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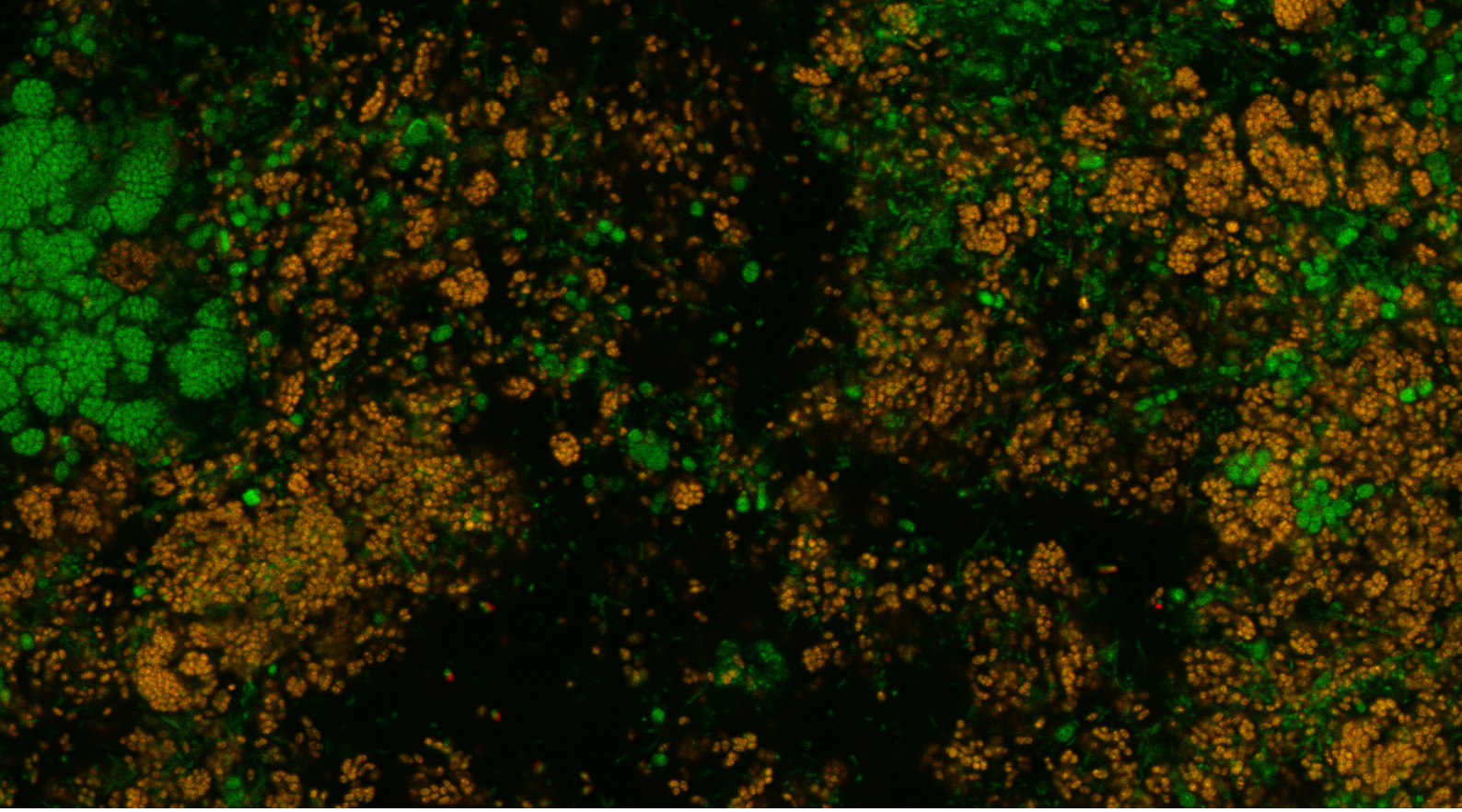
Publication date:
2019

Document Version
Publisher's PDF, also known as Version of record



REMOVAL OF PHOSPHATE BY POLY-P ACCUMULATING ORGANISMS IN ACTIVATED SLUDGE AND THEIR FATE IN DIGESTERS

BY
KAMILLA AGNETHE SMITH HANSEN

DISSERTATION SUBMITTED 2019

AALBORG UNIVERSITY
DENMARK

REMOVAL OF PHOSPHATE BY POLY-P ACCUMULATING ORGANISMS IN ACTIVATED SLUDGE AND THEIR FATE IN DIGESTERS

A dissertation submitted in partial fulfilment of requirements for obtaining the degree of

DOCTOR OF PHILOSOPHY

by

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December 2019

Dissertation submitted:	December 20, 2019
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PhD Series:	Faculty of Engineering and Science, Aalborg University
Institut:	Department of Chemistry and Bioscience

ISSN (online): 2446-1636
ISBN (online): 978-87-7210-571-0

Published by:
Aalborg University Press
Langagervej 2
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk

Printed in Denmark by Rosendahls, 2020

ENGLISH SUMMARY

Phosphorus (P) is a non-renewable resource and should be recovered when possible. One place to recover P could be from the digesters in wastewater treatment plants (WWTPs) which, depending on the size of the plant, receive large quantities of P each day. Polyphosphate accumulating organisms (PAOs) are organisms utilized in the enhanced biological phosphorus removal (EBPR) process in WWTPs and these microorganisms, storing P, are transferred from activated sludge and into digesters where P is released.

In this study it was investigated how the transfer from activated sludge into digesters would affect viability, substrate uptake and P release from known PAOs. A mass balance of the P pools found in the WWTPs was also conducted to get a better understanding of the P-pools available and an indication of how much of the P was stored in known PAOs. This was all done in an effort to better understand and optimize the recovery of P from WWTPs.

The survival of PAOs transferred from activated sludge into mesophilic and thermophilic digesters, was investigated with surveys in Danish WWTPs and digesters (Hansen et al., 2019a, 2019b). The surveys compared abundance of PAOs and potentially substrate competing glycogen accumulating organisms (GAOs) in activated sludge, with the abundance in the associated meso- and thermophilic digesters. The surveys showed decrease in abundance in almost all microorganisms investigated with a few exceptions showing a small increase in abundance in digesters. To confirm these results, long-term anaerobic batch experiments were conducted. Activated sludge high in *Tetrasphaera* PAO abundance and *Ca.* Accumulibacter PAO enriched sludge, was incubated under conditions mimicking meso- and thermophilic digester conditions and the survival of detectable known PAOs and GAOs was investigated using both 16S rRNA amplicon sequencing and fluorescent in situ hybridization (FISH). The results showed that *Tetrasphaera*, *Dechloromonas*, *Ca.* Accumulibacter and the polyphosphate containing organism *Ca.* Microthrix all decreased under digester conditions, but that 16S rRNA amplicon sequencing detected cells when they could not be detected with FISH. Both meso-and thermophilic digester conditions affected the PAOs ability to take up substrate negatively and P was released without an apparent uptake of substrate. Especially *Ca.* Accumulibacter was very sensitive to an increase in temperature when transferred to digester conditions and released all P instantly, when transferred to thermophilic digester conditions. The studies also showed that higher temperature resulted in faster and higher P release and an increased risk of P precipitation after a shorter period of anaerobic incubation. Room temperature anaerobic incubation without substrate addition lead to P-release comparable to the those observed under digester conditions. Therefore, prolonged anaerobic incubations in storage tanks before digesters should be taken into consideration, since it could cause P-release and precipitation in pipes, leading to operational problems. From these studies it was suggested that part of the P-recovery from WWTPs could be moved from the digesters and into storage tanks, placed before the digester, to minimize precipitation in pipes, precipitation in the digesters and increase dewaterability of the sludge before the digester.

In the P mass balance study (Petriglieri et al., 2019), the different pools of P found in a WWTP was investigated and it was found that most P was chemically bound and may not be available for P-recovery. A big fraction of the P was also found to be contained as poly-P in PAOs, primarily *Ca.* Accumulibacter, *Tetrasphaera* and *Dechloromonas*. Up to 50% of poly-P was stored in microorganisms not yet identified.

DANSK RESUME

Fosfor (P) er en ikke fornybar ressource og bør genindvindes når der er muligt. Et sted P kunne genindvindes kunne være fra rådnetanke på renseanlæg som, afhængig af størrelsen på anlægget, modtager store mængder af P hver dag. Poly-fosfat akkumulerende organismer (PAOer) er organismer der bliver udnyttet i den biologiske forfor fjernelses (EBPR) proces i renseanlæg og disse mikroorganismer, der lagrer P, overføres fra aktivt slam til rådnetanke hvor P frigives.

I dette studie blev det undersøgt, hvordan overførslen fra aktivt slam til rådnetanke ville påvirke overlevelse, substrat optag og P frigivelse fra kendte PAOer. En massebalance over de forskellige P fraktioner fundet i renseanlæg blev også lavet for at få en bedre forståelse for hvilke fraktioner der var til rådighed og en indikation af hvor meget P var opbevaret i kendte PAOer. Alt dette blev gjort for at opnå en bedre forståelse og optimering af genindvinding af P fra renseanlæg.

Overlevelsen af PAOer overført fra aktivt slam til mesofile og termofile rådnetanke blev undersøgt ved kortlægning af danske renseanlæg (Hansen et al., 2019b, 2019a). Kortlægningen sammenlignede forekomsten af PAOer og potentielt konkurrerende glykogen akkumulerende organismer (GAOer) i aktivt slam med forekomsten i efterfølgende meso- og termofile rådnetanke. Kortlægningen viste fald i forekomst ved næsten alle mikroorganismer undersøgt med få undtagelser der viste en stigning i forekomsten i rådnetankene. For at bekræfte disse resultater, blev lang tids batcheksperimenter udført. Aktivt slam med høj forekomst af *Tetrasphaera* PAO og beriget slam med *Ca.* Accumulibacter PAO, blev inkuberet under forhold der efterlignede meso-og termofile rådnetanks forhold og overlevelsen af kendte PAOer og GAOer blev undersøgt med både 16S rRNA amplicon sekventering og fluorescerende in situ hybridisering (FISH). Resultaterne viste at *Tetrasphaera*, *Dechloromonas*, *Ca.* Accumulibacter og den polyfosfatholdige *Ca.* Microthrix alle faldt i forekomst under rådnetanks forhold, men at 16S rRNA amplicon sekventering detekterede celler som ikke blev detekteret med FISH. Både meso-og thermofile rådnetanks forhold påvirkede PAOers evne til at optage substrat negativt og P blev frigivet uden nogen synlig optag af substrat. Især *Ca.* Accumulibacter var meget sensitiv over for temperatur forøgelsen, ved overførsel til rådnetanks forhold og frigav alt P med det samme, ved overførsel til termofil rådnetanks forhold. Studierne viste også at højere temperaturer, resulterede i hurtigere og højere P frigivelse samt en højere risiko for P udfældning, efter en kortere periode med anaerob inkubering. Rumtemperatur anaerob inkubering uden substrat tilsætning førte til P frigivelse der var sammenlignelig med dem observeret under rådnetanks forhold og forlænget anaerob inkubering i opbevarings tanke før rådnetankene, skal derfor tages med i overvejelserne, da dette kan lede til P-frigivelse og udfældning i rør og dermed skabe operationelle problemer. Fra disse studier blev det foreslået at P-genindvinding bør flyttes fra rådnetankene hen til opbevaringstankene, placeret direkte efter beluftningstankene, for at minimere udfældning i rør, udfældning i rådnetankene og give bedre afvanding af slammet til rådnetankene.

I P massebalance studiet (Petriglieri et al., 2019), blev de forskellige P fraktioner i renseanlæg undersøgt og det meste P fundet som kemisk bundet P og er derfor muligvis ikke til rådighed for genindvinding. En stor fraktion af P blev også fundet som poly-P i PAOer, hovedsageligt *Ca.* Accumulibacter, *Tetrasphaera* og *Dechloromonas*. Op til 50% af poly-P var i POAer endnu ikke identificeret.

ACKNOWLEDGEMENTS

In completing the requirements for obtaining a Doctor in Philosophy (PhD) degree, this dissertation was written. The dissertation is written as a collection of articles with an introduction on the subject and as well as a conclusion and perspectives.

The study was financed by the ReCoverP project funded by Innovation Fund Denmark and carried out at Center for Microbial Communities at Aalborg University.

I would like to thank my supervisor Per Halkjær Nielsen, for this opportunity to work on a project I personally find to be important for our environment and our future. It has been a great experience to participate in this project, given me lot of great memories from conferences and social gatherings and thought me to have pride in my work. Thank you!

I would also like to thank all the people working on the ReCoverP project. It has been a pleasure to share and discuss my research with all of you.

A big thank you to all my wonderful colleagues at Aalborg University. You have provided me with a network of fantastic people I could discuss everything with and shared my joy and frustrations on this project. Special thanks to Marta Nierychlo for always helping me with my over the top experimental setups, Marianne Stevenson and Susanne Bielidt for your help in the lap and motherly care and Kasper Anders for R support and patience with my stupid questions. A big thank you should also be extended to Aleksander Lie and Lars Pedersen for their help and guidance in maintaining the IC I was responsible for. Thanks to Jytte Dencker for her huge help with running the ICP. I would also like to thank Jette Petersen and Francesca Petriglieri for help with FISH analysis and mass-balance calculations. It is always more fun to do these things, when you have good people to discuss the results with.

I would like to thank all the people I have shared an office with the last four years, you have all been wonderful.

I would also like to thank my friends for being there for me: Nadieh, Malwina, Katie and Giulia, thanks for the nice talks, dinners and lunches, it has helped a lot in stressed times. Thank you Susan for always being caring, sharing your witty remarks and fantastic stories with me. Thanks to Ea and Camilla for being with me, from the start of our university journey and always know, you mean the world to me.

The biggest thanks go to my family, my wonderful husband Bjørn and daughter Alice that have supported me through this whole process. Your love knows no boundaries.

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OBJECTIVES OF THE PHD PROJECT

This project was a part of the ReCoverP project funded by Innovation Fund Denmark and took place from 2015 to 2019, in cooperation with universities and companies. The overall goal of the PhD project was to investigate the fate of known key PAOs when transferred from activated sludge, into meso- and thermophilic digesters and what would happen in regard to survival, phosphorus release and activity, with the goal of recommending optimization in regard to phosphorus recovery. The different P species in activated sludge was investigated to get a better understanding of the different P pools, available for recovery.

The specific objective of the papers in this thesis:

Paper 1	To describe the fate of known PAOs when transferred from activated sludge to thermophilic digesters, and how this affects the survival of the PAOs, the phosphorus release and the activity.
Paper 2	To describe the fate of known PAOs when transferred from activated sludge and into mesophilic digesters, and how it affects the survival of the PAOs, the phosphorus release and the activity.
Paper 3	To get a better understanding of the phosphorus distribution in activated sludge and elucidate how much of the phosphorus could be attributed to known PAOs, unknown PAOs, bound in inorganic and organic form.

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
EBPR	Enhanced biological phosphorus removal
ED	Entner Doudoroff
EMP	Embden–Meyerhof–Parnas
FISH	Fluorescence *in situ* hybridization
fGAO	Fermenting Glycogen-Accumulating organisms
fPAO	Fermenting Polyphosphate-Accumulating organisms
GAO	Glycogen-accumulating organisms
P	Phosphorus
PAO	Polyphosphate-accumulating organisms
PHA	Poly-β-hydroxyalkanoate
PHB	Poly-β-hydroxybutyrate
PHV	Polyhydroxyvalerate
Pit	Inorganic phosphate transporter
PMF	Proton motor force
Poly-P	Polyphosphate
Pst	Phosphate transport system
rRNA	Ribosomal ribonucleic acid
SS	Suspended solids
TCA	Tricarboxylic acid cycle
VFA	Volatile fatty acids
WWTPs	Wastewater treatment plants

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LIST OF SUPPORTING PAPERS

Papers included in the thesis:

Paper 1, Appendix A:

The fate of microorganisms involved in biological phosphorus removal in thermophilic digesters and the impact on survival, internal P storage and phosphorus recovery.

Kamilla A.S. Hansen, Marta Nierychlo, Jette Fisher Petersen, Adrian Oehmen, Simon McIlroy, Per Halkjær Nielsen

Paper 2, Appendix B:

The fate of microorganisms involved in biological phosphorus removal in mesophilic anaerobic digesters and the impact on phosphorus recovery.

Kamilla A.S. Hansen, Marta Nierychlo, Jette Fisher Petersen, Adrian Oehmen, Simon McIlroy, Per Halkjær Nielsen

Paper 3, Appendix C:

Quantificatioin of biologically and chemically bound P in activated sludge from EBPR plants.

Francesca Petriglieri, **Kamilla A. S. Hansen**, Jette Fisher Petersen, Marta Nierychlo, Miriam Peces, Cecilie E. Baastrand, Kasper Reitzel and Per Halkjær Nielsen

GENERAL INTRODUCTION

PHOSPHORUS A LIMITED RESOURCE

Phosphorus is a valuable and limited resource, essential for the production of fertilizer and thereby food. It has been pointed out by media and the scientific community, that phosphorus can cause eutrophication in aquatic environments when applied to fields, but the coming shortage of fertilizer has not been much debated. Current estimates indicate that in 50-100 years all phosphorus that can be extracted from mineswill be used (Cordell et al., 2009; Gilbert, 2009; Melia et al., 2017). Another problem is the lack of political control with the phosphorus mines, and currently the biggest mines are in Morocco and Western Sahara, with high political tension.

Different initiatives have been suggested to minimize the consumption of phosphorus, these include: banning phosphorus in detergents, minimizing food waste, recovering phosphorus from food waste, and recovering phosphorus from waste such as wastewater. Recovering phosphorus form wastewater has certain benefits: it will help meet legislations, reduce eutrophication in receiving water bodies, may be an income for the wastewater treatment plants, and minimize the need for mining of phosphorus.

Different techniques have been developed to recover phosphorus: sludge can be burned and phosphorus recovered chemically, chemicals can be used on the wet sludge, and lastly the sludge can be used directly as a fertilizer on the fields (Environmental Protection Agency, 2013). One of the problems with using this fertilizer directly on the fields is the risk of polluting the soil with different contaminants: heavy metals, pesticides, pharmaceuticals, nanoparticles, hormones, drugs and pathogens. The risk of introducing some of these into the fields have led to public concern and in many countries, using sludge as a fertilizer is illegal (Melia et al., 2017).

Another way to recover phosphorus from wastewater centers around the enhanced biological phosphorus removal (EBPR) process in combination with utilization of digesters.

PHOSPHORUS RECOVERY AND SLUDGE HANDLING

Different approaches are used when it comes to utilizing the phosphorus in wastewater sludge. In Denmark it is common practice to use digested sludge from WWTPs as a fertilizer directly on the fields (Jensen and Jepsen, 2005), but in most countries, such as Germany and the United States, this practice is banned. Rising concern regarding ground water pollution and the risk of pathogens and pharmaceuticals in the crops is pressing the WWTPs to find other solutions to handle the sludge. In Germany and in WWTPs in Denmark where the sludge is considered too contaminated to be distributed on fields, the sludge is incinerated and either used as landfill or stored temporarily. Different methods can be used to extract the phosphorus from the ashes including chemical extraction and electrical separation (Schaum et al., 2007; Viader et al., 2017). Common for both methods is that they require chemicals to dissolve the ashes, which is costly and the methods are not yet ready for full scale plants. Enhanced biological phosphorus removal in combination with digesters is another more economical way of removing and potentially recovering phosphorus.

ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL AND DIGESTERS

Removal of phosphorus from the liquid phase of wastewater, without use of chemical precipitations, is achievable through the enhanced biological phosphorus removal (EBPR) process. In activated sludge, microorganisms able to take up and store phosphorus as poly-phosphate, poly-phosphate accumulating organisms (PAO), remove the phosphorus from the liquid phase and store it in the biomass. The biomass can later be removed and the water discharged to receiving water bodies without the risk of eutrophication. The process is an asset to the wastewater treatment plant since it reduces the cost of removing phosphorus from the wastewater without the addition of costly chemicals. By trapping phosphorus in the biomass, potential recovery of the phosphorus is also possible. It is estimated that 15-25% of the phosphorus demand in Denmark can be met if phosphorus was to be recovered from Danish WWTPs (Environmental Protection Agency, 2013).

The EBPR process utilizes shifts between anaerobic and aerobic zones, to remove P in the liquid phase of wastewater (Fig. 1.1). The anaerobic tank, often placed right after the first settling step, is where PAO take up volatile fatty acids (VFAs) and replenish polyhydroxyalkanoate (PHA) storage. In the subsequent aerobic tank, PHA is converted to energy and poly-P and glycogen is formed. After secondary settling, some of the P-rich sludge is transported back to the anaerobic tank, to seed with PAO, and the rest is transported to the anaerobic digester.

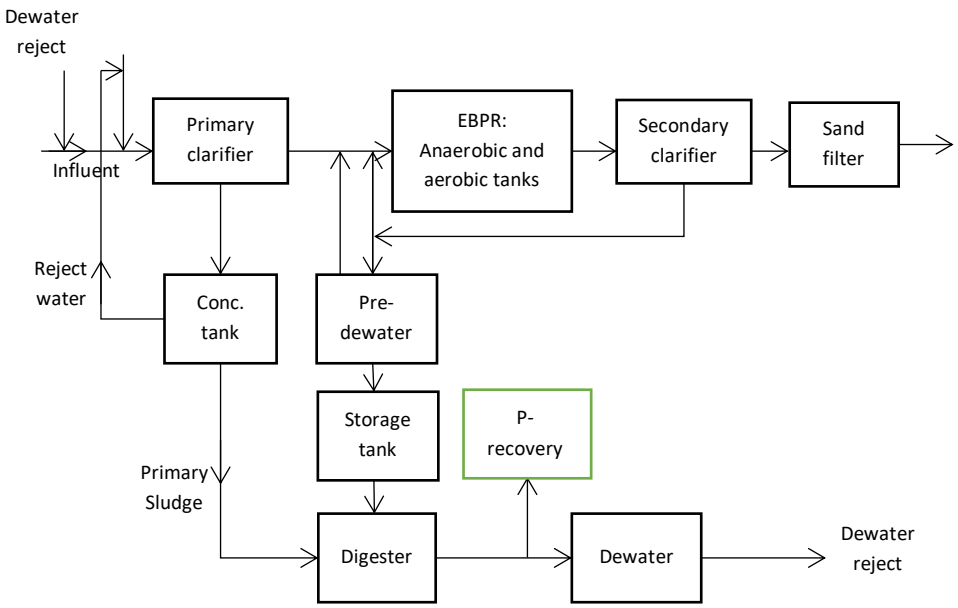

Figure 1.1: Schematic overview of a WWTP.

Anaerobic digestion is a process in which sludge is stabilized, the sludge volume is reduced, and biogas is produced (Nizami, 2012). It also helps reduce the amounts of pathogens in the sludge and reduce odors (Appels et al., 2008). When sludge from the EBPR process is transferred to the digester, P is transferred from the biomass into the liquid phase, which results in reject water with high phosphorus content (Xavier et al., 2014). The reject water can then be used in commercial processes, which extract the phosphorus and produce products that can be sold by the WWTPs, creating value (further described on page 30).

PAOS, GAOS AND PHOSPHORUS RECOVERY

Two of the most studied types of microorganisms related to the EBPR process are PAOs and the glycogen accumulating organisms (GAOs). PAO are responsible for the removal of P from the liquid phase of wastewater in the EBPR process and take up different substrates such as VFAs to facilitate the uptake of P. GAO do not take up P and store it as PAOs, but they do utilize some of the same substrate as PAO and could therefore be competing with PAO. The effect of transferring PAOs and GAOs from the EBPR process into digesters has not been studied and the effect on survivability, substrate competition, and P-release could be important for optimal P-recovery from digesters.

POLYPHOSPHATE ACCUMULATING ORGANISMS

The classic definition of a PAO is a microorganism able to take up P and store it as poly-P under aerobic conditions and under anaerobic conditions take up organic carbon from the liquid phase and store it in the form of PHA, with the energy from poly-P and glycogen consumption (Comeau et al., 1986). It has now been suggested that some PAO are able to ferment substrates more complex than VFAs and they have been termed fermenting PAO (fPAO) (McIlroy et al., 2018a). These microorganisms are the workhorses of the EBPR process and how they function is of great importance when discussing EBPR and later recovery of P from digesters.

PAO METABOLISM

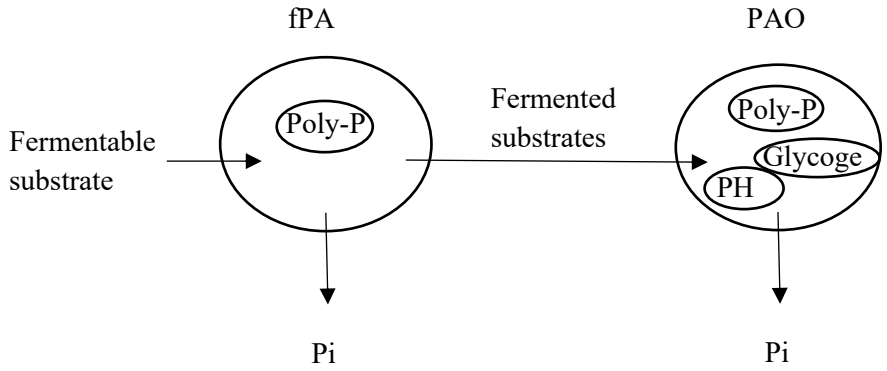

Figure 2.1: Simplified model of fPAO and PAO interaction and metabolism. Inspired by (Nielsen et al., 2019).

In the WWTP classic-PAOs take up carbon substrates under anaerobic conditions, in the form of e.g. simple VFAs and store them as intercellular storage compounds. These compounds known as PHAs are polymers produced under anaerobic conditions when carbon sources are plentiful (Mino et al., 1998). The amount of polymer stored in the cells varies between 30-80% of the total dry weight of the cells (Brandl et al., 1990) and the type of PHA produced is dependent on the type of substrate available and the PHA synthase present in the microorganism. The presence of PHA in a cell can be examined using Nile Red for staining and visualization by microscopy (Spiekermann et al., 1999) and Raman spectroscopy can be used to quantify the amount of PHA in the cell (Fernando et al., 2019a). The most studied and simplest PHA is poly-β-hydroxybutyrate (PHB) (Khanna and Srivastava, 2005), which is formed from two acetyl-CoA molecules and the second simplest molecule is polyhydroxyvalerate (PHV) formed from one molecule acetyl-CoA and one molecule propionyl-CoA (Mino et al., 1998). The energy needed for the transformation of substrate into PHA comes from the consumption of poly-P and partly from the degradation of glycogen.

The pathway used for reducing power for the formation of PHA has been discussed for some years, and is still ongoing. The two original models used, the Comeau/Wentzel model (Comeau et al., 1986; Wentzel et al., 1986) and the Mino model (Mino et al., 1987) have discussed whether the tricarboxylic acid cycle (TCA) cycle is active under the process. The Comeau/Wentzel model suggested that the TCA cycle was actively used, while Minos model suggested that the TCA cycle was inactive, and that reducing equivalents were made from degradation of glycogen. Later research have confirmed that glycogen is used, but that some organisms may be able to utilize the TCA circle fully, partially, or in split mode (He and McMahon, 2011).

The pathway which has been used for the production of reducing equivalents through glycogen has also been discussed. The two pathways suggested have been the Embden–Meyerhof–Parnas (EMP) pathway and the Entner Doudoroff (ED) pathway. The amount of energy produced from the two pathways differ, EMP (3 ATP) and ED (2 ATP), and the pathway available will have impact on the amount of energy available for the cell (He and Mcmahon, 2011).

The ability to transport acetate into the cells at the cost of P comes from the presence of P transporters. The inorganic phosphate transporter (Pit) is a transporting protein, which sends P over the cell membrane along with a stabilizing metal ion (Mg^{2+}) and a proton (H^{+}). This generates a proton motor force (PMF) that can be used for the transport of acetate into the cell. Another way to transport P in and out of the cell is through the high affinity specific phosphate transport system (Pst). The Pst system is composed of several proteins and requires ATP to transport P, it is a far more complex system than the Pit transporter (Hendrik, 1997; Saunders et al., 2007).

As PAO is transferred to anoxic/aerobic conditions, the PHA stored under anaerobic conditions is oxidized to produce ATP and the energy from ATP is used for production of biomass and replenishment of poly-P and glycogen (Smolders et al., 1994).

DIFFERENT PAOS

Candidatus Accumulibacter is an example of a classical PAO, which has been believed to be responsible for most of the P removal in EBPR systems. *Ca.* Accumulibacter is able to take up substrates such as acetate, propionate, pyruvate, glutamic acid, store them as PHA and form glycogen (Kong et al., 2004). The limited number of substrates *Ca.* Accumulibacter is able to take up makes them dependent on other microorganisms able to ferment, such as *Tetrasphaera*. *Ca.* Accumulibacter has been reported to be present in EBPR systems in different abundance, from 1.0-10.8% of bio-volume in Danish WWTPs (Nielsen et al., 2010) to 13-18% in Australian EBPR systems (Zilles et al., 2002). The difference in *Ca.* Accumulibacter abundance could be found in the use of different probes for FISH, and it is recommended that the genus FISH probe PAO651 is used, since overestimation of *Ca.* Accumulibacter has been attributed to the traditional probes PAOmix also targeting the GAO *Propionvibrio* (Albertsen et al., 2016; Gregory R. Crocetti et al., 2000). *Ca.* Accumulibacter has been separated into two major groups (I and II) based on the sequenced polyphosphate kinase 1 (ppk1) gene (He et al., 2007). These two genotypes are suggested to be able to take up P along with doing denitrification. Clade I is only able to reduce nitrate along with taking up P, while clade II is able to convert nitrite and take up P.

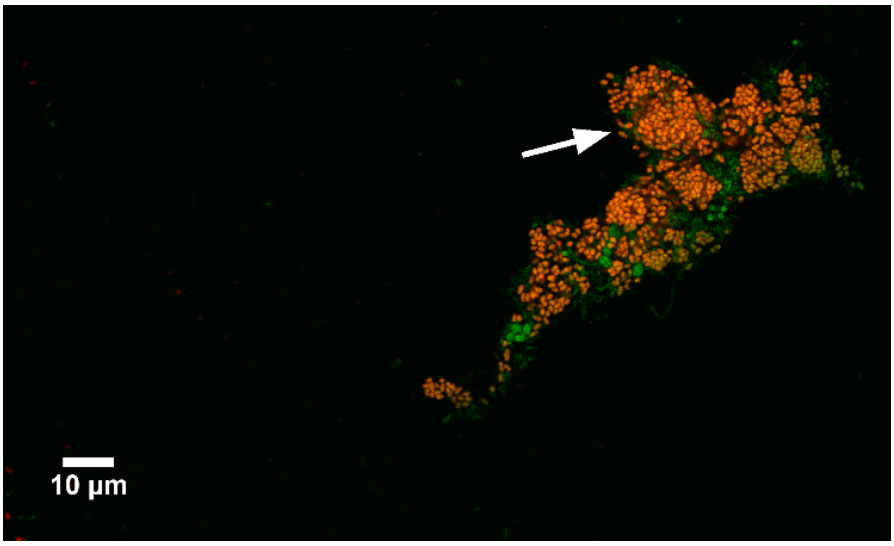

Figure 3.1: FISH image of *Ca.* Accumulibacter. Orange cells are *Ca.* Accumulibacter and green cells are other cells in the biomass.

Tetrasphaera is a non-classical PAO able to store phosphate, but does not produce the storage compounds PHA and glycogen (Fernando et al., 2019a). In Danish WWTPs *Tetrasphaera* is the most abundant PAO and is observed to constitute up to 20-30% of the microbial community based on both amplicon sequencing and FISH analysis (Herbst et al., 2019; Kristiansen et al., 2013). In a survey of 32 full-scale EBPR plants, divided over 32 countries, it was shown that Tetrasphaera was more abundant than *Ca.* Accumulibacter in most plants (Nielsen et al., 2019). *Tetrasphaera* is able to ferment (Herbst et al., 2019) and could be producing products, e.g. acetate, that could be used by classic PAOs such as *Ca.* Accumulibacter (Nielsen et al., 2010). *Tetrasphaera* also have the advantage over classic PAO that they are able to grow under anaerobic conditions (Nielsen et al., 2019). Substrates that *Tetrasphaera* is able to take up include amino acids and glucose (Ricardo Marques et al., 2017). Besides being able to ferment, *Tetrasphaera* is different from classic PAO in its lack of storage compounds. Tetrasphaera is not able to store PHA (Kong et al., 2005) and they do not store glycogen, even though they have the metabolic potential for it (Fernando et al., 2019a). *Tetrasphaera* has, like *Ca.* Accumulibacter, the genetic potential to do nitrification (Kristiansen et al., 2013; Marques et al., 2018).

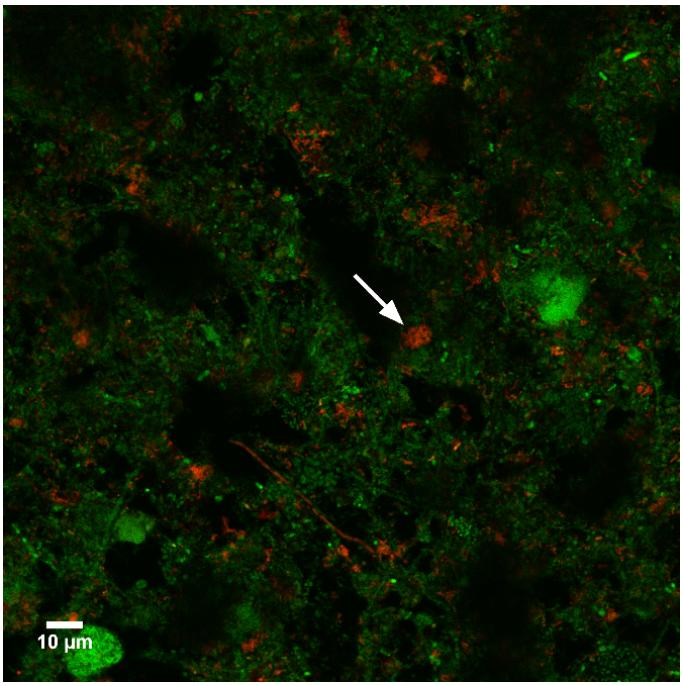

Figure 4.1: FISH image of *Tetrasphaera* cells. Orange cells are *Tetrasphaera* and green cells are other cells in the biomass.

Other putative PAO include *Dechloromonas*, *Tessaracoccus*, *Candidatus* Obscuribacter and *Microlunatus*. *Dechloromonas* and *Tessaracoccus* are often found in rather high abundance in WWTPs (Nielsen et al., 2019; Stokholm-Bjerregaard et al., 2017).

Another microorganism that is not considered a PAO, but is taking up P, is *Ca.* Microthrix. *Ca.* Microthrix is a filamentous bacteria often associated with foaming and bulking problems in WWTPs (Rossetti et al., 2005). *Ca.* Microthrix store poly-P but they do not cycle it under anaerobic/ aerobic conditions like PAOs. It has been suggested that *Ca.* Microthrix use it to supply energy under anaerobic conditions for the uptake and storage of lipids (Blackall et al., 1995; McIlroy et al., 2013). Other microorganisms could potentially be storing P, but these are so far unknown.

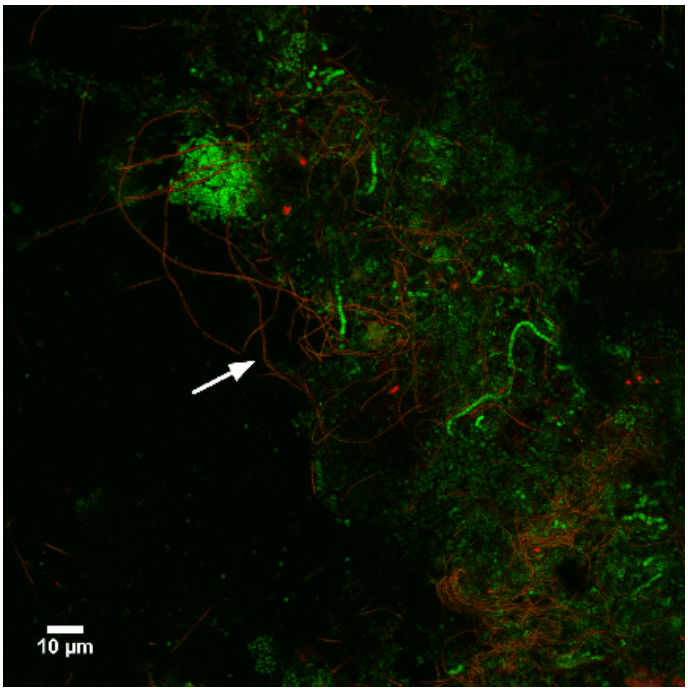

Figure 5.1: FISH image of *Ca.* Microthrix cells. Orange cells are *Ca.* Microthrix and green cells are other cells in the biomass.

GLYCOGEN ACCUMULATING ORGANISMS

Microorganisms able to take up glycogen and other substrates found in the WWTP, but not cycle P are known as glycogen accumulating organisms (GAO). These microorganisms do not store poly-P, cannot make a PMF from transporting P out of the cells, and therefore get most of their energy from glycogen (Oehmen et al., 2007). It is believed that these microorganisms are competing with PAO for substrate and could potentially take important substrate from PAO in the digesters, if both PAO and GAO survive the transition to digesters, thereby minimizing the amount of substrate ready to be traded for internal poly-P.

GAO METABOLISM

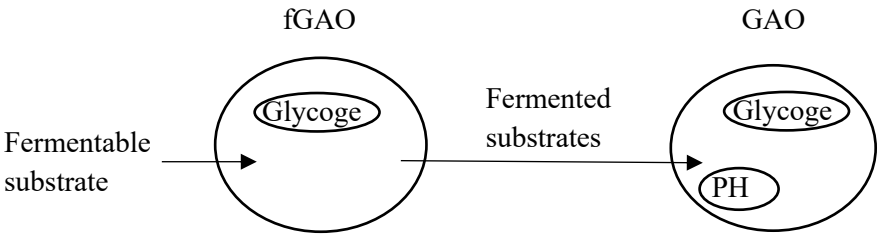

Figure 6.1: Simplified model of fGAO and GAO interaction and metabolism. Inspired by (Nielsen et al., 2019).

Besides the lack of poly-P accumulation, GAO metabolism is very similar to PAO metabolism. Under anaerobic conditions VFAs e.g. acetate is taken up and transformed into acetyl-CoA along with the degradation of glycogen to produce propionyl-CoA. These two compounds are condensed and transformed into PHA (Zeng et al., 2002). Under aerobic conditions PHA is oxidized, glycogen in restored and biomass is produced (Liu et al., 1994; Zeng et al., 2003). The lack of poly-P storage in GAO make them unable to utilize it for building up a PMF, this is instead done by efflux of protons through ATPase in the membrane (Saunders, 2005).

DIFFERENT GAOS

A survey carried out by Nielsen et al., (2019), shows the abundance of different GAOs, distributed over 12 countries and 5 continents. The two genera *Micropruina* and *Defluviicoccus* were most abundant, with *Ca.* Competibacter coming in at fourth place. The genus *Competibacter* is a classic GAO able to store both PHA and glycogen (Kong et al., 2006; Oehmen et al., 2004). Studies do not seem to agree on the use of the EMP pathway for glycolysis or the ED pathway. A newer study by Mcilroy et al., (2013) have shown that some *Ca.* Competibacter species have the genomic potential to use both the EMP and the ED pathway, but that some genes for the transformation of products from the glycogen catabolism to the start products in the ED pathway was missing. Further studies using e.g. proteomics are needed to elucidate this.

Micropruina, the most abundant GAO in Danish WWTPs, is a fermenting GAO with the ability to take up sugars and amino acids and produce VFAs such as acetate and lactate, that can be used by non-fermenting GAO and PAO. Like the fermenting PAO *Tetrasphaera* it does not store PHA and glycogen (McIlroy et al., 2018b). It is able to utilize acetate, propionate, pyruvate, and succinate and grow at 20-30°C (Shintani et al., 2000).

Defluviicoccus is the third most abundant GAO in Danish WWTPs with an average relative 16S rRNA abundance of 0.5%, but was found to constitute up to 13% (Stokholm-Bjerregaard et al., 2017). *Defluviicoccus* is able to produce PHA and glycogen and is able to take up acetate and glucose (Burow et al., 2007; Nobu et al., 2014; Wong and Liu, 2007). Several studies have shown *Defluviicoccus* present in failing EBPR systems and even though most *Defluviicoccus* clusters are tetrad forming organisms (TFO), cluster III have been shown to form filaments and could potentially be causing foaming problems in digesters (McIlroy and Seviour, 2009; Nittami et al., 2009). *Ca.* Propioninvibrio was determined to be a GAO in the study by Albertsen et al., (2016). *Ca.* Propionivibrio is a classic GAO able to synthesize both glycogen and PHA. It has a fermentative metabolism and is able to produce both acetate and propionate. In activated sludge they have been shown to constitute up to 3% of the bio-volume measured with FISH and up to 0.3% relative abundance measured with 16S rRNA amplicon sequencing, in a survey of 12 different countries (Nielsen et al., 2019). *Ca.* Propionivibrio may have caused overestimation of the PAO *Ca.* Accumulibacter, since the two 16S rRNA gene sequences are 97% identical. This has caused FISH probes meant for *Ca.* Accumulibacter to hybridize with *Ca.* Propionivibrio, and *Ca.* Accumulibacter has thus been overestimated (Albertsen et al., 2016).

PAOS IN DIGESTERS

How PAOs react to the transfer from activated sludge and into digesters will potentially affect how much P is available for recovery from the digesters. Not many studies have been carried out regarding PAOs in digesters Kirkegaard et al. (2017) did a survey of 32 full-scale mesophilic and thermophilic digesters in Denmark and could show that PAO *Tetrasphaera,* along with poly-P containing *Ca.* Microthrix, was present in high abundance in the digesters. They also showed that digesters with thermal pre-treatment did not contain these microorganisms, indicating that they are not native to digesters but were coming with the feed. Findings in this

study (Hansen et al., 2019a, 2019b) supported these results and showed that both *Tetrasphaera* and *Ca.* Microthrix decreased in abundance when activated sludge was exposed to digester conditions. There was some discrepancy between 16S rRNA amplicon results and FISH results, where amplicon results showed positive signal under thermophilic digester conditions for both *Tetrasphaera* and *Ca.* Microthrix, but no signal was observed with FISH. The same trend was observed with *Ca.* Accumulibacter at thermophilic temperature (Hansen et al., 2019a) and it was seen that the signal from *Ca.* Accumulibacter disappeared when anaerobic sludge was added to the activated sludge, indicating that the signal observed with amplicon sequencing was from DNA contained in dead cells or free DNA that could be digested by anaerobic microorganisms. This finding stresses the importance of validating the amplicon results with other methods such as FISH.

Regarding PAO activity in the digesters, no apparent substrate uptake could be seen with the release of P in these experiments (Hansen et al., 2019b, 2019a). This would indicate that the PAO are not actively cycling P for substrate, but releasing the P either as a stress response or as they are dying and being degraded.

ACTIVATED SLUDGE COMPOSITION AND DIGESTER TRANSFORMATIONS

The quantity of P available for recovery is dependent on many factors with the first being how much P is present in the sludge. In incoming wastewater the concentration of P can range from 6-25 g P/ m^3 and is up-concentrated in the activated sludge and the digesters (Mogensen and Comeau, 2008). In Table 1.1 the amount of P found in activated sludge in four different EBPR plants in Denmark is listed and varies between 36-50 mg P/ g SS (Petriglieri et al., 2019). And the amount of P found in digester supernatant can vary between 15-300 g P/ m^3 (Mogensen and Comeau, 2008).

Table 1.1: Concentration of total-P and Mg in activated sludge in Danish EBPR systems (unit in mg/ g SS). Modified from (Petriglieri et al., 2019).

WWTP	Total-P	Mg
Lynetten	37.10 ± 1.74	13.44 ± 1.55
Ejby Mølle	35.85 ± 2.87	10.18 ± 1.92
Viby	45.18 ± 2.26	8.64 ± 0.28
Aalborg West	49.68 ± 1.89	14.09 ± 0.43

The amount of P being stored in the activated sludge also depends on the abundance of PAO and which PAOs are present in the activated sludge. From this study is was shown that up to 50% of P was found in known PAOs while some PAO are still unknown and further research is needed. It was also shown that up to almost 60% of P is stored as inorganic P, and may therefore not be available for recovery.

PHOPHORUS RECOVERY SETUP

When the activated sludge enters the digester (mesophilic and thermophilic) P is released from the PAOs transferred from the activated sludge, and the released P is then available to react with other chemical components in the digesters (Hansen et al., 2019b, 2019a). Under the right conditions P reacts in equal parts with magnesium (Mg^{2+}) and ammonium (NH_4) to form struvite ($MgNH_4PO_4 \cdot 6H_2O$). Different commercial setups are available for P recovery from digesters and many produce stuvite as a final product (Table 1.2).

Table 2.1: Commercial systems used for recovery of P from digester reject-water. Adjusted from (Environmental Protection Agency, 2013).

Process name	Product	Placement of unit
NuReSys®	Struvite fertilizer	After digester
Ostara's Pearl® process	"Crystal Green®" fertilizer	After digester
Phosnix®	Struvite fertilizer	After digester
Airprex®	Struvite fertilizer	After digester

The commercial setups have the drawback that the unit for recovery is placed after the digester. As was showed in (Hansen et al., 2019a, 2019b), this is not the optimal place to recover P, with problems such as increased risk of struvite precipitation in the digesters. From this study, it is suggested that a storage tank is placed right after the aeration tank and that activated sludge is stored under anaerobic thermophilic conditions a week or at 20°C for more than 3 weeks, before reject water is removed, for partial P recovery and dewatered sludge is moved to digester for further degradation and P-recovery.

CONCLUSION AND FUTURE PERSPECTIVES

From this PhD study, new knowledge was obtained on PAOs essential for the EBPR process and how transfer into meso- and thermophilic digesters affected survival, P-release, and substrate uptake. P-mass balances on PAO in the WWTPs was also conducted:

- Survey of mesophilic digesters in Denmark showed that PAOs and GAOs coming with the feed into the digesters do not survive. This was supported by the study of Hansen et al., (2019b) that showed a decrease in the PAOs investigated under mesophilic conditions with both 16S rRNA amplicon sequencing and FISH analysis.
- For the survey of Danish thermophilic digesters it was found that all PAO and GAO died upon entering the digesters, confirmed with results from Hansen et al., (2019a).
- In mesophilic batch experiment, it was shown that some PAO such as *Tetrasphaera* was able to withstand the increased temperature for some time and intact cells could still be found after almost 2 weeks of anaerobic storage. Other PAO such as *Dechloromonas* and *Ca.* Accumulibacter quickly decreased in abundance and was not detectable with FISH. In regard to P-release and substrate uptake, PAOs were not taking up acetate and P was released slowly as cells were degraded. Other P-containing species such as *Ca.* Microthrix were also observed to decrease in abundance through the experiment.
- In thermophilic batch experiments PAO were all dying and intact cells were not detected at day 2 of the experiment. *Ca.* Microthrix was also observed to quickly decrease in abundance.
- From the survey of mesophilic and thermophilic digesters, GAO was seen to decrease in abundance when going from activated sludge to digesters. The only GAO showing an increase in abundance was *Micropruina*. The increase in relative abundance could not be confirmed in the batch experiment, due to low abundance.
- In Hansen et al., (2019a, 2019b), it was showed that free DNA in samples can be a problem since methods such as 16S rRNA amplicon sequencing may sequence fragments of free DNA and give a positive signal for organisms not present as living cells. Other methods such as FISH should be used to verify the presence of whole cells, but to ensure that cells are still active, other methods such as microautoradiography - fluorescent in situ hybridization should be applied.
- From the P-mass balance it was found that a large fraction, almost 50%, of Poly-P is stored in PAOs still not identified. Up to 60% of total P was also found as inorganic P and may therefore not be available for P-recovery.

When designing WWTPs for the recovery of P, one should take into account the abundance of different key PAOs. From the study it was shown that P is released from the PAO under all conditions and that incubation time is key. If storage is needed before sludge enters the digesters, recovery of P could be done directly after the storage tank, before sludge enters the digester. If sludge is transported directly into digesters, the abundance of different PAOs should be taken into account along with the temperature of the digester. The P-mass balance showed that a large fraction of PAOs are still uncharacterized and further studies need to be conducted to identify them. This study also shows that 16S rRNA results should be supported by other independent techniques, such as FISH, to eliminate false positives from free DNA or DNA in dead cells.

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ISSN (online): 2446-1636
ISBN (online): 978-87-7210-571-0

AALBORG UNIVERSITY PRESS