

COD	***Unit***	***for 20L of feed solution***
Sodium acetate tri-hydrate	g	9
Magnesium sulphate hepta- hydrate	g	1.186
Potassium Chloride	g	0.468
Nutrients		
Dipotassium phosphate	g	0.977
Potassium dihydrogen phosphate	g	0.191
Trace Elements	ml	225
+Water		

4.2.1 Sampling from the reactor

Sampling during the cycles was conducted at a height of 56.9 cm from the bottom.

Cycle readings were taken at a rate of one cycle per day. Two simultaneous cycle measurements were taken on day three for experiment 1. The sample volume was 5.6 ml, taken every 10 minutes during both the aerobic and anaerobic periods. After hour 1 of the aerobic phase, the subsequent samples were collected every 20 minutes.

The samples were centrifuged (LABOFUGE 400e) for 3 minutes at 10,000 rpm and 4ºC for ease of filtration. It was then filtered through a 0.45 μm filter (Whatman FP30/0.2 CA-S) before conducting analytical tests on the samples.
If centrifuge was not possible, the samples were kept on ice to limit the reaction after sampling.

A triplicate volume of 50ml was taken from the reactor before and after the cycles were completed for reactor biomass measurement.

4.3Batch Tests

There were two batch experiments conducted in triplicates. One was to measure the anaerobic maintenance values and the other was to measure the activity of the crushed granules.

4.3.1 Sampling from batch tests

The samples from the batch test were collected in 2 ml Eppendorf vials and centrifuged immediately to limit any reactions after sampling. If immediate centrifuge was not possible, the samples were kept on ice and centrifuged as soon as possible. After this process, the supernatant was filtered through a 0.45 μm filter (Whatman Spartan 30/0.45RC Rinse filter) before testing them.

4.3.2 Anaerobic maintenance test

The total reaction volume for the batch test was 80 ml (10 ml granular bed + tris HCl).
pH was fixed at 7±0.1 with solutions of 20 mmol Tris HCl (Alfa Aesar, Germany) replacing tap water.

Nitrogen gas was passed into the system for 105 seconds, ensuring anaerobic conditions. The bottle was then placed on the orbital shaker for the entirety of the batch test at 135 rpm for complete homogeneous mixing of the sludge. The test was conducted for four hours, during which the test samples were taken every 60 minutes. The first sample at time 0 was taken right before the nitrogen gas was passed. A sample volume of 2.270 ml was taken at each time step and stored for phosphate measurement.

4.3.3 Activity Test

The total reaction volume for the activity test was 80 ml (10 ml granular bed + tris HCl). pH was fixed at 7±0.1 with a solution of 20 mmol tris HCl replacing tap water.
30 ml of $CH_3COONa.3H_2O$ (>98%, Sigma-Aldrich, Switzerland) was added to the bottle such that there was a final acetate concentration of 400 mg/L. Nitrogen gas was passed into the bottle for 105 seconds, ensuring anaerobic conditions. The bottle was then placed on the orbital shaker at 135 rpm for complete homogeneous mixing of the sludge. The test was conducted for two hours. Test samples were taken every 10 minutes for the first hour and the last reading at the end of the second hour. The first sample at time 0 right after the acetate was mixed in with the biomass and before the nitrogen gas was passed. A sample volume of 2.581 ml was taken at each time step and stored for phosphate and acetate measurement.

4.4 Analysis

4.4.1 Biomass measurement

The crushed granules were centrifuged to drain the excess water volume. They were then transferred to filter over a 0.7 μm glass fibre (Whatman) and kept for sequential drying in the 105-degree Celsius oven for the TSS measurement. After 24 hours, the containers were transferred to a high-temperature oven of 550 degrees and VSS was measured.

4.4.2 Acetate and Phosphate measurements

The samples were measured for PO_4-P concentrations by photometric tests using a Gallery™ Discrete Analyzer. The sample volume was 300 μL, with 10% of the volume being 69% nitric acid to make the sample slightly acidic.
Acetate concentrations were measured using gas chromatography (Agilent tech 7890A, US) equipped with a capillary HP-FFAP column, a runtime of 10 minutes, and an injection volume of 20μL. 1.5 mL of sample was collected into glass vials and was acidified by adding 10 μL of formic acid to cut the microbiological activity and reduce the pH of the prepared samples for analysis.

4.4.3 Numerical Analysis

To determine if there is a significant statistical difference among different sets of experimental data obtained, an analysis of variance (ANOVA) test was applied using Microsoft excel. The alpha level in ANOVA analysis was set as $p=0.05$ in this study. If the p-value is less than the alpha level, the null hypothesis can be rejected, and it can be said that there is a statistically significant difference among the data groups.

Slope calculation for maintenance and activity rates are conducted with the function LINEST() in Microsoft excel. The slope value is fixed such that the highest R^2 value is achieved.

4.4.4 Biomass mass balance

The mass balance for the biomass entering and exiting the lab-scale reactor during the acclimatization cycles is given by equation 1.

$$X_{beforetreatment} = X_{aftertreatment} + X_{effluent} + X_{sampling} + X_{error} \quad (1)$$

Where:
$X_{beforetreatment}$ = biomass in reactor before acclimatization (gVSS)
$X_{aftertreatment}$ = biomass in reactor after acclimatization (gVSS)
$X_{effluent}$ = biomass in the effluent after acclimatization (gVSS)
X_{error} = biomass missing or biomass growth (gVSS)

4.4.5 Maximum substrate calculation

The maximum acetate concentration that can be fed to the reactor without having an abundance of the substrate in the aeration phase is calculated by equation 2.

$$Hac_{max} = \frac{Hac_{\max cap} * M_{ir}}{V_{in}} \quad (2)$$

Where:
Hac_{max} = Maximum acetate concentration M_{ir} can take up in mg-Hac/$L_{influent}$
$Hac_{\max cap}$ = Maximum acetate capacity given in the research of Schouteren (2019) in mg-Hac/gVSS
M_{ir} = Mass of biomass in the reactor before the cycle in gVSS
V_{in} = Volume of the influent in $L_{influent}$

4.4.6 Maximum biomass growth in the lab-scale reactor

The maximum biomass growth in the reactor is calculated by equation 3.

$$X = \frac{Y * (S_i - S)}{1000} \quad (3)$$

Where:
X = Biomass production in gVSS/$L_{influent}$
Y = Biomass yield in $gVSS_{created}/gCOD_{consumed}$
S_I = Substrate at the beginning of the cycle in mgCOD/$L_{influent}$
S = Substrate at the end of the cycle in mgCOD/$L_{influent}$

4.4.7 Growth in Utrecht NEREDA® wastewater treatment plant

Equation 4 is used to calculate the growth in Utrecht Nereda® wastewater treatment plant.

$$b_w = \frac{b_t * r_v}{srt} \quad (4)$$

Where:
b_w = Biomass wasted per day in Kg VSS/d
b_t = Biomass load in Kg VSS/m3
r_v = reactor volume in m3
srt = sludge retention time in day

5. METHOD DEVELOPMENT

In section 2.6, a research paper studying the effect of starvation and potential dormancy in AOB was reviewed (Geets et al., 2006b). In the research, Geets et al. (2006b) observed that nutrient starving AOB cells responded to ammonium addition within minutes to days (different AOB strains reacted differently) to regain their ammonium-oxidizing activity. This observation was used to design the methodology to understand starvation and dormancy in the PAO cells.

During the method development, entry and exit conditions of PAO from dormancy was studied and designed. The entry into dormancy was simulated by anaerobic nutrient starvation, just like the AOB and by lowering the storage temperature to 4º, which added extra stress on the PAOs. As a result of the stresses, the PAO cells periodically decreased their metabolic rates. This decrease in rate with increased storage time can be observed in table 3 (1a,2a,3a,4a & 5a). In this research thesis, the definition of dormancy is "*a reversible state of lowered maintenance and activity rates with no growth".* A state of lowered maintenance and activity rates were achieved with the induced stresses. However, for the PAO cells to be dormant, it was vital that they were able to regain their optimum metabolic rate. For the revival process, excess substrate addition (acetate as the source of VFA) was used to resuscitate the PAO cells.

Table 2: Different experimental condition that were altered to achieve parameters for the highest performance.

SL.No	Experiment	No of days after sampling	Type of sludge	Size of granules	Cycle condition time (mins)			Feed amount (mg-Hac/L / mg-P/L)	No of cycles	DO (mg/L)
					Settling	Feed	Aeration			
1a	Before manual cycle	18	Intact	>2.5mm						
1b	After Manual cycle	25	Intact	>2.5mm	5	45	60	400 / -	2	*
2a	Before manual/ reactor cycle	22	Intact	>2.5mm						
2b	After Manual cycle	35	Intact	>2.5mm	15	60	120	400 / -	3	*
2c	After reactor cycle	44	Intact	>2.5mm	5	60	110	352 / 15	5	2
2d	After reactor cycle	48	Intact	>2.5mm	5	60	110	353 / 16	5	10
3a	Before reactor cycle	55	Intact	>2.5mm						
3b	After reactor cycle	55	Intact	>2.5mm	5	60	110	201 / 21	8	10
4a	Before reactor cycle	59	Intact	1mm to 2mm						
4b	After reactor cycle	59	Intact	1mm to 2mm	5	60	110	359 / 27	20	10
5a	Before reactor cycle	76	Crushed	>2.5mm						
5b	After reactor cycle	76	Crushed	>2.5mm	30	60	145	323 / 21	17	10
6a	Before reactor cycle	7	Crushed	>2.5mm						
6b	After reactor cycle	7	Crushed	>2.5mm	30	60	145	408 / 22	16	2

**Constant aeration without DO control.*

Further, in this method development chapter, different conditions that were used to revive the dormant PAOs will be discussed and finally the optimal conditions will be stated for future experiments. Table 2 shows the different reactor parameters that were calibrated to observe a change in the metabolic rates during batch experiments (given in Table 3).

During method development, acclimatization cycles were used to revive the dormant PAOs. The cycles were a method of introducing the substrate to the PAOs while simulating the PAO metabolism. The acclimatization cycles had the same sequence as that of an EBPR system. The anaerobic feeding and aerobic reaction cycles were performed to mimic the NEREDA® treatment cycles.

The granule size chosen for the experiments were the most significant fraction size (> 2.24 mm). This granule size was initially chosen because it was observed that larger intact granules stay in the reactor for the longest time (SRT 142 days)(Ali et al., 2019) and can possibly hold a greater number of PAOs in a state of dormancy. Therefore, probability of reviving dormant PAOs were greater in larger granules.

Initially, the acclimatization cycles were conducted manually in a 1 L beaker (Table 2, 1b and 2b). This entailed manually switching on/off the aeration for the aerobic period and nitrogen sparging during anaerobic feeding. The feed amount was provided at an assumed concentration of 400 mg/L to eliminate any substrate limiting condition in the system. Manual cycles, however faced time limitations as only three continuous cycles could be carried out in a day. Nevertheless, during the manual cycles, a rise in anaerobic maintenance and acetate uptake rates was observed (Table 3, 2b). It was an initial indication as to the validity of the method. The increase in the rates could have been because of the substrate addition that woke the dormant PAO cells in the granules.

Subsequently (Table 2, 2c to 6b), the cycles were conducted in a lab-scale Sequencing Batch Reactor (SBR) to eliminate the cycle restriction and reduce manual errors. In the SBR, parameters like temperature, pH, DO concentration, length of each phase and feeding conditions could be controlled. To mimic the NEREDA® treatment conditions, the DO was set at 2 mg/L initially (Table 2, 2c), with 60 minutes of bed feeding and 110 minutes of aeration, which would allow for production of storage polymers like glycogen and polyP. The acclimatization cycles performed with the above conditions failed to increase the metabolic rates of the PAO cells (Table 3, 2c). Since the experiment was conducted with intact granules, a theory was postulated that a DO of 2mg/L might have caused oxygen diffusion limitations to the granules thereby failing to revive the PAOs. To eliminate this problem the setpoint was increased 10 mg/L. A more significant increase in metabolic rates were observed after the increase. Hence the higher setpoint was used for all further experiments (Table 2, 2d to 5b). In later experiments (not shown in table 2), the DO was decreased to 5mg/L since comparable metabolic rates were obtained at this DO concentration as well. The need for increased DO suggest that larger granules host PAOs deeper in the granules and they face substrate and DO limitations. The increased DO concentration might have helped with easier oxygen diffusion into the granules and faster degradation of PHA to produce the storage polymer glycogen.

The batch experiments conducted with granules after storage and after the acclimatization cycles were not entirely comparable since they were conducted on different days Therefore, going forward, all the batch experiments were conducted on the same day to provide a better metabolic rate comparison.

However, after implementing these changes, an increase in metabolic rates could not be detected (Table 3, 3b). It was suggested that the reason might be that there were too few cycles. Further, the number of cycles was increased. Four days of acclimatization cycles were seen to eliminate any lag experienced by the starved or dormant PAOs (Table 2, 4b and 5b).
Subsequently, two experiments were conducted, one with smaller granules (1 – 2 mm) (Table 2, 4a,b) and one with crushed granules (>2.24 mm) (Table 2, 5a,b), both chosen to eliminate any substrate or oxygen diffusion limitation to the centre of the granules. These experiments showed an increase in metabolic rates, with crushed granules displaying the most prominent effect. The increased effect in larger granules could indicate the former theory of larger granules hosting larger dormant PAOs than smaller granules.

Even though metabolic rates were increasing, it was difficult to know what was happening in the cycles, making it challenging to evaluate the potential increase or decrease (as was observed in some rates) of the metabolic rates. Therefore, the acclimatization cycles were further conducted with measurements of acetate and phosphate over multiple cycles, which could potentially explain the change in storage polymers.
Due to the plug flow feeding system during the revival cycles, it was unfeasible to get accurate acetate and phosphate readings. This made the calculation of acetate uptake and phosphate release rates impossible. It was then decided that pulse feeding with N2 mixing would be a better method to measure concentrations in the anaerobic phase.
During cycle measurements, it was observed that the concentration of acetate provided was vastly overestimated, and it could not be entirely taken up in the anaerobic feed period. This meant that other heterotrophs used the excess acetate in the aerobic phase. The aerobic feed to the other heterotrophs was eliminated by carefully estimating the biomass concentration and providing the maximum substrate concentration that the PAOs could take up using equation 2.

Although no nitrogen compounds were added in this experiment to prevent growth, it was important to create a mass balance of the biomass to verify the lack of growth. For this reason, the effluent biomass that was wasted after the settling period was collected. The acetate and phosphate concentrations were measured to provide an estimate of their abundance in the effluent. A cooling jacket was used to limit any reactions between the biomass and substrate in the effluent bottle.

The lab-scale reactor had three different sampling ports. Triplicate readings of biomass were collected and analyzed from 3 different heights in the reactor. First from the bottom of the reactor, second 56.9cm from the bottom and third 101.62cm from the bottom. The triplicate VSS measurements were analyzed with ANOVA, and a *p-value* > 0.05 showed no statistical difference between the data sets. However, the values from height 56.9cm gave the least relative standard deviation and hence was chosen for this methodology.

Once the reactor characteristics and sampling procedures were optimized, the final two experiments were conducted with a DO of 5 mg/L, crushed granules (size > 2.24 mm) and four days of acclimatization cycles with controlled feeding. The first experiment was conducted to test the developed method on AGS granules that were anaerobically stored without feed at 4º. The second experiment was conducted to study if fresh granules from NEREDA® reactors have dormant PAOs.

Table 3: Metabolic rates of crushed (green and grey) and intact (blue, orange, and yellow) granules from batch experiments after storage and after the acclimatization cycles to compare the difference in rates.

SL. No	Experiment	No of days after sampling	No of cycles	DO (mg /L)	Anaerobic maintanance term	Phosphate release	Acetate consumption	Phosphate release/Acetate consumption
					mg P/g VSS/hr	mg P/g VSS/hr	mg Hac/g VSS/hr	mg P/ mg Hac
1a	Before reactor cycle	18			0.43	1.18	24.82	0.05
1b	After manual cycle	25	2	*	0.24	0.31	13.68	0.02
2a	Before reactor cycle	22			0.37	4.28	23.02	0.19
2b	After manual cycle	35	3	*	0.52	3.64	24.08	0.15
2c	After reactor cycle	44	5	2	0.41	0.82	6.93	0.12
2d	After reactor cycle	48	5	10	0.53	1.06	9.36	0.11
3a	Before reactor cycle	55			0.36	0.36	8.92	0.04
3b	After reactor cycle	55	8	10	0.38	0.38	6.80	0.06
4a	Before reactor cycle	59			0.25	0.21	8.50	0.02
4b	After reactor cycle	59	20	10	0.42	0.32	5.92	0.05
5a	Before reactor cycle	76			0.17	2.91	1.62	1.80
5b	After reactor cycle	76	17	10	1.35	4.65	3.31	1.41
6a	Before reactor cycle	7			0.58	2.21	2.80	0.79
6b	After reactor cycle	7	16	2	0.69	0.62	1.51	0.41

**Constant aeration without DO control.*

6. RESULTS

6.1 Experiment 1: Stored AGS sludge

The developed method from Chapter 5 was tested on AGS that was sampled from the Utrecht plant and stored in the fridge for 27 days at 4ºC without feed. The focus of this experiment is to test if the developed method can identify and revive dormant PAO cells that are formed during the 27-day storage.
The batch experiments after anaerobic storage and after running the acclimatization cycles were conducted together on day 31.

There are three sections to this result. The first will show the analytical and numerical results of what happened during the acclimatization cycles of crushed granules. The second section will display the results of anaerobic maintenance tests for both the crushed granules after they were stored in the refrigerator and after acclimatization cycles, to be compared. Subsequently, the third section will display the activity test results for the crushed granules after storage and after acclimatization cycles for comparison.

6.1.1 Acclimatization cycles

This subsection will focus on the measurements obtained during different cycles when the 27-day old, crushed granules were acclimatized in the lab-scale AGS reactor. Table 4 displays the aerobic and anaerobic period of the reactor and sludge and effluent data. Fig.5 is a graphical representation of a typical cycle in the reactor running in a mixed aerobic and anaerobic condition in line with the PAO metabolism. The substrate addition per cycle and carefully fixed reactor conditions was expected to reverse the effect of starvation and dormancy, as seen in the study of *Nitrosomonas europaea* (Geets et al., 2006b).

From equation 1, the biomass mass balance gives an error of 0.528 gVSS. This gives an error percentage of -6.6% implying wastage of biomass either during sampling or during the mixing phase, where the biomass enters small crevices of the reactor or due to decay of biomass. If we calculate the amount of biomass lost in 4 days due to decay (Kd = 0.1/d; same as that of AS) a value of 1.18 gVSS is observed. No growth of the crushed granules was observed from the biomass mass balance.
If we consider maximum growth without decay in the system with equation 2, the biomass in the reactor would have increased by 22% in four days.

It can be seen from Fig.5 that the phosphate was not fully taken up during the aerobic phase. This can be due to two reasons: the aeration period was not long enough to take up all the phosphate, or the maximum phosphate uptake capacity was reached. Since no tapering of the phosphate value was observed by the end of the aeration period, it is most likely that the crushed granules could take up more phosphate but needed more time to do so. This residual phosphate is observed in the effluent as well.

Table 4: Reactor characteristics for method testing experiment

	Units	*Before Treatment*	*After treatment*
Reactor sludge	gVSS/L	2.893	2.178
VSS/TSS	gVSS/gTSS	0.819	0.836
Settle time	mins	60	
Decant time	mins	10	
Feed time	mins	60	
Aerobic mixing time	mins	110	
Reactor Volume	L	2.950 ± 0.02	
Influent COD	mg HAc/L	153	
Influent P	mg-P/L	7.2	
Effluent COD	mg HAc/L	0	
Effluent PO4+	mg-P/L	1.502	
Effluent Volume	L	33.46	
Effluent VSS	gVSS/L	0.019	
No of cycles	No	23	

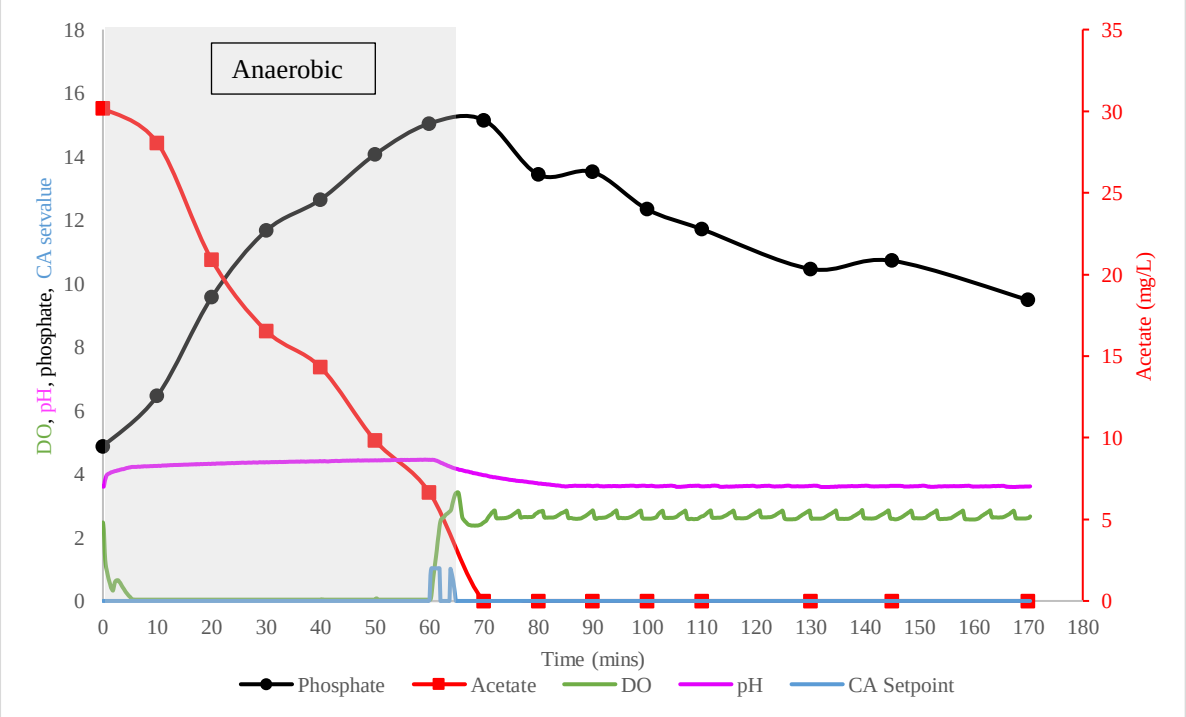


Figure 5: Sample cycle measurement for experiment conducted with crushed granules from the Utrecht plant after anaerobic storage. For cycles where acetate and phosphate measurements were analyzed plus feeding was provided.

Table 5 provides a numeric analysis of the phosphate and acetate readings measured during the cycles. The acetate and phosphate readings are given in Appendix 2. The slopes were analyzed with the maximum R2 value, and the calculations are given in Appendix 2.

A consistent increase of rates for acetate uptake and phosphate release rate were observed. The increase between cycle 1 and cycle 6 for all the rates is the highest and might owe to the fact that the system takes time to adjust to the new operating conditions. In cycle 13, the phosphate release and consumption decreased by 6% and 36% respectively.

The amount of phosphate released per acetate taken up during anaerobic conditions can indicate whether other organisms like GAOs are taking up acetate. The Typical AGS from the Utrecht wastewater treatment plant have a P/Hac ratio of 0.57 for pulse fed reactors (Schouteren, 2019). In the cycles, the P/Hac ratio stays between 0.36-0.45, indicating that at least 70% of the acetate uptake was due to PAOs.
The ratio also signifies that glycogen instead of the TCA cycle was preferred for ATP generation in the anaerobic period (Smolders et al., 1994).

Table 5: Results for numerical analysis of cycle measurements for the method testing experiment

	Units	*Cycle 1*	*Cycle 6*	*Cycle 12*	*Cycle 13*
Acetate uptake rate	mg/gVSS/min	0.097	0.135	0.152	0.167
PO4-P release rate	mg/gVSS/min	0.035	0.061	0.070	0.066
PO4-P uptake rate	mg/gVSS/min	0.044	0.048	0.031	0.020
P release/Hac uptake	mg-P/ mg-Hac	0.364	0.454	0.464	0.399

6.1.2 *Anaerobic maintenance test*

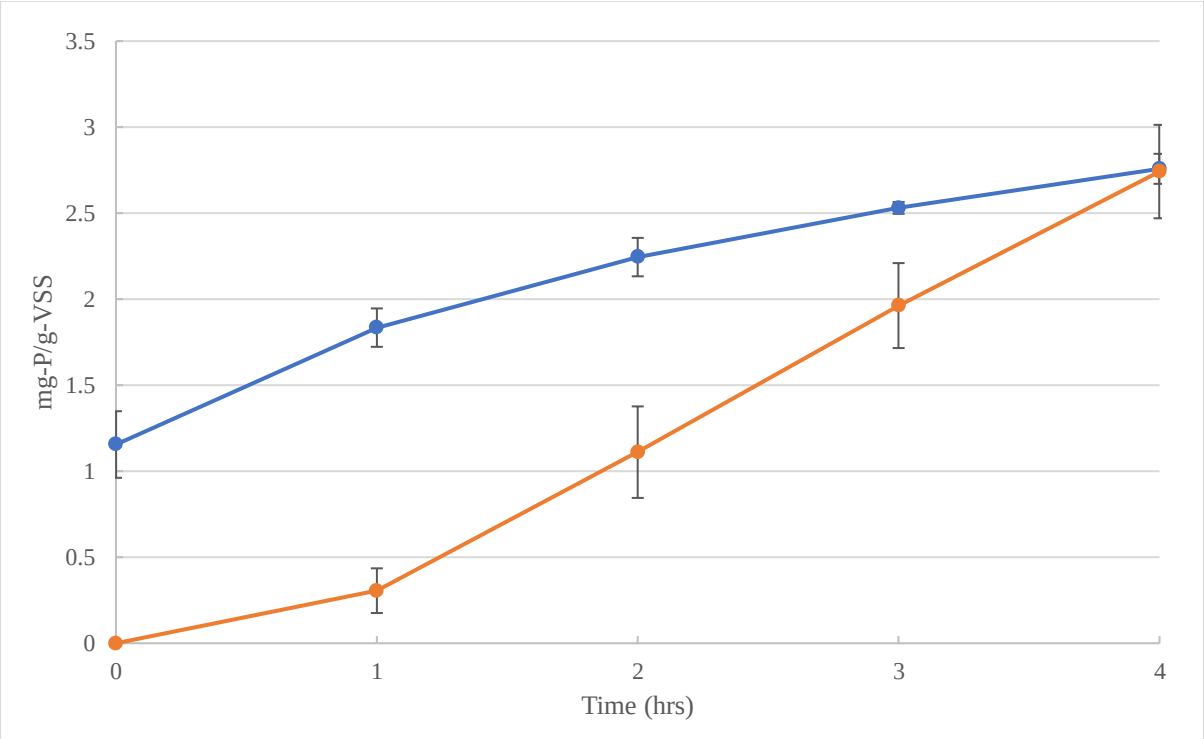


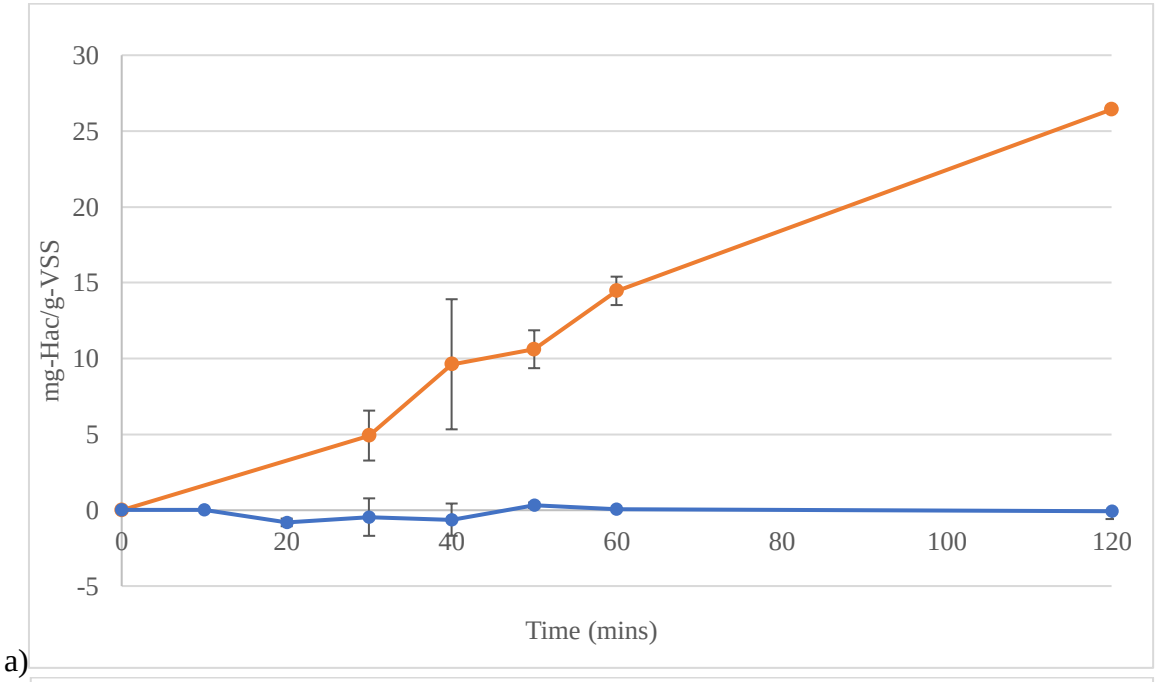
Figure 6: Shows the anaerobic maintenance values for granules after 31 days of storage in the fridge (blue) and after the acclimatization cycles (orange). The graph is the average of triplicate batch experiments, with the standard deviation error. These values are for experiment conducted with crushed granules from the Utrecht plant after anaerobic storage.

The batch test for anaerobic maintenance was conducted on the same day after they were stored in the fridge and after the acclimatization cycles. This subsection will describe the results for the anaerobic maintenance tests for both the crushed granules.

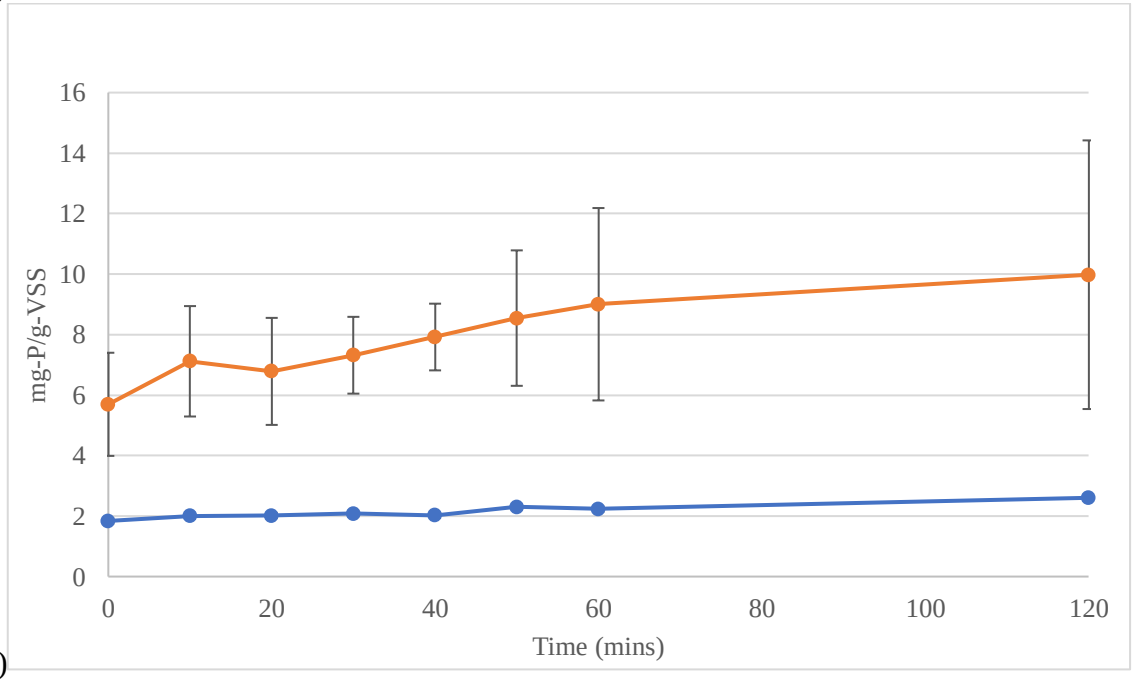
The average anaerobic maintenance rate was *0.005 mg-P/g-VSS/min* after anaerobic storage and *0.014 mg-P/g-VSS/m*in after the acclimatization cycles. There is a 167% increase in the anaerobic maintenance terms. The maintenance rates were calculated from hour one since the slope from 0 to 1 hour in Fig. 6 shows a different slope than the rest. If we extend the slope from hour 1 to 4 hours, the line crosses the x-axis at around 35 minutes. This could signify that the crushed granules take this time to dispel any remaining oxygen from the system.

As observed from fig. 6, there seems to be a significant amount of phosphate at time 0 for the crushed granules after the storage. Since the granules were crushed and stored back in the fridge for four extra days, they could have released a significant amount of phosphate for anaerobic maintenance purposes. Using these granules directly for the experiment could explain the raised phosphate value at time 0.
Measurement of supernatant of the AGS sample from Utrecht, after 27 days, displayed a substantial amount of phosphate and negligible amount of NH_4-N compared to day 0.

6.1.3 *Activity test*



a)



b)

Figure 7: a) Acetate uptake for crushed granules after storage in the fridge for 31 days (blue) and after the acclimatization cycles (orange) b) phosphate release for crushed granules after storage in the fridge for 31 days (blue) and after the acclimatization cycles (orange). These values are for experiment conducted with crushed granules from the Utrecht plant after anaerobic storage.

In this subsection, the results of the batch activity experiments conducted for granules after the storage in the fridge and after the acclimatization cycles are presented.

The average acetate uptake rate was *0.021 mg-Hac/g-VSS/min* after anaerobic storage and *0.222 mg-Hac/g-VSS/min* after the acclimatization cycles. The average phosphate release rate was *0.006 mg-P/g-VSS/min* after storage and *0.050 mg-P/g-VSS/mi*n after the acclimatization cycles.

After the cycles (Fig. 7(b)), the acetate uptake has a relative standard deviation of 32%. Here, one of the triplicate readings had to be ignored due to an experimental error in phosphate calculations. Therefore, considering the other two of the triplicate readings as individual readings, we get an increase between 500 to 900% after storage and after the acclimatization cycles. This acetate uptake rate after the cycles is closely related to the reactor values in subsection 6.1.1. During cycle 13, the rate was 0.167 mg-Hac/g-VSS/min. If we plot a graph as seen in Fig. 8 to give us an estimated value at cycle 23, we get a similar value of 0.22 mg-Hac/g-VSS/min from the batch experiments. However, the phosphate release rate in the batch experiment was lower than what would have been expected at cycle 23.

The acetate uptake of crushed granules after storage (Fig. 7(a)) is very small or relatively non-existent. The rates are lower than what is shown for cycle 1 from table 5. Since the crushed granules were in the refrigerator for four extra days, there might have been further lowering of the activity. Alternatively, it could be because the crushed granules in cycle 1 had received some aeration at the start of the cycles during biomass collection from the reactor. This aeration could have increased the polyP and glycogen contents of the cells, which increased the activity rates. The phosphate release rate is similar to the anaerobic maintenance term seen in subsection 6.1.2. This indicates that no activity took place, and there was only maintenance even though substrate was present.

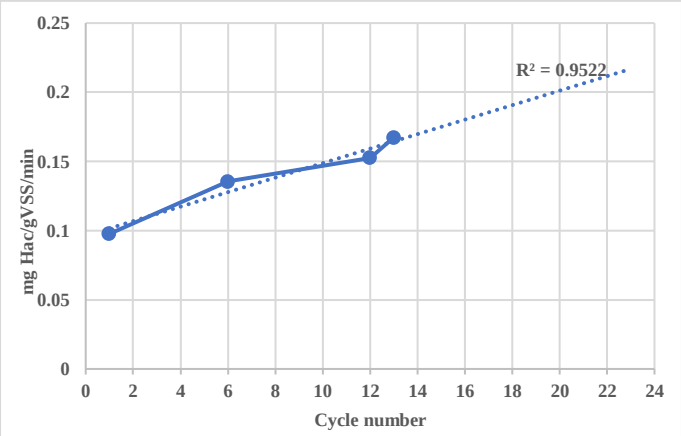


Figure 8: Extended graph of acetate uptake rate vs the cycle number

6.2 Experiment 2: New AGS sludge

In experiment 2, the newly developed and tested method was used to study the physiology of PAO in fresh AGS and to understand the conditions prevailing in the NEREDA® reactor.
For experiment 2, aerobic granular sludge was sampled from the Utrecht plant and stored in the fridge for three days at 4ºC before the acclimatization cycles were conducted.
Batch experiments were conducted on the fresh AGS on the same day as sampling to calculate metabolic rates at day 0 (before any induced starvation/dormancy to the granules).

There are three sections to this result. The first will present the analytical and numerical results of what happened during the cycles in the lab-scale reactor. The second section will display the results of anaerobic maintenance tests for the crushed fresh granules and after the acclimatization cycles in the reactor for comparison. Furthermore, the third section will display the activity test results for crushed fresh granules and after the acclimatization cycles in the reactor for comparison.

6.2.1 Acclimatization cycles

This subsection will focus on the measurements obtained during different cycles when the new crushed granules were acclimatized in the lab-scale AGS reactor. Table 6 gives the aerobic and anaerobic reaction time with the reactor, sludge, and effluent data. Fig. 9 is a graphical representation of a typical cycle in the reactor running in a mixed aerobic and anaerobic condition in line with the PAO metabolism. The acetate uptake in anaerobic conditions and the phosphate uptake in the aerobic condition is representative of a typical EBRP system.

As seen from Fig.9, the acetate load was not completely taken up by the crushed granules during the 60 minutes of feeding. This was due to the overestimation of the biomass, thereby overestimation of influent substrate concentration. The influent substrate concentration was corrected in the subsequent cycles to fit the 60 minutes of anaerobic feeding. It was also observed that the phosphate was not fully taken up during the aerobic phase. This can be due to two reasons: the aeration period was not enough to take up all the phosphate, or the maximum phosphate uptake capacity was reached. Since no tapering of the phosphate value was observed by the end of the aeration period, it is most likely that the crushed granules could take up more phosphate but needed more time to do so.

Looking at the mass balance of the biomass from equation 1, we get an error of 0.062 gVSS. This gives a difference of -0.843%. The negative difference indicates some wastage of biomass either during sampling or during the mixing phase, where the biomass enters small crevices of the reactor. No growth of the crushed granules was observed from this data.

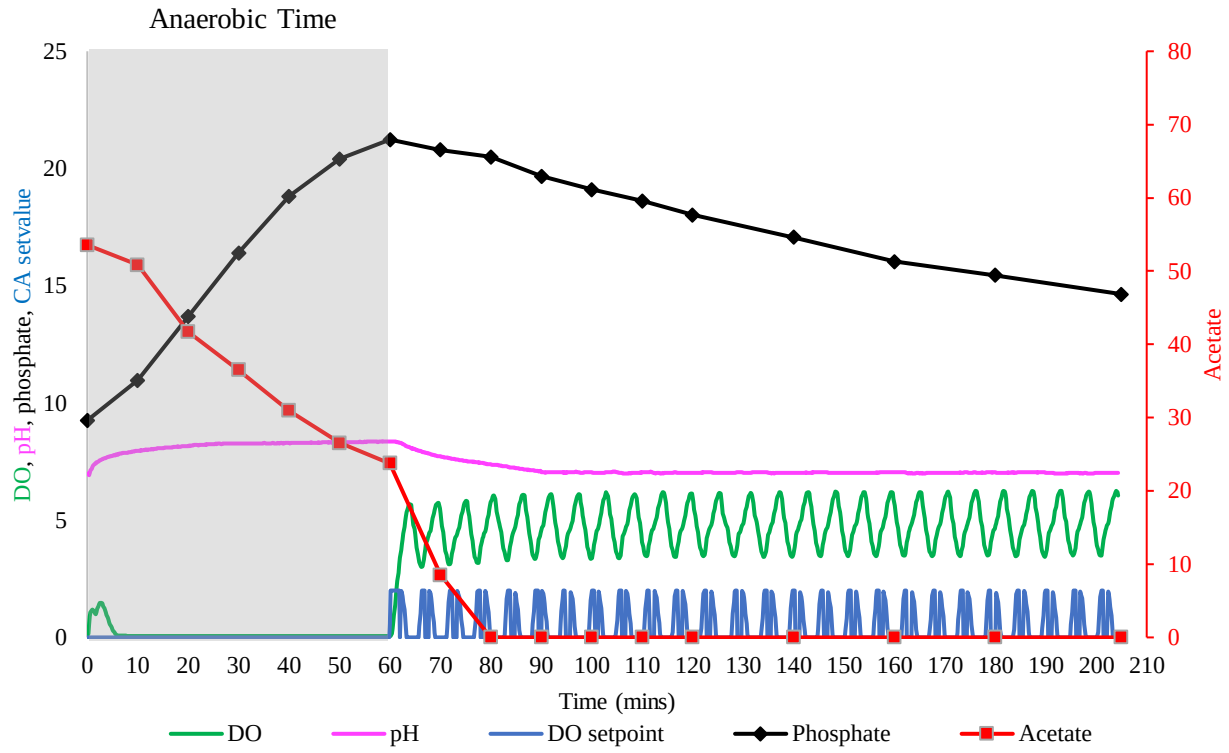


Figure 9: Sample cycle dynamics during experiment conducted with crushed granules from the Utrecht plant after 3 days of refrigeration. For cycles where acetate and phosphate measurements were analyzed plus feeding was provided.

*Table 6: Reactor characteristics for experiment with newly sampled AGS. *concentration was changed to 122mg/L after 2 days (6 cycles) ** the aerobic time was later increased for larger phosphate uptake.*

	Units	*Before Treatment*	*After treatment*
Reactor sludge	gVSS/L	2.538	1.968
VSS/TSS	gVSS/gTSS	0.793	0.818
Settle time	mins	60	
Decant time	mins	10	
Feed time	mins	60	
Aerobic mixing time	mins	110/145**	
Influent COD/P	mg HAc/L	179*	
Influent P	mg-P/L	9.6	
Effluent COD	mg HAc/L	0	
Effluent P	mg-P/L	6.66	
Reactor Volume	L	2.958	
Effluent Volume	L	31.036	
Effluent VSS	gVSS/L	0.028	
No of cycles	No	19	

Table 7 provides the result of numeric analysis from the phosphate and acetate readings measured during the cycles. The acetate and phosphate readings are given in Appendix 3. The slopes were analyzed with a maximum R2 value, and the calculations are also provided in Appendix 3.

The acetate uptake and phosphate release rate were higher for cycle 1 than any other cycle. This indicates that the crushed granules were being adjusted to the cycle conditions and reached a steady-state by cycle 6. Even though the acetate and phosphate release rates are statistically significant for cycle 6, 11 and 16 the relative standard deviation of the values were between 5 and 8%.

Table 7: Results for numerical analysis of cycle measurements for experiment with newly sampled AGS

	Units	*Cycle 1*	*Cycle 6*	*Cycle 11*	*Cycle 16*
Acetate uptake rate	mg/gVSS/min	0.361	0.245	0.225	0.214
PO4-P release rate	mg/gVSS/min	0.145	0.123	0.104	0.111
PO4-P uptake rate	mg/gVSS/min	0.075	0.028	0.020	0.020
P release/Hac uptake	mg-P/ mg-Hac	0.401	0.501	0.463	0.521

The amount of phosphate released per acetate taken up during anaerobic conditions can indicate whether other organisms like GAOs are taking up acetate. The Typical AGS from the Utrecht wastewater treatment plant have a P/Hac ratio of 0.57 for pulse fed reactor (Schouteren, 2019). In the cycles, the P/Hac ratio stays between 0.4-0.5, indicating that at least 80% of the acetate uptake was due to PAOs. The ratio also signifies that glycogen instead of the TCA cycle was preferred for ATP generation in the anaerobic period. According to Smolders et al. (1994) P/Hac ratio between 0.25 and 0.77 g-P/g-Hac, signifies glycogen degradation for ATP generation.

6.2.2 Anaerobic maintenance test

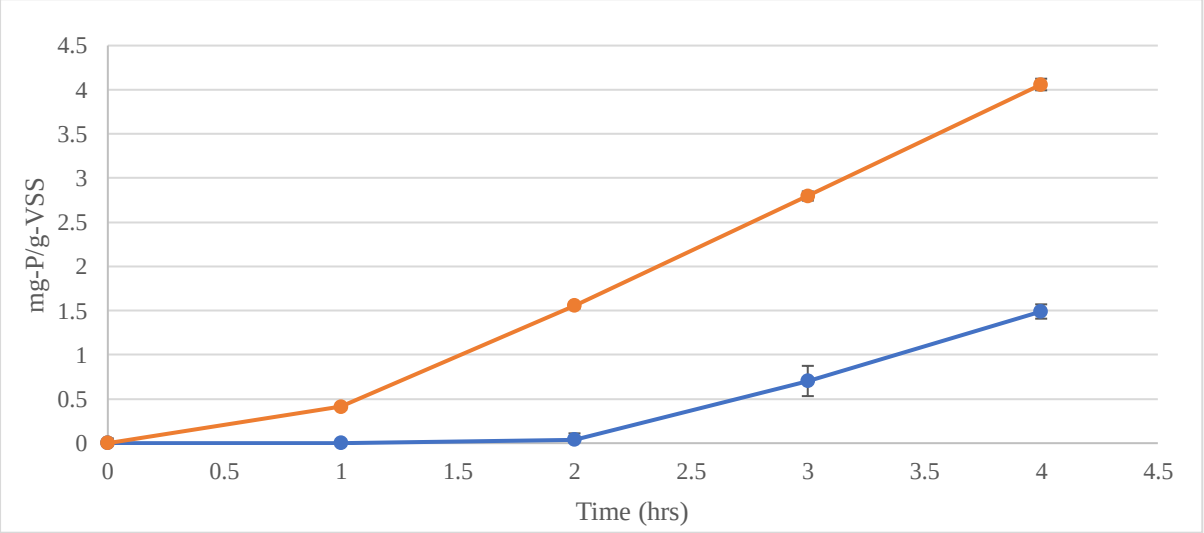
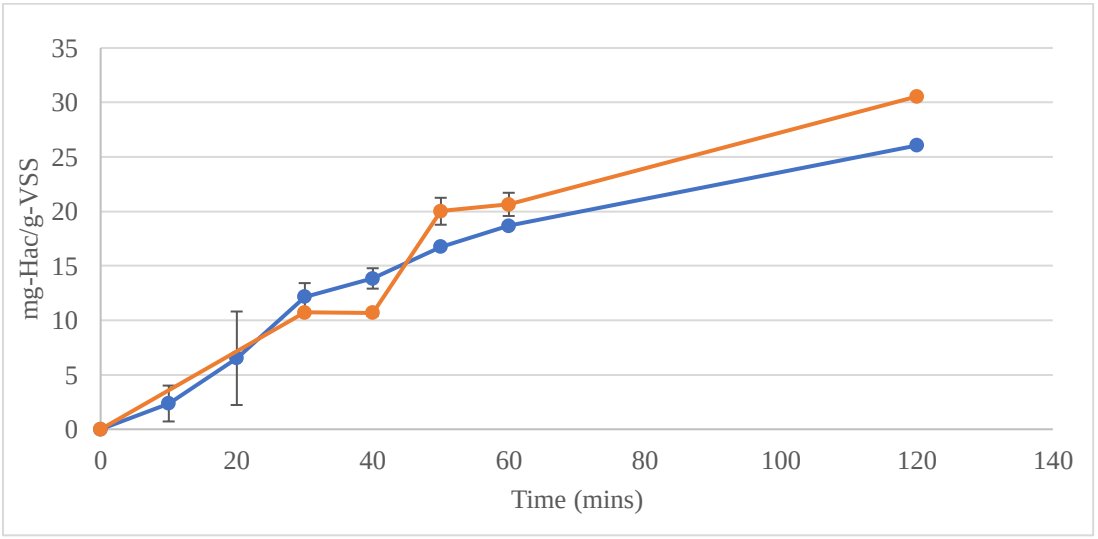


Figure 10: Shows the mean of triplicate anaerobic maintenance values for granules that were newly sampled from the AGS reactor (blue) and after the acclimatization cycles (orange). These values are for experiment conducted with new crushed granules from the Utrecht plant.

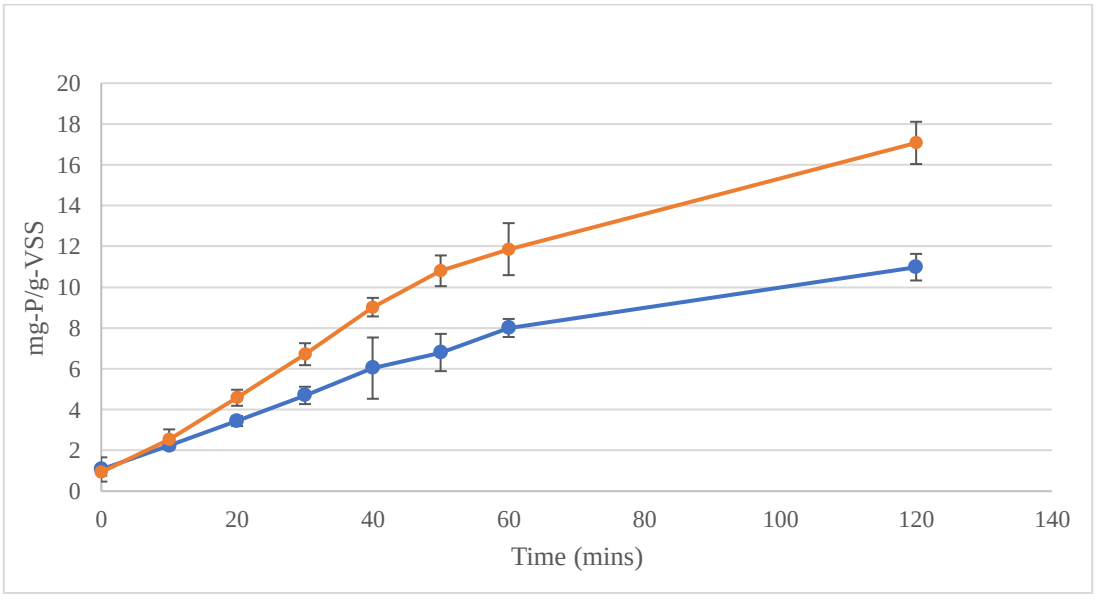
The average anaerobic maintenance coefficient was *0.725 mg-P/g-VSS/h* for the crushed newly sampled AGS and *1.218 mg-P/g-VSS/h* after the cycle. For the newly sampled AGS, the anaerobic maintenance rate was calculated from hour 2. After the acclimatization cycles, the anaerobic maintenance rate was calculated from hour 1 as the slope from 0 to 1 has a different value.

In Fig. 10, the slope of the anaerobic maintenance rate before the cycles has a lag of 2 hours. Since the granules were in a sample container (anaerobic climate) for few hours after it was sampled from the Utrecht plant, the granules were sparged with oxygen for 15 minutes before they were crushed. Due to this, the 3-minute sparging with nitrogen gas might not have been enough, and they took 2 hours to expunge all the oxygen and then start their anaerobic maintenance.

6.2.3 Activity test



a)



b)

Figure 11: a) Acetate uptake for granules that were newly sampled from the AGS reactor (blue) and after the acclimatization cycles (orange) b) phosphate release for granules that were newly sampled from the AGS reactor (blue) and after the acclimatization cycles (orange). These values are for experiment conducted with new crushed granules from the Utrecht plant.

The average acetate uptake rate was calculated as *0.360 mg-Hac/g-VSS/min* for new crushed granules and *0.355 mg-Hac/g-VSS/min* after the acclimatization cycle. Before and after acclimatization acetate uptake rate respectively has a relative standard deviation of 17%. The ANOVA was then conducted for the triplicate readings separately for each batch experiment for each time point. It was shown to be statistically insignificant or part of the same population. Therefore, the acetate uptake rate remained the same before and after the acclimatization cycles.

The average phosphate release rate was *0.192 mg-P/g-VSS/min* for new crushed granules and *0.123 mg-P/g-VSS/min* after the acclimatization cycle. There was a 36% decrease in phosphate release. This is indicative of lowered polyP use for ATP for similar acetate uptake rates.

The acetate uptake rate for batch tests for crushed newly sampled AGS was similar to the values from cycle one. However, the phosphate release rate in cycle 1 was greater for the batch test. This can be because the granules used for acclimatization in the reactor were stored in the fridge for three days more than the crushed granules used before cycle batch tests. During anaerobic storage with no substrate, the polyP in the AGS would have been slowly released for maintenance purposes so that the acetate rate would not be changed due to PHB and glycogen present, but since they are low on polyP, the release rate decreased to the value calculated in cycle 1.

6.3Growth calculations based on full-scale reactor

If dormant PAO cells are present in the Utrecht NEREDA® reactors, they can decrease the treatment efficiency by decreasing the phosphate uptake capacity of the biomass. When the treatment efficiency of the reactors is affected, it is important to find a method to improve them to their optimal working conditions. In section 6.2, the revival efficiency of the acclimatization cycles was discussed, which if effective improves the treatment efficiency. In the same period as the acclimatization cycles (4 days) there is new biomass growth replacing the old granules which also increases the treatment efficiency.
The best method for improving treatment efficiency among the above two can be discussed and effectively used. Since table 9 provides % apparent growth in 4 days, it can be compared to the results of the acclimatization cycles.

Table 8: Parameters of Aerobic Granular Sludge from Utrecht, Netherlands used for calculation of the granule growth

Values	*Units*	*Utrecht Plant*
Si	$mg/L_{influent}$	707
S	$mg/L_{influent}$	41
Biomass Load	$kg\ VSS/m^3$	6.4
Inflow	$m^3{}_{influent}/d$	18000
reactor volume	m^3	12000

In this subsection of the result chapter, table 9 displays the apparent biomass growth in the Utrecht NEREDA® reactors for different granule sizes. The Utrecht reactor characteristics

given in table 8, are from the research paper of van Dijk et al. (2018) and the SRT for different granule sizes are obtained from the paper of Ali et al. (2019).

Table 9: Apparent growth in Utrecht full-scale Nereda® reactor based on equation 4

Granule size	*srt*	b_w	*% apparent growth in 4 days*
0.2 to 1mm	7.7	9974	52%
>1mm	142.6	539	3%

7. DISCUSSIONS

7.1 Method testing with AGS that was induced with external stress to simulate dormancy

To test the validity and the scope of the developed method, an experiment was performed with 27-day old AGS. The granules were induced with external stress to simulate dormancy for the PAOs. From literature, it was found that substrate limitation and low temperature could be great external stresses leading to dormancy.
Therefore, the AGS was conditioned at 4ºC anaerobic climate without feed to induce dormancy in the PAOs. Observations for this experiment will be discussed in this section.

In this experiment, the metabolic rates of the crushed granules decreased after 27 days of storage compared to the fresh granules. The activity and anaerobic maintenance rate of the crushed granules after storage was only 6% and 42% of the freshly crushed granules, respectively. After the acclimatization cycles of four days, the activity recovered to 60%, and the anaerobic maintenance term increased to 122% of the freshly crushed granules. According to section 2.2 of the literature survey, this reversible state of reduced metabolic rates in cells defines dormancy in this research thesis.
During the literature survey in section 2.4, it was discussed that there were two pathways to dormancy. One is due to starvation that can slowly lead to dormancy, and the other is due to responsive switching leading to dormancy. Even though the developed method shows the stored PAOs in a state of dormancy, accurately identifying the pathway to this dormancy is not possible with the current methodology. However, with the acclimatization cycles and the batch experiment results, we can speculate the probability of the pathways mentioned above.

In the research of Vargas et al. (2013), where similar resuscitation cycles were provided to 21 days starved PAO cells, the acetate uptake rate increased to the initial rate in just one cycle. This trend was not seen during the acclimatization cycle in this experiment. The activity increased only by 60% of the initial rate in four days of the acclimatization cycle. In the research paper of Vargas et al. (2013), it was also noticed that the storage polymers: polyP and glycogen, which were drastically decreased during the 21-day starvation period, were restocked in the first and fourth day of the resuscitation cycle, respectively. Even though storage polymers were not measured for this experiment, the change in the storage polymers could be postulated with the help of the acclimatization and batch experiment results.
During the acclimatization cycles, the acetate uptake and phosphate release rates increased in response to the added substrate. Cycle 1, 6 and 12 showed an increase in the P/Hac ratio. For cycle 13, the ratio decreased, suggesting less dependence on polyP for acetate uptake. It can point towards an increase in glycogen content, which provides the energy for acetate uptake, thereby decreasing the need for polyP release for energy. However, the P/HAc ratio for both the batch experiments stayed between 0.282 and 0.221. These values do not show a shift from TCA to glycogen for the dependence of energy during acetate uptake, according to the research of Smolders et al. (1994).
During the batch experiments, a 167% increase in anaerobic maintenance rate was observed after the acclimatization cycle compared to the rate after anaerobic storage. This increase in anaerobic maintenance energy can have two potential explanations. The first explanation might be that the anaerobic maintenance increases due to increased polyP storage in the cells. However, the VSS/TSS ratio after storage and after the acclimatization cycles does not increase, suggesting a negligible increase in the cell's polyP content.

The second explanation can be that the granules were stressed due to substrate limitations during the anaerobic storage. In this stressed state, there could have been the formation of guanosine 3′,5′-bis-diphosphate (ppGpp), which is responsible for the stringent response (He & McMahon, 2011). The stringent response activates the stress response and lowers cell growth and metabolic rates to adjust to nutrient starvation in bacterial cells (Boutte & Crosson, 2013). During the acclimatization cycle, the PAO cells could have sensed that they were no longer facing nutrient starvation and eliminated the stringent response. The elimination of stringent response can be seen as a transition from starved to live cells, which increases the cells anaerobic maintenance requirement to their optimal rate, thereby increasing the phosphate release.

During this experiment, a significant increase in the storage polymers after the acclimatization cycles could not be identified through the batch and acclimatization results. However, the research of Vargas et al. (2013) clearly shows an increase of polyP and glycogen during the revival of starved PAO. Therefore, solely through a theoretical observation of the change in storage polymers of the PAOs, starvation as the cause of decreased rates of crushed granules might not be the case in this experiment.

If we hypothesize that the PAOs go into a state of dormancy through responsive switching, then a periodic increase in rates as observed in the acclimatization cycles can be expected. The periodic decrease in metabolic rates due to low temperature and absence of feed (as seen during method development) could be due to PAO cells turning dormant at different rates. As observed in Cesar et al. (2020), an increase in the dormancy period increases the lag between dormant and live-cell resuscitation. Therefore, if cells are turning dormant at different time during storage, it only makes sense that during the cycles, they are being resuscitated at different times as well. According to the research of Cesar et al. (2020), the cells that were dormant for an extended period will be expected to take a longer time to become alive, and the recently dormant cells will be expected to wake up the fastest. Hypothetically, the dormancy observed in the PAO cells of 27-days anaerobically stored AGS could be through responsive switching.

To check for responsive switching, it is essential to investigate the signalling molecules of PAO. K+ is a signalling ion for PAO to adapt to osmotic pressure (Wang et al., 2018). In the same way, when dormancy is induced, it will produce signalling molecules to indicate when to go in and out of dormancy. In *Nitrosomonas europaea,* gram-negative signalling molecules, acyl homoserine lactones (AHLs), were added to starved cells, which reduced the resuscitation time by five times (Batchelor et al., 1997). Since AHL is found in many gram-negative bacteria, it is likely present during responsive dormancy of PAOs. The periodic increase in rates (during the acclimatization cycle) could also have been due to an increase in the signalling molecules as the dormant cells become live. An increase in the number of live cells increases the signalling molecules and therefore increases the metabolic rates, as seen in the acclimatization cycles.

The four-day acclimatization cycles were efficient in increasing the activity and anaerobic maintenance of the AGS by 130% and 167%, respectively, compared to the rates after 27 days of storage. Even with a maximum growth of crushed granules without any decay, there would only be a biomass increase of 22% (see results 6.1.1). This result can suggest that during the acclimatization cycle, influent can include nitrogen compounds (which was initially not considered to avoid the growth of biomass) as the effect of the cycles are higher than the maximum growth. This new experiment can observe the effect of ammonium or new cell growth in the exit of dormancy or starvation. New granule growth is essential because, in

bacteria, the growth of new cells is one of the methods to signal dormant cells to exit their state of dormancy (Dworkin & Shah, 2010). Cells that undergo responsive switching have sensory mechanisms to monitor their environment continuously, which helps them quickly switch in and out of dormancy (Kussell, 2005). The presence of new growing cells can indicate a nutrient-rich climate forcing the dormant cells to become live.

Overall, two main lessons can be interpreted from the method testing experiment with 27-day old granules. Firstly, the developed methodology can reverse the effect of induced dormancy in PAOs of AGS, and second, the method is unable to distinguish which type of dormancy might be affecting the PAOs of the anaerobically stored AGS.

7.2 Physiological state of newly sampled AGS from NEREDA® reactors

An experiment was conducted to observe if the PAOs undergo dormancy in full-scale NEREDA® reactors. Observations of this experiment will be discussed in this section.
In this experiment, the activity of the crushed AGS did not change after the acclimatization cycles, but the anaerobic maintenance terms increased by 68%. Three interpretations for this observation are given below.

1. There might be starvation observed for AGS in full-scale reactors.
2. The granules from the full-scale Utrecht NEREDA® reactor do not have dormancy, or the methodology used here is not efficient to identify this type of dormancy.
3. The current methodology does not increase the activity of the crushed granules.

The first interpretation states that the AGS granules in full-scale NEREDA® reactor might face starvation condition. It was observed by Vargas et al. (2013) that during starvation in PAOs, storage polymers were quickly taken up for energy purposes. Since storage polymers were not measured for this experiment, the change in storage polymers was analyzed theoretically.
The increase in polyP storage of the PAO cells can potentially explain the increase in anaerobic maintenance rates observed in this experiment. However, the VSS/TSS ratio of fresh granules and after the acclimatization cycles did not increase suggesting a negligible increase in the cell's polyP content.
The second explanation can be that the granules sampled from the NEREDA® plant were already stressed, which might have activated the stringent response. This stringent response could have been eliminated by unlimited substrate availability during the cycles, which might have been the reason for the increased anaerobic maintenance rates and phosphate release. This decrease in maintenance energy was also observed in starved PAOs by Lopez et al. (2006) after the storage polymers were used for energy.

In the batch experiment conducted for newly sampled AGS, the P/Hac ratio of 0.533 mg-P/mg-Hac is in the range (1.03 – 0.51 mg-P/mg-Hac) of phosphate release during a Tricarboxylic Acid (TCA) cycle (Smolders et al., 1994). After the acclimatization cycles, the P/Hac ratio decreased to a value of 0.345 mg-P/mg-Hac, which is in the range (0.78 – 0.26 mg-P/mg-Hac) of phosphate release during glycogen degradation of ATP production. The decrease in the P/Hac ratio signifies a shift from TCA to glycogen for energy. Since glycogen content was not experimentally measured, it is hypothesized here solely based on decreased P/Hac ratio that glycogen might have been restocked in the PAO cells during the acclimatization cycles. The glycogen polymer could have further provided energy for acetate consumption, decreasing the dependency on polyP for ATP. If the acclimatization cycles potentially increased the glycogen content, it would mean that the PAO cells are starved in full-scale reactors. The possibility of

starvation in a full-scale AGS reactor is not unlikely. It is observed from the research of Lopez et al. (2006) that the storage polymers can be depleted in one day as a result of anaerobic starvation conditions. So even if the NEREDA® reactors are typically not without influent for an extended period, short-term fluctuations in the influent hydraulic and organic load can potentially reduce the cell's glycogen and polyP content, resulting in starvation of the PAO cells. Starvation in a full-scale reactor could be a cumulative result of short-term influent fluctuations.

From the experiment and the literature survey conducted for this master thesis, it is not evident if PAOs of newly sampled AGS can go into a state of dormancy due to long term starvation. Even though starvation is a pathway to dormancy in other bacteria like *E.Coli,* the current methodology does not indicate dormant PAO cells. This leads to the second interpretation that either dormant PAOs do not exist in full-scale reactors, or they cannot be detected or revived with the current methodology. When compared to the previous method testing experiment, the acetate uptake values does not increase after the acclimatization of crushed fresh granules. Since the PAO cells do not observe a reversible state of lowered metabolic state, according to the definition of this master thesis, the cells are not in a state of dormancy.
The differences between the two experiments: method testing and experiment with fresh AGS, were the storage conditions of the granules. The anaerobic storage of the AGS without feed at a temperature of 4ºC was probably the reason for the dormancy of PAOs during method testing, and starvation in fresh AGS could be caused by influent substrate fluctuations in the full-scale NEREDA® reactor.

The third interpretation states that the current methodology is not efficient in increasing the acetate uptake rate of the granules. A potential explanation for the decreased P/Hac ratio in this experiment was the increase of the glycogen pool. This increase of glycogen decreased the dependency of polyP release for similar acetate uptake. Either the restored glycogen was not enough to increase the acetate uptake, or there is another reason hindering the increase. To further understand the inability of the method to increase the acetate uptake rate, the abundance of PAO in the newly sampled AGS was studied. The anaerobic maintenance rate from batch experiments for newly sampled AGS granules were 25% of the value from an enriched PAO system described in Kuba et al. (1993). If the research paper of Kuba et al. (1993) deals with biomass of close to 100% PAO, it signifies that the abundance of PAO in AGS was only 25%. Interestingly, the maximum acetate uptake rate was only 5% of the maximum acetate uptake rate mentioned in a PAO enriched system (Murnleitner, 1997).
This is an interesting observation, as the acetate uptake would be expected to be carried out by the same fraction of biomass that is active during the anaerobic maintenance. This is not the case. Based on the anaerobic maintenance, the abundance of PAOs would be 25%. Based on the acetate uptake activity, the abundance would only be 5%. This forces us to explain the 20%-point difference. The 20-point difference could be due to two potential reasons. One, the activity per PAO could be lowered, where all the live PAO might not function with their optimal activity. During starvation, the bacteria experience physiological changes. Therefore the activity just after the starvation period might be limited due to certain enzymes and not necessarily be directly proportional to the population size (Lu et al., 2007). Two, some of the PAO cells might not undergo acetate uptake due to limited glycogen content in the system. Currently, the methodology suggests a shift from TCA to glycogen for acetate uptake, directing towards low glycogen content in the newly sampled AGS.
During the study of PAO abundance in AGS, it was also observed that the anaerobic maintenance energy increased from 25% to 40% of the value from an enriched PAO system after the acclimatization cycles. This increase suggests two possible explanations. One, the

PAO cells increased their maintenance energy demand in response to substrate addition or two, there was an increase in active PAOs contributing to the increased maintenance energy. As it is experimentally unclear which option might be valid, the active PAO fraction from this method does not necessarily reflect the actual live PAO population in the AGS. Measurement of the live PAO population would be advantageous because it would indicate the difference in active PAO fractions participating in maintenance and activity. Currently, assessing an accurate PAO population is not straightforward. A combination of different DNA sequencing techniques with proteomics can estimate this fraction. To find out the dead or inert portion that might be dormant in the system, a different combination of techniques along with a LIVE/DEAD® BacLight™ method can be used. Therefore, the methodology to assess PAO abundance in the full-scale reactor is complex.

It is therefore hard to study the physiological state of the AGS due to the unknown live PAO population and added complexity of storage polymers. The developed methodology indicates a decrease in glycogen content, but it is inefficient in increasing the acetate uptake rate. If starvation occurs, maybe providing growing conditions for the cells (by including nitrogen compound in the feed) can induce a faster increase in activity, as seen in the research of Vargas et al. (2013). To experimentally observe starvation, it is necessary to measure the storage polymers (polyP, glycogen, and PHA) of PAOs. Reduced storage polymer is an established observation of starvation in PAO (Lopez et al., 2006; Lu et al., 2007; Vargas et al., 2013). The complexity only starts here as measuring storage polymers of PAO accurately in an AGS system is not easy. All the different types of PHA and glycogen must be measured.

Two main lessons can be interpreted from this experiment conducted on newly sampled AGS. Firstly, an increase in the PAO cells maintenance energy and potential glycogen replenishment can point towards starvation rather than dormancy for newly sampled AGS. Secondly, the current methodology is not capable of increasing the activity of the starved PAOs.

7.3 Implications of dormancy on NEREDA® reactors

In a full-scale AGS plant, granule size stratification is observed during the settling phase. This means that the granules with larger sizes settle the fastest at the bottom, and the smaller sized granules settle on top of them (van Dijk et al., 2020). Due to this stratification, during anaerobic bed feeding, the larger granules are fed first, consuming a large portion of the substrate (van Dijk et al., 2020), and the rest travel upwards to the smaller sized granules. In this case, smaller granules (size 0.2 to 1 mm) can experience starvation if the rest is not enough to feed all the smaller granules. For larger granules (size > 1mm), starvation can occur due to substrate diffusion limitation affecting PAOs resting deeper in the granules.
Larger granules are greater in number than smaller ones in the Utrecht reactor (van Dijk et al., 2020) and have a higher amount of *Ca.* Accumulibacter phosphatis, than smaller granules (Ali et al., 2019). This could mean that larger granules can have a greater amount of starved PAOs.

Hypothetically if these starved PAO cells were to enter a state of dormancy, there could appear a loss in phosphate removal capacity due to the loss in their cell activity. Two different methods can compensate for the loss in treatment capacity due to dormant PAOs. The first method is by resuscitating the dormant PAOs, thereby increasing their activity and hence the treatment capacity. The second method is by new biomass growth that produces new PAOs, which will increase the treatment capacity. From method testing, it was observed that dormant cells show resuscitation during the 4-day acclimatization cycles. Even though newly sampled AGS did not show resuscitation during the same period, a minimum of 4 days was required to revive the

activity of dormant cells to at least 50% of their optimal rate. In this same four days of acclimatization cycle, the apparent biomass growth was calculated in the result section 6.3. The SRT of small granules were observed to be 8 days. The apparent biomass growth was calculated as 50%, which means 50% of the biomass is replaced in the 4 days (same time as the acclimatization cycles). Therefore, new biomass growth in the reactor is the easier choice to increase the lost treatment capacity due to dormant PAOs.

This would mean that the treatment efficiency of the granules in the full-scale reactor might not be affected due to starvation. This can be because of the new growth of PAOs compensating for the potential decrease in the treatment capacity due to dormant cells. Even though the exact reason for lowered maintenance is not understood with this methodology, it might not be valuable from a practical view.

8. CONCLUSIONS

This research involved developing a method to detect dormancy in AGS from a full-scale NEREDA® reactor. The conclusions of the same are given below.

1. The methodology developed in this research thesis is successful in identifying at least one pathway to dormancy, which is most likely the responsive switching pathway.

2. Due to the absence of lowered metabolic state that is reversible, dormancy in PAOs is unlikely to happen in the NEREDA® full-scale reactors. However, it is possible that the granules in the full-scale reactors, face starvation condition.

3. The methodology developed in this thesis does not revive the starved granules in the NEREDA® full-scale reactors.

4. The abundance of PAO calculated by the anaerobic maintenance rate and the maximum acetate uptake rate is observed to differ greatly. This suggests that the full-scale aerobic granular sludge has a different PAO physiology compared to enriched laboratory cultures. However, the physiology of the PAO in the NEREDA® reactors cannot be easily studied due to the complexity of storage polymers and live PAO fraction measurement.

9. RECOMMENDATIONS

The following recommendations are made based on the conclusions of this research. It will validate the current methodology and close the research gaps of this thesis.

1. The method developed in this thesis can likely detect responsive switching in AGS where dormancy was induced. It is essential to measure and identify signalling molecules to validate the method. This will also answer if dormancy is possible in PAOs and hence in full-scale NEREDA® reactors.

2. Starvation in PAO observes a reduction in storage polymers. Here, the change in storage polymers were abstracted from the results of batch experiments, as no measured quantities were present. Therefore, it is recommended to measure storage polymers which include polyP, glycogen, PHV and PHB. The change in these storage polymers can confirm the effect of starvation in full-scale reactors.

3. It was observed that although 25% of the PAOs were involved in maintenance, only 5% of the PAOs assisted during activity. There can be many reasons for this, including reduced storage polymers or reduced activity of the PAOs. To differentiate between physiological issues and environmental causes, it is vital to have an accurate measurement of live and dead PAOs in the system. It is currently not easy to get an accurate measurement, so combining different methods (i.e. DNA sequencing and proteomics or proteomics, LIVE/DEAD® BacLight™ and FISH) can give a reasonable estimate of the same.

4. Currently, it is not possible to quickly revive the PAOs in the full-scale reactor. It would therefore be helpful to operate the NEREDA® reactors at a lower Total Solids Concentration (TSS). With this change in operation, the amount of PAOs that are currently functioning with lowered maintenance can function at full capacity. The reduction in TSS can also increase the oxygen transfer efficiency during the treatment.

5. If fermentation of the influent takes place in NEREDA® wastewater treatment plants, it could be a possible explanation as to how the PAOs survive with low VFA in the feed. Therefore, further research to study the fermentation efficiency of the influent feed during the anaerobic phase of the NEREDA® cycle can be advantageous from a research perspective.

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Annexure 1 *(Calculation of acetate requirement for PAO maintenance purpose)*

Based on the paper "Kinetic model of a granular sludge {SBR}: Influences on nutrient removal" for PAOs and Murnleitner (1997):

Anaerobic maintenance = 0.05 $gP/gCOD_X/d$
Aerobic maintenance = 0.06 $gO2/gCOD_X/d$
Overall maintenance term = 0.053 $gCOD_{PHB}/gCOD_X/d$
From overall stoichiometry equation of

$$1.00\ Ac \rightarrow 0.\ 72\ PHA.$$

we can calculate that for 0.053 $gCOD_{PHB}$ needs 0.10 $gCOD_{Ac/}gCOD_X/d$.
= 0.074*1.42/1.07 = 0.0976 gAc/gX/d
=97.68 mgAc/gX/d
Based on different models, the PAO fraction in the reactor varied from 2g/L to a max of 7g/L. Therefore, for this calculation we take a value of 2.7g/L which is seen in typical models.
= 97.68*2.7 = 263.73 $mgAc/L_R/d$
Volume of influent is 1.2 times the volume of the reactor if we consider four batches per day. Each batch volume is 30% of the reactor volume:
=263.73/1.2=219.78 $mgAc/L_{influent}$

Annexure 2 (Cycle readings and slope calculation for method testing experiment)

Table 10: Acetate and phosphate measurements in each cycle for method testing experiment

CYCLE 1			CYCLE 6			CYCLE 12			CYCLE 13		
Time (mins)	Acetate (mg-Hac/L)	Phosphate (mg-P/L)	Time (mins)	Acetate (mg-Hac/L)	Phosphate (mg-P/L)	Time (mins)	Acetate (mg-Hac/L)	Phosphate (mg-P/L)	Time (mins)	Acetate (mg-Hac/L)	Phosphate (mg-P/L)
0	50.6	3.3	0	39.7	5.2	0	30.2	4.9	0	49.8	8.4
10	57.7	4.8	15	36.7	1.2	10	28.1	6.5	10	45.5	9.9
20	50.9	5.3	20	29.3	9.2	20	20.9	9.6	20	38.2	12.3
30	50.4	5.4	30	29.7	10.6	30	16.5	11.7	30	35.1	13.4
40	46.1	5.9	40	23.0	12.0	40	14.3	12.6	40	31.6	13.7
50	51.4	5.9	50	23.0	12.3	50	9.8	14.1	50	28.9	15.1
60	48.5	5.9	60	18.0	13.2	60	6.6	15.0	60	24.3	15.7
70	27.7	5.0	70	4.5	12.2	70	0.0	15.1	70	7.8	15.0
80	12.6	4.4	80	0.0	10.4	80	0.0	13.4	80	0.0	14.0
90	0.0	3.1	90	0.0	8.8	90	0.0	13.5	90	0.0	14.4
100	0.0	0.6	100	0.0	7.6	100	0.0	12.3	100	0.0	14.1
110	0.0	-0.572	110	0.0	6.4	110	0.0	11.7	110	0.0	12.8
130	0.0	-0.5	130	0.0	5.5	130	0.0	10.5	130	0.0	12.7
145	0.0	-0.5	145	0.0	4.8	145	0.0	10.7	145	0.0	10.7
170	0.0	-0.5	170	0.0	1.7	170	0.0	9.5	170	0.0	9.8

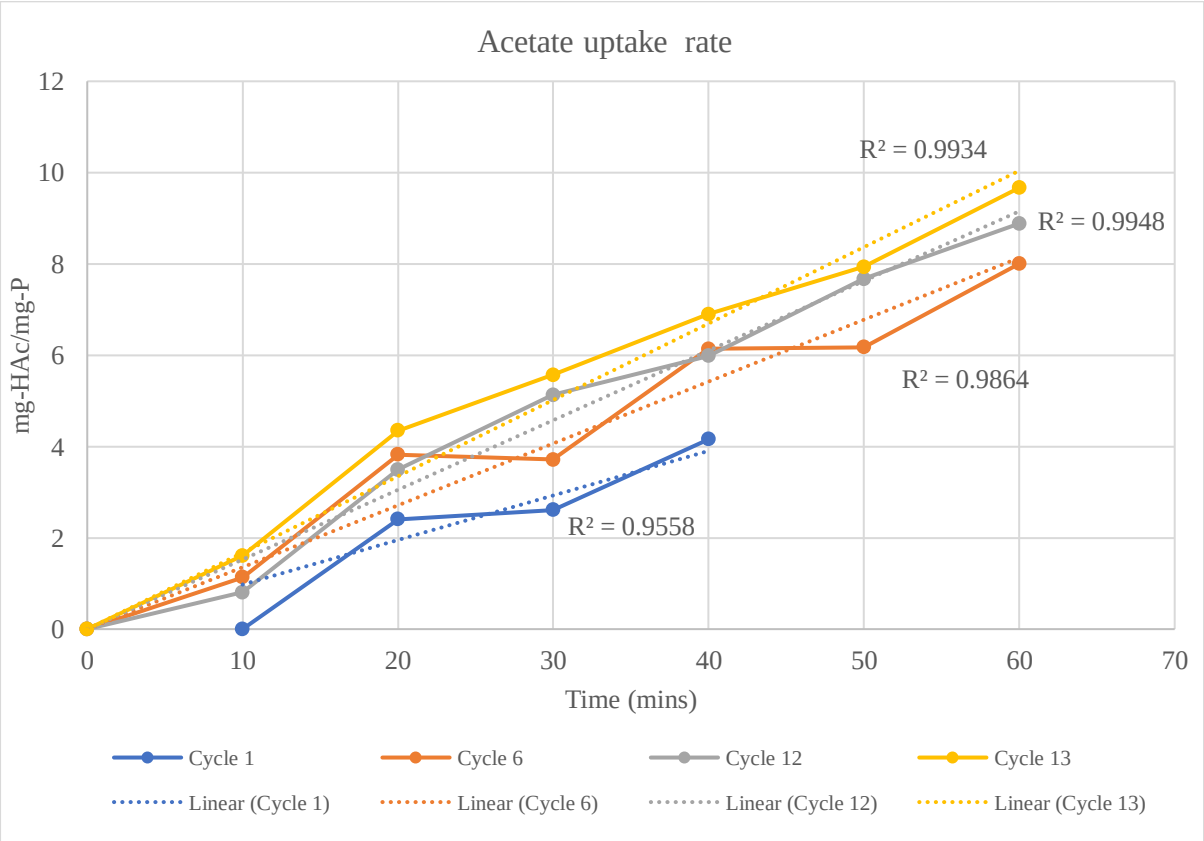

Figure 12: Acetate uptake for 4 cycles in the reactor for method testing experiment

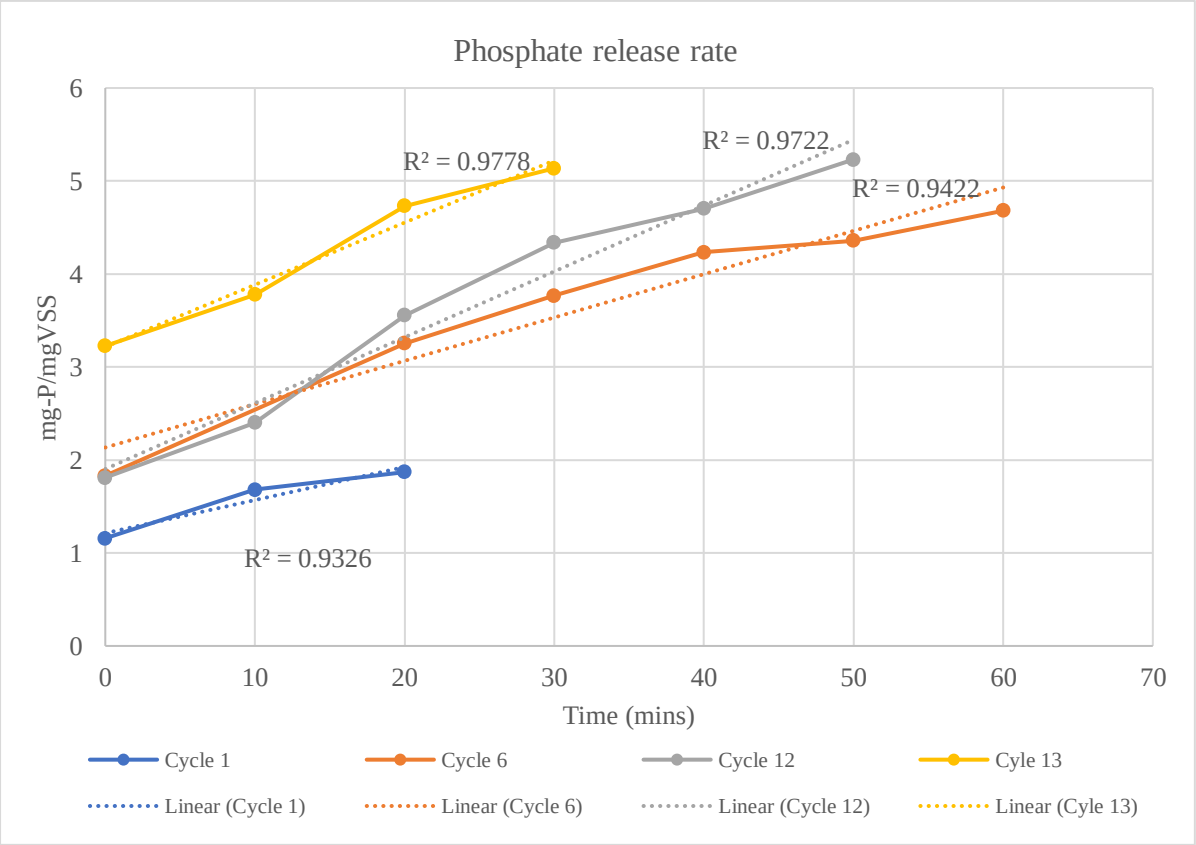

Figure 13: Phosphate release for 4 cycles in the reactor for method testing experiment

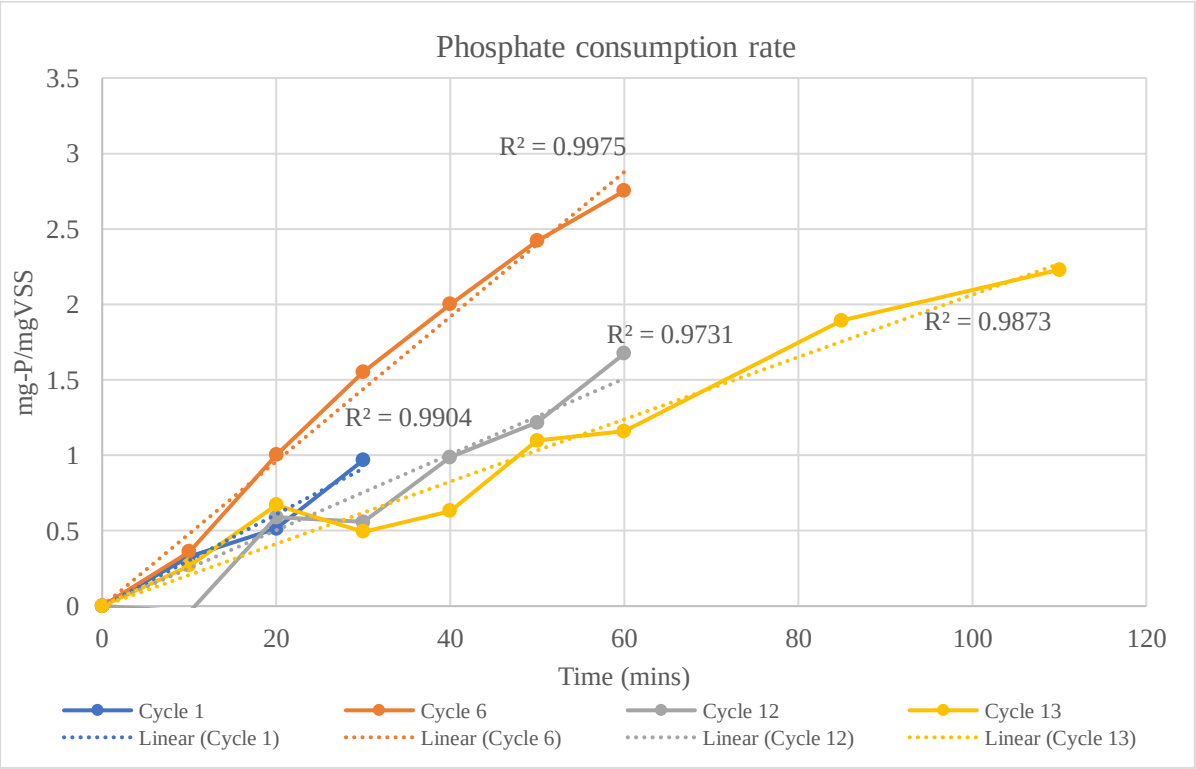

Figure 14: Phosphate consumption for 4 cycles in the reactor for method testing experiment

Annexure 3 (Cycle reading and slope calculation for experiment with fresh granules)

Table 11: Acetate and phosphate measurements in each cycle for experiment with fresh AGS

CYCLE 1			CYCLE 6			CYCLE 11			CYCLE 16		
Time	Acetate	Phosphate	Time	Acetate	Phosphate	Time	Acetate	Phosphate	Time	Acetate	Phosphate
0	83.8	4.1	0.0	81.5	9.5	0.0	53.6	9.3	0.0	52.1	7.3
10	75.4	6.9	10.0	80.0	12.0	10.0	50.8	11.0	10.0	49.5	10.1
20	65.4	11.3	20.0	74.6	15.3	20.0	41.7	13.7	20.0	43.5	13.4
30	54.8	14.9	30.0	63.9	18.8	30.0	36.5	16.4	30.0	35.8	16.2
40	48.8	18.3	40.0	54.5	21.1	40.0	30.9	18.8	40.0	30.4	17.4
50	43.3	20.7	50.0	54.0	23.0	50.0	26.5	20.4	50.0	27.9	19.0
60	35.8	22.6	60.0	49.7	24.5	60.0	23.7	21.2	60.0	23.6	20.2
70	25.7	23.1	70.0	37.3	24.6	70.0	8.5	20.8	70.0	6.8	20.5
80	14.1	23.0	80.0	20.0	24.4	80.0	0.0	20.5	80.0	0.0	19.4
90	0.0	22.4	90.0	0.0	24.3	90.0	0.0	19.7	90.0	0.0	19.0
100	0.0	19.9	100.0	0.0	23.5	100.0	0.0	19.1	100.0	0.0	18.4
110	0.0	17.2	110.0	0.0	22.9	110.0	0.0	18.6	110.0	0.0	17.8
120	0.0	15.0	120.0	0.0	21.6	120.0	0.0	18.0	120.0	0.0	17.0
140	0.0	9.8	140.0	0.0	20.1	140.0	0.0	17.1	140.0	0.0	16.2
165	0.0	6.5	160.0	0.0	18.8	160.0	0.0	16.0	160.0	0.0	15.2
			180.0	0.0	17.7	180.0	0.0	15.4	180.0	0.0	14.6
			205.0	0.0	16.9	205.0	0.0	14.6	205.0	0.0	13.9

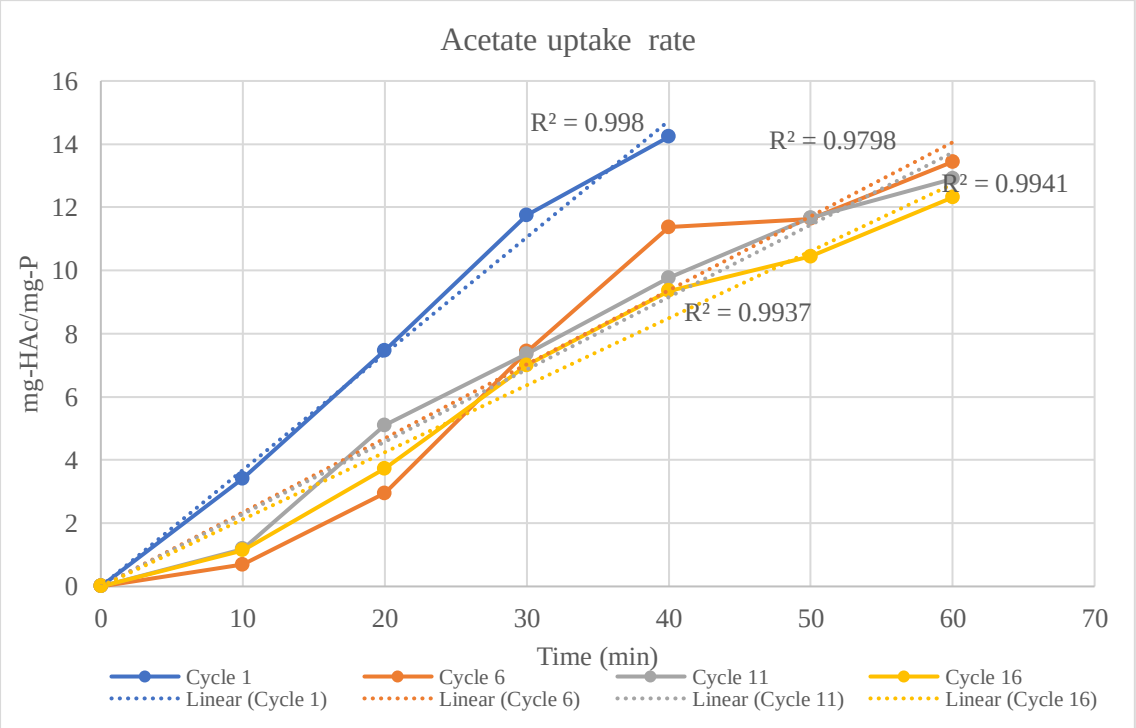

Figure 15: Acetate uptake for 4 cycles in the reactor for experiment with fresh AGS

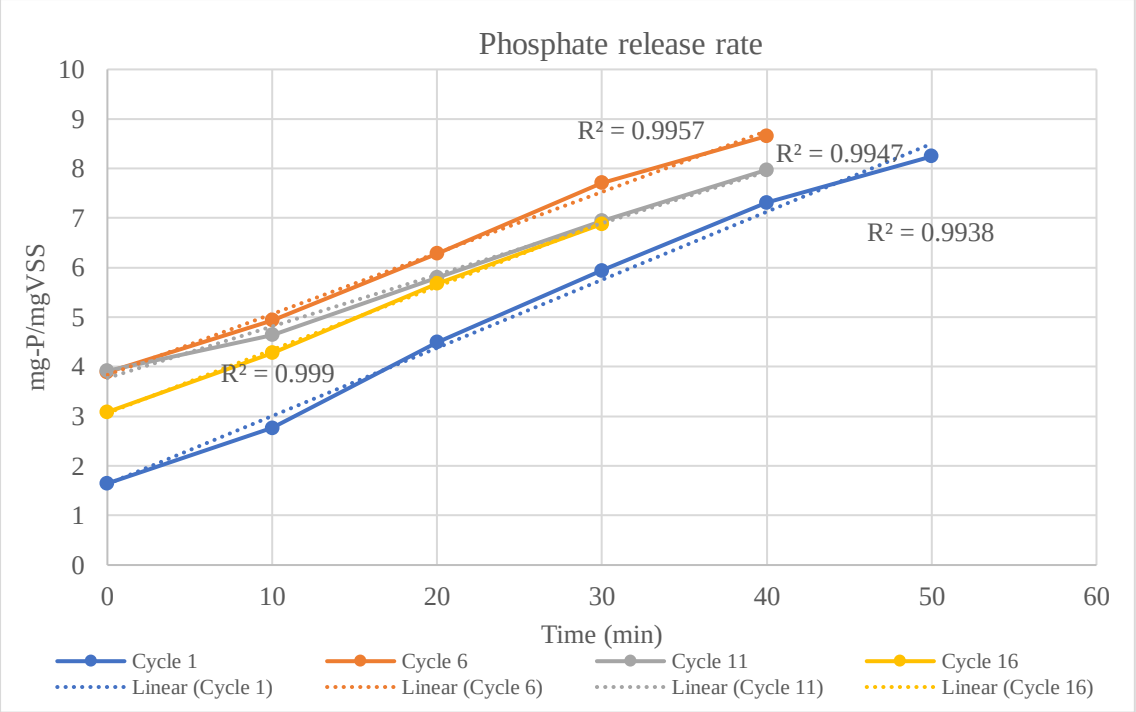

Figure 16: Phosphate release for 4 cycles in the reactor for experiment with fresh AGS

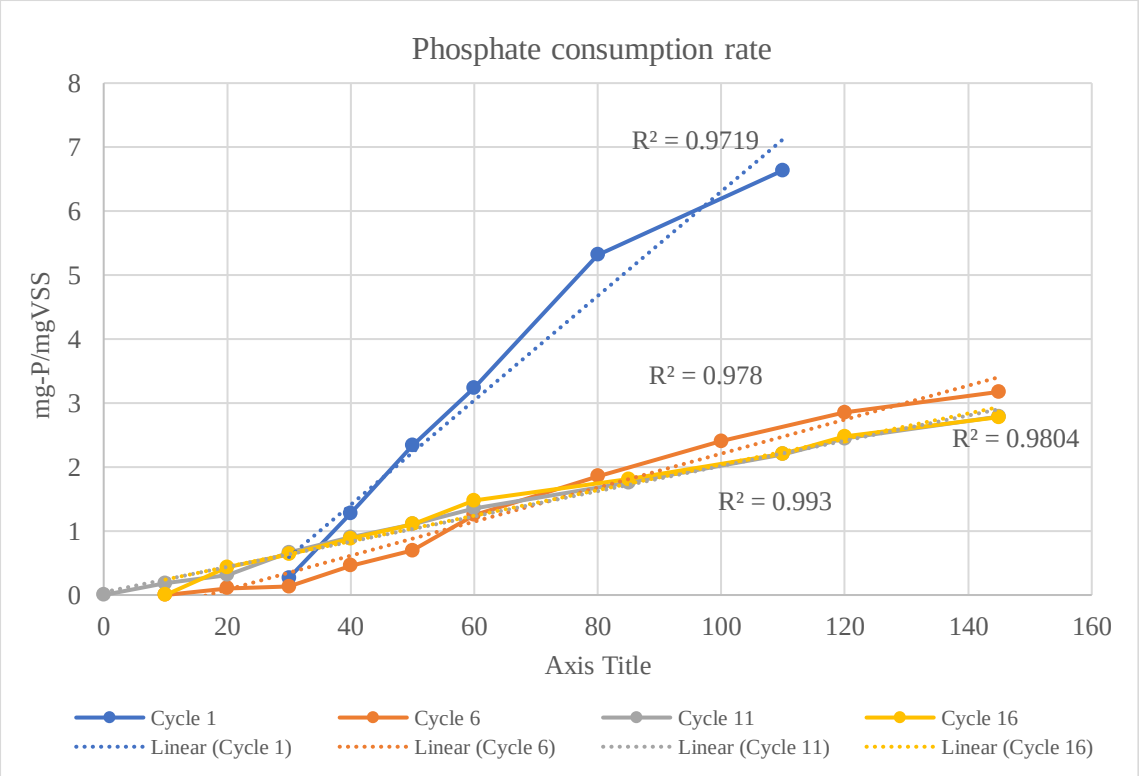

Figure 17: Phosphate consumption for 4 cycles in the reactor for experiment with fresh AGS