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Master of Environmental engineering

Investigation of key operational factors impacting phosphorus removal and recovery from wastewater treatment plants

Dissertation for obtaining the Degree
of Doctor in Chemical and Biochemical Engineering

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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КЉУЧНА ДОКУМЕНТАЦИЈСКА ИНФОРМАЦИЈА[1]

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Име и презиме аутора:	Srđana Kolaković Oliveira Barreiros
Ментор (титула, име, презиме, звање, институција)	Doktor nauka, Maria Ascensão, Miranda Reis, redovni profesor, Fakultet za Nauku i Tehnologiju, Nova Univerzitet u Lisabonu, Portugalija Doktor nauka, Maja, Turk Sekulić, redovni profesor, Fakultet Tehničkih Nauka, Univerzitet u Novom Sadu, Srbija Doktor nauka, Adrian Michael, Oehmen, vanredni profesor, Univerzitet u Kvinslendu, Brizbejn, Australija
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Кључне речи / предметна одредница:	uklanjanje i rekuperacija fosfora, fosfor akumulišući organizmi, mikropolutanti, diklofenak, valorizacija otpada
Резиме на српском језику:	Uklanjanje nutrijenata i mikropolutanata, kao i rekuperacija resursa, neki su od glavnih izazova u oblasti tretmana otpadnih voda. Postrojenjima za prečišćavanje otpadnih voda potrebna su rešenja za efikasno prevazilaženje novonastalih problema i uspostavljanje održivog rada postrojenja. Iz navedenih razloga, fokus doktorske disertacije usmeren je na istraživanje nekih od glavnih izazova u oblasti tretmana voda, kao što su: i) parametri koji utiču na efikasnost procesa biološkog uklanjanja fosfora i strukturu mikrobne zajednice; ii) biotransformacija značajnog mikropolutanta (diklofenaka) u procesu unapređenog biološkog uklanjanja fosfora i iii) solubilizacija fosfora i ugljenika iz mulja sa postrojenja za prečišćavanje otpadnih voda u cilju rekuperacije resursa. Biološko uklanjanje fosfora je složen proces, u kome se za uklanjanje polutanta iz otpadne vode koriste specifični sojevi bakterija. Najvažniji organizam koji

[1] Аутор докторске дисертације потписао је и приложио следеће Обрасце:
5б – Изјава о ауторству;
5в – Изјава о истоветности штампане и електронске верзије и о личним подацима;
5г – Изјава о коришћењу.
Ове Изјаве се чувају на факултету у штампаном и електронском облику и не кориче се са тезом.

	akumulira polifosfate (*phosphate accumulating organisms - PAO*) je „*Candidatus* Accumulibacter phosphatis“, koji se sastoji od filogenetski različitih tipova, klasa i podklasa, gde određeni uslovi mogu uticati na metabolizam specifičnih sojeva *Accumulibacter*. Eksperimenti u šaržnom reaktoru, sa visokim rastom *Accumulibacter* bakterija (> 85 %), rađeni su više od godinu dana, pri čemu je dalja identifikacija na nivou podklase bila neophodna, kako bi se povezao specifični identitet grupe *Accumulibacter* sa posmatranim performansama reaktora. Brzina hranjenja ugljenikom pokazala se ključnom za ekspresiju različitih metabolizama, gde je brzo hranjenje dovelo do mešovitog metabolizma usvajanja polifosfata/glikogena, rezultujući neefikasnim uklanjanjem P (< 30 %), povezanog sa klasterima ii i iii *Accumulibacter* IIc podklase. Sa druge strane, mala brzina hranjenja ugljenikom dovela je do tipičnog metabolizma usvajanja polifosfata, koji je rezultirao potpunom separacijom fosfora kao rezultat metaboličkih procesa najbrojnijeg prisutnog klastera (klaster i) *Accumulibacter* IIc podklase. Rezultati ekperimentalnog rada u okviru teze pokazali su da određeni organizmi, trenutno prepoznati kao PAO organizmi, neefikasno uklanjaju fosfor i da postoji opravdana potreba za reklasifikacijom organizama unutar grupe *Accumulibacter*. Uklanjanje mikropolutanta dodatni je problem u domenu savremenih tretmana voda, naročito iz razloga što su neki mikropolutanti, poput diklofenaka, veoma rezistentni na tretmane u konvencionalnim postrojenjima za prečišćavanje otpadnih voda. Sa druge strane, određeni fizički/hemijski procesi rezultuju generisanjem produkata koji su još toksičniji od matičnog jedinjenja. Biotransformacija diklofenaka ispitivana je simultano sa biološkim uklanjanjem fosfora i procenjen je metabolički put i toksičnost formiranih nusproizvoda diklofenaka. Iako *Accumulibacter* ne razlaže diklofenak tako efikasno kao neki drugi sojevi bakterija (npr. nitrifikujuće bakterije), eksperimentalno je potvrđeno da i dalje doprinosi redukciji toksičnosti, proizvodnjom nusproizvoda manje toksičnosti. Takođe, utvrđena je korelacija između količine biotransformacije diklofenaka i tipa *Accumulibacter* bakterija, što potencijalno ukazuje da stimulacija rasta *Accumulibacter* tipa II može intenzivirati biorazgradnju diklofenaka u procesima prečišćavanja otpadnih voda. Na kraju, rekuperacija resursa jedna je od prioritetnih inicijativa u oblasti prečišćavanja otpadnih voda. Postrojenja za prečišćavanje otpadnih voda više se ne posmatraju samo kao postrojenja za prečišćavanje, već predstavljaju i mogućnost za rekuperaciju resursa i pružanje održivih rešenja za rad prečistača. Fosfor je nezamenljiv nutrijent i oskudan resurs koji je neophodno ponovno iskoristiti iz otpadnih tokova bogatih fosforom. Bioacidifikacija je testirana u ovoj tezi kao dodatni proces u okviru konvencionalnih postrojenja za prečišćavanje otpadnih voda i pokazalo se da ima potencijal da rastvori do 80 % fosfora i proizvede 0,35 g COD_{VFA}/g VS koji bi se mogli koristiti u kasnijim procesima za proizvodnju fosforom bogatih đubriva i bioplastike.
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	Члан: Doktor nauka, Maria Ascensão, Miranda Reis, redovni profesor, Fakultet za Nauku i Tehnologiju, Nova Univerzitet u Lisabonu, Portugalija Члан: Doktor nauka, Nídia Dana, Mariano Lourenço, senior istraživač, Fakultet za Nauku i tehnologiju, Nova Univerzitet u Lisabonu, Portugalija
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KEY WORD DOCUMENTATION[2]

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Subject, Key words:	phosphorus removal and recovery, enhanced biological phosphorus removal (EBPR), polyphosphate accumulating organisms (PAOs), micropollutants, diclofenac, waste valorisation
Abstract in English language:	Nutrient and micropollutant removal, as well as resource recovery, are some of the major current concerns in the wastewater treatment field. Wastewater treatment plants (WWTPs) need solutions to quickly tackle these emerging problems and operate as sustainably as possible. Therefore, this thesis focused on providing insight into some of the major challenges in these fields, such as: i) factors influencing efficient enhanced biological phosphorus removal (EBPR) and its microbial community structure; ii) the biotransformation of an important micropollutant (diclofenac) in the EBPR process and iii) phosphorus and carbon solubilisation from WWTP sludge for resource recovery. EBPR is a complex process where specific bacteria are used for phosphorus removal from the bulk liquid. The most important polyphosphate accumulating organism (PAO) is "*Candidatus* Accumulibacter phosphatis", that comprises of phylogenetically different Types, clades and subclades, whose metabolism can

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	be influenced by specific conditions. A reactor enriched with *Accumulibacter (*> 85 %) was operated for over a one-year period, and identification at the sub-clade level was necessary to correlate the specific identity of the *Accumulibacter* group with the observed reactor performance. The carbon feeding rate proved to be crucial for the expression of different metabolisms, where a fast-feeding rate resulted in a mixed phosphate/glycogen accumulating metabolism leading to poor P removal (< 30 %) that correlated with clusters ii and iii of *Accumulibacter* IIc, while a slow carbon feeding rate resulted in complete phosphorus removal and an abundance of cluster i. This work showed that some organisms commonly recognised as PAO do not promote efficient phosphorus removal and there is a need to recharacterize the organisms within the *Accumulibacter* group. Removal of micropollutants is another emerging concern, especially since some micropollutants such as diclofenac have been shown to be near-recalcitrant in conventional WWTPs. Additionally, some physical/chemical processes have been shown to produce transformation products that are more toxic than the parent compound. Diclofenac biotransformation was investigated in the EBPR process, where the metabolic pathway and toxicity of diclofenac by-products were also assessed. Although *Accumulibacter* did not appear to transform diclofenac as efficiently as other bacteria (e.g., nitrifiers), it was found to still contribute towards its detoxification. Furthermore, a correlation was observed between the quantity of diclofenac biotransformation and *Accumulibacter* Type, which could suggest that enrichment of Type II *Accumulibacter* can stimulate diclofenac biodegradation in wastewater treatment. Finally, resource recovery is one of the major initiatives in the wastewater treatment field. WWTPs are no longer seen as just treatment facilities, but also present opportunities to recover other added-value products and provide more sustainable solutions. Phosphorus is an irreplaceable nutrient and a scarce resource that must be recovered from phosphorus rich waste streams. Biological acidification was tested in this study as an additional step within conventional WWTPs and was shown to have great potential to solubilise up to 80 % of phosphorus and produce on average 0.35 g COD_{VFA}/gVS that could be used in subsequent processes for the production of phosphorus rich fertilizers or bioplastics, respectively.
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Thesis Defend Board: (title, first name, last name, position, institution)	President: PhD, João, Goulão Crespo, Full professor, NOVA School of Science and Technology, University Nova of Lisbon, Portugal Member: PhD, Maria Teresa, Pijuan Vilalta, Senior Researcher, Catalan Institute for Water Research (ICRA), Spain Member: PhD, Pedro Ricardo, Neto Póvoa, partner, Company Positive Benefits, Lda Member: PhD, Ramón, Barat Baviera, Associate professor, Polytechnical University of Valencia, Spain Member: PhD, Maria Ascensão, Miranda Reis, Full professor, NOVA School of Science and Technology, University Nova of Lisbon, Portugal Member: Nídia Dana Mariano Lourenço, Senior researcher, NOVA School of Science and Technology, University Nova of Lisbon, Portugal
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Investigation of key operational factors impacting phosphorus removal and recovery from wastewater treatment plants

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Hvala and obrigada!

Abstract

Nutrient and micropollutant removal, as well as resource recovery, are some of the major current concerns in the wastewater treatment field. Wastewater treatment plants (WWTPs) need solutions to quickly tackle these emerging problems and operate as sustainably as possible. Therefore, this thesis focused on providing insight into some of the major challenges in these fields, such as: i) factors influencing efficient enhanced biological phosphorus removal (EBPR) and its microbial community structure; ii) the biotransformation of an important micropollutant (diclofenac) in the EBPR process and iii) phosphorus and carbon solubilisation from WWTP sludge for resource recovery.

EBPR is a complex process where specific bacteria are used for phosphorus removal from the bulk liquid. The most important polyphosphate accumulating organism (PAO) is "*Candidatus* Accumulibacter phosphatis", that comprises of phylogenetically different Types, clades and subclades, whose metabolism can be influenced by specific conditions. A reactor enriched with *Accumulibacter (*> 85 %) was operated for over a one-year period, and identification at the sub-clade level was necessary to correlate the specific identity of the *Accumulibacter* group with the observed reactor performance. The carbon feeding rate proved to be crucial for the expression of different metabolisms, where a fast-feeding rate resulted in a mixed phosphate/glycogen accumulating metabolism leading to poor P removal (< 30 %) that correlated with clusters ii and iii of *Accumulibacter* IIc, while a slow carbon feeding rate resulted in complete phosphorus removal and an abundance of cluster i. This work showed that some organisms commonly recognised as PAO do not promote efficient phosphorus removal and there is a need to recharacterize the organisms within the *Accumulibacter* group.

Removal of micropollutants is another emerging concern, especially since some micropollutants such as diclofenac have been shown to be near-recalcitrant in conventional WWTPs. Additionally, some physical/chemical processes have been shown to produce transformation products that are more toxic than the parent compound. Diclofenac biotransformation was investigated in the EBPR process, where the metabolic pathway and toxicity of diclofenac by-products were also assessed. Although *Accumulibacter* did not appear to transform diclofenac as efficiently as other bacteria (e.g., nitrifiers), it was found to still contribute towards its detoxification. Furthermore, a correlation was observed between the quantity of diclofenac biotransformation and *Accumulibacter* Type, which could suggest that enrichment of Type II *Accumulibacter* can stimulate diclofenac biodegradation in wastewater treatment.

Finally, resource recovery is one of the major initiatives in the wastewater treatment field. WWTPs are no longer seen as just treatment facilities, but also present opportunities to recover other added-value products and provide more sustainable solutions. Phosphorus is an irreplaceable nutrient and a scarce resource that must be recovered from phosphorus rich waste streams. Biological acidification was tested in this study as an additional step within conventional WWTPs and was shown to have great potential to solubilise up to 80 % of phosphorus and produce on average 0.35 g

COD_{VFA}/gVS that could be used in subsequent processes for the production of phosphorus rich fertilizers or bioplastics, respectively.

Keywords: phosphorus removal and recovery, enhanced biological phosphorus removal (EBPR), polyphosphate accumulating organisms (PAOs), micropollutants, diclofenac, waste valorisation.

Resumo

A remoção de nutrientes e micropoluentes, bem como a recuperação de recursos, são algumas das principais preocupações atuais na área do tratamento de águas residuais. As estações de tratamento de águas residuais precisam de soluções para combater rapidamente esses problemas emergentes e operar da forma mais sustentável possível. Por esse motivo, o objectivo desta tese foi identificar alguns dos principais desafios nestas áreas, tais como: i) fatores que influenciam a remoção biológica eficiente de fósforo e respetiva composição da comunidade microbiana; ii) a biotransformação de um importante micropoluente (diclofenac) no processo de remoção biológica de fósforo e iii) solubilização de fósforo e carbono de lamas de tratamento de águas residuais com objectivo de recuperação de recursos.

A remoção biológica do fósforo é um processo complexo em que bactérias específicas são usadas para a remoção do fósforo da parte liquida. O organismo acumulador de polifosfato (PAO) mais importante é a "*Candidatus* Accumulibacter phosphatis", composta por diferentes tipos filogenéticos, *clades* e *subclades*, cujo metabolismo das diferentes espécies é influenciado por condições especificas. Neste trabalho, um reator enriquecido em Accumulibacter (> 85%) foi operado por um período superior a um ano, durante o qual a identificação de uma espécie , ao nível do *subclade*, foi feita de forma a correlacionar a identidade específica do grupo *Accumulibacter* com o desempenho do reator. A taxa de alimentação de carbono provou ser crucial para a expressão de diferentes metabolismos. Uma taxa de alimentação rápida resultou em um metabolismo misto de acumulação de fosfato/glicogênio, levando a uma baixa remoção de P (< 30%), o que se correlacionou com os clusters ii e iii, enquanto uma taxa de alimentação de carbono lenta levou a um metabolismo de acumulação de polifosfato típico, resultando na remoção completa do fósforo e de uma abundância do cluster i dentro da mesma *Accumulibacter* IIc. Este trabalho mostrou que alguns organismos habitualmente reconhecidos como PAO não promovem uma remoção eficiente de fósforo, verificando-se assim a necessidade de reclassificar os organismos que são PAOs dentro do grupo *Accumulibacter*.

A remoção de micropoluentes é outra preocupação emergente, especialmente porque alguns micropoluentes, como o diclofenaco, mostraram ser recalcitrantes em estações de tratamento de águas residuais convencionais. Foi demonstrado que alguns processos físicos/químicos produzem produtos de transformação mais tóxicos do que o composto original. A biotransformação do diclofenaco foi investigada simultaneamente com a remoção biológica do fósforo, onde a via metabólica e a toxicidade dos subprodutos do diclofenaco também foram avaliadas. Embora a *Accumulibacter* não pareça biotransformar o diclofenaco tão eficientemente quanto outras bactérias (por exemplo, nitrificantes), ainda assim contribui para a sua desintoxicação ao produzir subprodutos de menor toxicidade do que o próprio diclofenaco. Além disso, foi observada uma correlação entre a quantidade de biotransformação de diclofenaco e o tipo de *Accumulibacter*, o que pode sugerir que o enriquecimento de *Accumulibacter* do tipo II pode estimular a biodegradação do diclofenaco no tratamento de águas residuais.

Por fim, a recuperação de recursos é uma das principais iniciativas na área de tratamento de águas residuais. As estações de tratamento de águas residuais não são apenas vistas como estações de tratamento, apresentando também oportunidades de recuperação de outros produtos de valor acrescentado e de soluções mais sustentáveis. O fósforo é um nutriente insubstituível e um recurso escasso que deve ser recuperado de correntes de resíduos ricos em fósforo. A acidificação biológica foi testada neste estudo como uma etapa adicional a incluir em estações de tratamento de águas residuais convencionais e demonstrou ter o potencial de solubilizar até 80 % do fósforo e produzir 0,35 g COD_{VFA}/g VS, que poderá ser usado em processos subsequentes para a produção de fertilizantes ricos em fósforo ou bioplásticos, respetivamente.

Palavras-chave: remoção e recuperação do fósforo, organismos acumuladores do fósforo, micropolutantes, diclofenac, valorisação dos residuos

Apstrakt

Uklanjanje nutrijenata i mikropolutanata, kao i rekuperacija resursa, neki su od glavnih izazova u oblasti tretmana otpadnih voda. Postrojenjima za prečišćavanje otpadnih voda potrebna su rešenja za efikasno prevazilaženje novonastalih problema i uspostavljanje održivog rada postrojenja. Iz navedenih razloga, fokus doktorske disertacije usmeren je na istraživanje nekih od glavnih izazova u oblasti tretmana voda, kao što su: i) parametri koji utiču na efikasnost procesa biološkog uklanjanja fosfora i strukturu mikrobne zajednice; ii) biotransformacija značajnog mikropolutanta (diklofenaka) u procesu unapređenog biološkog uklanjanja fosfora i iii) solubilizacija fosfora i ugljenika iz mulja sa postrojenja za prečišćavanje otpadnih voda u cilju rekuperacije resursa.

Biološko uklanjanje fosfora je složen proces, u kome se za uklanjanje polutanta iz otpadne vode koriste specifični sojevi bakterija. Najvažniji organizam koji akumulira polifosfate (*phosphate accumulating organisms - PAO*) je „*Candidatus* Accumulibacter phosphatis“, koji se sastoji od filogenetski različitih tipova, klasa i podklasa, gde određeni uslovi mogu uticati na metabolizam specifičnih sojeva *Accumulibacter*. Eksperimenti u šaržnom reaktoru, sa visokim rastom *Accumulibacter* bakterija (> 85 %), rađeni su više od godinu dana, pri čemu je dalja identifikacija na nivou podklase bila neophodna, kako bi se povezao specifični identitet grupe *Accumulibacter* sa posmatranim performansama reaktora. Brzina hranjenja ugljenikom pokazala se ključnom za ekspresiju različitih metabolizama, gde je brzo hranjenje dovelo do mešovitog metabolizma usvajanja polifosfata/glikogena, rezultujući neefikasnim uklanjanjem P (< 30 %), povezanog sa klasterima ii i iii *Accumulibacter* IIc podklase. Sa druge strane, mala brzina hranjenja ugljenikom dovela je do tipičnog metabolizma usvajanja polifosfata, koji je rezultirao potpunom separacijom fosfora kao rezultat metaboličkih procesa najbrojnijeg prisutnog klastera (klaster i) *Accumulibacter* IIc podklase. Rezultati ekperimentalnog rada u okviru teze pokazali su da određeni organizmi, trenutno prepoznati kao PAO organizmi, neefikasno uklanjaju fosfor i da postoji opravdana potreba za reklasifikacijom organizama unutar grupe *Accumulibacter*.

Uklanjanje mikropolutanta dodatni je problem u domenu savremenih tretmana voda, naročito iz razloga što su neki mikropolutanti, poput diklofenaka, veoma rezistentni na tretmane u konvencionalnim postrojenjima za prečišćavanje otpadnih voda. Sa druge strane, određeni fizički/hemijski procesi rezultuju generisanjem produkata koji su još toksičniji od matičnog jedinjenja. Biotransformacija diklofenaka ispitivana je simultano sa biološkim uklanjanjem fosfora i procenjen je metabolički put i toksičnost formiranih nusproizvoda diklofenaka. Iako *Accumulibacter* ne razlaže diklofenak tako efikasno kao neki drugi sojevi bakterija (npr. nitrifikujuće bakterije), eksperimentalno je potvrđeno da i dalje doprinosi redukciji toksičnosti, proizvodnjom nusproizvoda manje toksičnosti. Takođe, utvrđena je korelacija između količine biotransformacije diklofenaka i tipa *Accumulibacter* bakterija, što potencijalno ukazuje da stimulacija rasta *Accumulibacter* tipa II može intenzivirati biorazgradnju diklofenaka u procesima prečišćavanja otpadnih voda.

Na kraju, rekuperacija resursa jedna je od prioritetnih inicijativa u oblasti prečišćavanja otpadnih voda. Postrojenja za prečišćavanje otpadnih voda više se ne posmatraju samo kao postrojenja za prečišćavanje, već predstavljaju i mogućnost za rekuperaciju resursa i pružanje održivih rešenja za rad prečistača. Fosfor je nezamenljiv nutrijent i oskudan resurs koji je neophodno ponovno iskoristiti iz otpadnih tokova bogatih fosforom. Bioacidifikacija je testirana u ovoj tezi kao dodatni proces u okviru konvencionalnih postrojenja za prečišćavanje otpadnih voda i pokazalo se da ima potencijal da rastvori do 80 % fosfora i proizvede 0,35 g COD_{VFA}/g VS koji bi se mogli koristiti u kasnijim procesima za proizvodnju fosforom bogatih đubriva i bioplastike.

Ključne reči: uklanjanje i rekuperacija fosfora, fosfor akumulišući organizmi, mikropolutanti, diklofenak, valorizacija otpada.

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Notations and abbreviations

A^2O – anaerobic/anoxic/aerobic configuration

BOD – biological oxygen demand

C – carbon

Ca_{tot} and **Ca_{sol}** – total and soluble Calcium

COD_{tot} and **COD_{sol}** – total and soluble chemical oxygen demand

DCF - diclofenac

EBPR – enhanced biological phosphorus removal

Fe_{tot} and **Fe_{sol}** – total and soluble Iron

FISH – Fluorescence *in situ* hybridisation

GAO – glycogen accumulating organism

GC – gas chromatography

Gly – glycogen

HPLC – high-performance liquid chromatography

HRT – hydraulic retention time

Mg_{tot} and **Mg_{sol}** – total and soluble Magnesium

NH_x-N - ammonium plus ammonia nitrogen

N_{tot} – total nitrogen

OLR – organic loading rate

P – phosphorus

PAO – polyphosphate accumulating organism

PHA – polyhydroxyalkanoates

PHB - Polyhydroxybutyrate

PHV - Polyhydroxyvalerate

PO_4^{3-}-P – soluble phosphorus

P_{tot} – total phosphorus

SRT – sludge retention time

TS(S) - total (suspended) solids

VFA – volatile fatty acid

VS(S) – volatile (suspended) solids

WAS – waste activated sludge

WRRF – water resource recovery facility

WWTP – wastewater treatment plant

1. MOTIVATION AND THESIS OUTLINE

1.1 Motivation and objectives

An increasing number of challenges for wastewater treatment plants emerging in recent years require a lot of effort and investigation to rapidly provide answers and give solutions that would lead to more sustainable operation of modern wastewater treatment plants (WWTP), converting them to water resource recovery facilities (WRRF).

Activated sludge processes are widely implemented in conventional wastewater treatment plants, designed to remove organic matter, nitrogen and phosphorus from wastewater. Phosphorus removal is widely investigated, and phosphorus is long recognized as an important pollutant that should be removed from wastewater due to possible eutrophication when discharged in excess concentrations to water bodies. Phosphorus can be removed by biological or chemical processes in wastewater treatment plants. The enhanced biological phosphorus removal (EBPR) process is an activated sludge process which requires alternating anaerobic and aerobic conditions that stimulate *Accumulibacter* polyphosphate accumulating organisms (PAOs) to take up phosphorus in excess concentrations from the bulk liquid. Chemical processes comprise of iron or aluminium salts addition, that bind with phosphorus and form precipitates, also removing it from the bulk liquid. However, even with a lot of scientific contributions in this field, the real practice in wastewater treatment plants often leads to inefficient biological phosphorus removal, thus requiring chemical addition for complete phosphorus removal. Although a high number of studies have investigated reasons for EBPR process failure in WWTPs, much remains unknown, such as why do EBPR processes fail even with a high abundance of PAOs and without limiting phosphorus in the influent. The complex metabolism of these bacteria is widely studied; however, recent studies continue to raise questions about important aspects in metabolic differences between different types of *Accumulibacter* PAOs.

In addition to phosphorus removal, activated sludge processes have received some attention for micropollutant removal at conventional WWTPs. A high number of micropollutants entering the aquatic environment have raised concern and led to the search for solutions of their removal that could be easily implemented in existing WWTP. However, the fate of some micropollutants and its impacts is unknown in EBPR systems, especially for the resistant pharmaceutical compounds, such as diclofenac, often detected in the aquatic environment. It has been found that diclofenac concentrations of 1 μg/L can pose harmful effects on living species, thus its detoxification is important prior to its introduction to the environment. Some studies have investigated diclofenac transformation with nitrifying biomass, however it´s transformation

pathways as well as the toxicity of the transformation products in EBPR processes remains unknown.

In addition to micropollutant removal, in recent years, modern WWTPs are searching for solutions for more sustainable operation and the possibility of not only treating the wastewater, but also recovering resources and producing added-value products that could help both economic and environmental aspects of wastewater treatment process. In addition to being a pollutant, phosphorus is a valuable resource that gained a lot of attention in recent years. It is an indispensable nutrient for all living species; however, the reserves of phosphorus are limited. Furthermore, phosphorus reserves are distributed within a small number of countries (e.g., Morocco, China, USA), additionally leading to geopolitical dependence of other countries and putting it on the list of critical raw materials. Europe has invested and encouraged research in the area of phosphorus recovery due to its high dependence on phosphorus imports. Although, much research is done in this area, contrasting results, efficiencies, as well as the cost of some solutions have made challenging the implementation of phosphorus recovery in full-scale WWTP.

Therefore, this thesis' objective is to help address the problems introduced by conducting the following research:

- Bioreactor operation for enhanced biological phosphorus removal enriched with *Accumulibacter* polyphosphate accumulating organisms to study their metabolism as well as fine-scale differences of different *Accumulibacter* Types and sub-clades on the phosphorus removal performance.
- Bioreactor operation enriched with *Accumulibacter* polyphosphate accumulating organisms acclimatized with low influent concentrations (i.e., micropollutant levels) of diclofenac, to investigate their potential to biotransform diclofenac, identify transformation products and predict their toxicity.
- Anaerobic bioreactor operation at acidic pH conditions (bioacidification reactor) in order to select acidogenic bacteria and assess the efficiency of this process and optimal conditions to solubilise phosphorus and carbon from three different sludge substrates.
- Applying the bioacidification process at the pilot scale in order to provide proof of concept and investigate the potential of full-scale implementation.

1.2 Thesis outline

The content of this thesis is divided in six chapters, which describe the work performed during this PhD project:

Chapter 1 includes Motivation and objectives for PhD project.

Chapter 2 comprises the background necessary to understand the complexity of the problems addressed in this thesis, as well as the bibliography review that includes current solutions and ideas for these modern wastewater treatment plants to be transformed in the future to water resource recovery facilities. The thesis addresses phosphorus as a pollutant and as a resource, as well as the micropollutants removal in conventional wastewater treatment plants.

Chapter 3 comprises the investigation performed in the area of *Accumulibacter*, known polyphosphate accumulating organisms, in order to clarify possible differences in the metabolism used between different *Accumulibacter* Types and sub-clades on the performance of enhanced biological phosphorus removal. Long-term sequencing batch reactor was operated with alternating carbon feeding conditions in order to select for different microbial community profiles. The steady state periods were identified, and the present microbial community was linked to the performance of biological phosphorus removal.

This work was published in an international peer reviewed scientific journal article: Srdana Kolakovic, Elisabete B. Freitas, Maria A. M. Reis, Gilda Carvalho, Adrian Oehmen, 2021. *Accumulibacter* diversity at the sub-clade level impacts enhanced biological phosphorus removal performance, Water Research (2021) 117210. https://doi.org/10.1016/j.watres.2021.117210.

Chapter 4 comprises the investigation of diclofenac biotransformation in the enhanced biological phosphorus removal. The *Accumulibacter* polyphosphate accumulating organisms were acclimatized with low concentrations of diclofenac in the influent, and diclofenac biotransformation was followed in various periods. The transformation products and their toxicity were also addressed within this chapter.

This work was published in an international peer reviewed scientific journal article: Srdana Kolakovic, Ricardo Salgado, Elisabete B. Freitas, Maria R. Bronze, Maja Turk Sekulic, Gilda Carvalho, Maria A. M. Reis, Adrian Oehmen, 2022. Diclofenac biotransformation in the enhanced biological phosphorus removal process. Sci. Total Environ. (2022) 806, 151232. https://doi.org/10.1016/j.scitotenv.2021.151232.

Chapter 5 comprises the investigation of the bioacidification process with the objective to solubilise phosphorus and carbon. For this purpose, a semi-continuous

anaerobic reactor was operated, and several operational conditions were investigated in order to achieve the optimal conditions for process implementation in full-scale water resource recovery facilities. The conditions tested in the kinetic tests were further tested on a pilot scale reactor, in order to provide proof of concept and encourage full-scale implementation.

Part of this work was published in an international peer reviewed scientific journal article: Srdana Kolakovic, Jorge M. M. Santos, Maria A. M. Reis, 2021. Phosphorus and carbon solubilisation strategies for wastewater sludge valorisation, Journal of Environmental Chemical Engineering (2021). https://doi.org/10.1016/j.jece.2021.106261.

The other part will be submitted with additional modelling work that is in progress.

Chapter 6 comprises a summary of the main findings within this thesis along with some questions that should be addressed in future work.

2. INTRODUCTION

Water is the most important resource in human life; thus, its supply is of crucial importance for the development of the humanity. The preservation and sustainable use of water bodies as sources of the water supply is the absolute priority for modern societies. The contamination of water bodies mostly comes from anthropogenic activity, through wastewater discharge or agricultural runoff. Although wastewater was considered in the past as only a pollution source that needs to be treated, in recent years it finally started to be considered as a resource rather than a waste stream, and wastewater treatment plants (WWTP) as water resource recovery facilities (WRRF) (Cornejo et al., 2019) that could be continuous and sustainable sources of chemical energy and resources (Fernández-Arévalo et al., 2017).

Transforming WWTPs into WRRFs has prompted much research and ideas for more sustainable operation and added-value products that could be reused within WRRF or even bring revenue. Properly treated wastewater can be a high-quality fresh water source that is climate-independent and can be locally controlled (Diaz-Elsayed et al., 2019). In addition to the solutions for energy recovery, value-added products, such as fertilizers, bioproducts and chemicals can be obtained. However, modernization of WWTP and their transformation to WRRF does not present an easy task, especially without proper decision-making tools and socio-political barriers often encountered in environmental technologies management. Technologies that provide big waste streams that require further treatment or disposal should be avoided and substituted for more sustainable technologies. Solving one problem but creating another one is a common issue in the environmental area, but in recent years more research is done to tackle not only the primary problem but to avoid creating additional hazardous streams.

To be able to transform WWTPs into WRRFs, resource recovery technologies must be implemented in the existing wastewater treatment schemes. For this purpose, the investigation of innovative and sustainable technologies must be accelerated by creating frameworks for systematic evaluation of the technologies, simultaneously linking the real testing facilities and researchers, investors and other stakeholders (Cornejo et al., 2019). To achieve more sustainable operation, the energy generation should be maximized and/or energy consumption should be minimized. Furthermore, WWTP were recognized as some of the largest greenhouse gas (GHG) emitters from the processes to remove organic matter, phosphorus, nitrogen, sludge management as well as the indirect emissions from imported energy, chemicals, and fuels (Lu et al., 2018). Therefore, in addition to investigation of novel technologies, the most immediate step for resource recovery is updating the existing WWTPs in order to reduce operating costs, decrease GHG emissions and recover resources (Fernández-Arévalo et al., 2017).

A new application and combination of conventional mature technologies could make WRRFs energy self-sufficient or even energy-positive (Solon et al., 2019). For instance, changing conventional plant layout by including thermal hydrolysis to improve sludge biodegradability and decoupling chemical oxygen demand (COD) and nutrients treatment has prompted energy neutral WRRF (Fernández-Arévalo et al., 2017). Other techniques separating COD and nutrient treatment are also investigated so that organic fraction is concentrated and treated in the anaerobic digestor. For this purpose, high rate activated sludge systems or chemically enhanced primary treatment were investigated (Solon et al., 2019). This approach is used as well to minimize the organic fraction to be treated in the aerobic biological treatment, as it is one of the most energy consuming steps of the process. However, if excessive COD is removed in early stages, nutrient removal processes that require organic carbon might be affected. In these cases, external carbon source should be added for the biological treatment. Since good nutrient removal is very important, different configurations such as partial nitrification-denitrification by intermittent aeration control (Regmi et al., 2015) were investigated for nutrient removal in order to overcome lack of carbon source, or high energy consumption. Carbon capture and utilization wastewater treatment pathways such as microalgae cultivation, constructed wetlands, biochar production and microbial electrolytic carbon capture could help contribute to negative emission practices without compromising aquatic environment and public health (Lu et al., 2018). Anaerobic digestion provides energy recovery that reduces energy consumption of WWTPs where produced biogas can be used to provide electric and thermal energy or biomethane that could be an economical solution due to attractive economic subsidies in recent regulations (Borzooei et al., 2020). Therefore, many problems present in wastewater treatment difficult the modernization of conventional WWTPs, which is why this thesis focused on investigation of some of the existing challenges.

2.1 Phosphorus as a pollutant

Phosphorus (P) present in wastewater is a pollutant that can cause eutrophication if discharged in excessive concentrations. Phosphorus removal has been widely investigated in the past with a big focus on enhanced biological phosphorus removal (EBPR) processes due to the high P removal efficiencies achieved through a chemical-free approach. EBPR employs polyphosphate accumulating organisms (PAOs) that store excess amounts of P as polyphosphate by incorporating it to cell flocs and removing it from the bulk liquid. Therefore, in addition to high P removal, it is simultaneously creating a valuable P rich sludge (Wang et al., 2016; Yang et al., 2017).

EBPR processes require alternating anaerobic and aerobic conditions, where *Candidatus* Accumulibacter phosphatis, commonly known PAO organisms, are subjected to specific conditions in order to take up excess concentrations of phosphorus. PAOs use organic carbon source (volatile fatty acids (VFAs)) in anaerobic conditions and store them as poly-β-hydroxyalkanoates (PHAs), using polyphosphate cleavage as main energy source, while glycogen stored in cells provides additional energy and a reducing power source for PHA synthesis. Carbon and energy source for biomass growth, polyphosphate storage and glycogen regeneration is obtained from PHA oxidation under aerobic conditions (Oehmen et al., 2007; Zhou et al., 2008). Glycogen accumulating organisms (GAOs) are the competitors of PAOs, as they can consume VFAs in anaerobic conditions without performing P removal (Oehmen et al., 2007). The main differences between PAO and GAO metabolism are presented in Figure 2.1. The competition between the two organisms is widely studied, and operational conditions to enrich PAOs and inhibit GAOs were widely reported.

However, the research focus in recent years was on the metabolism of different *Accumulibacter* Types, as this PAO group consists of phylogenetically closely related bacteria that have shown different metabolic expression under specific conditions. *Accumulibacter* consists of two distinct Types (Type I and Type II), based on the analysis of the 16S rRNA genes and the ppk1 gene (He et al., 2007). Within these two Types, several clades were identified (IA-I_E and IIA-II_I), reflecting fine differences in the genetic sequence of the ppk1 gene (Camejo et al., 2016). Carvalho et al. (2007) detected that rod morphotypes were able to use nitrate as the electron acceptor, while cocci were not. Rod morphotypes in that study were later linked to *Accumulibacter* Type I, and the cocci to *Accumulibacter* Type II (Oehmen et al., 2010). Furthermore, PAO and GAO metabolic expression as well as the toxicity tolerance were investigated and compared between different *Accumulibacter* clades. More recently, Camejo et al. (2019) and Rubio-Rincón et al. (2019a) investigated the differences in denitrification pathways between the same *Accumulibacter* clade IC, where intra-clade diversity analysis showed that phylogenetic diversity at the sub-clade level can influence *Accumulibacter* metabolism, as Ca. *Accumulibacter* UW-LDO-IC was capable of the full denitrification pathway, while Ca. *Accumulibacter* delftensis IC could not use nitrate as the electron acceptor.

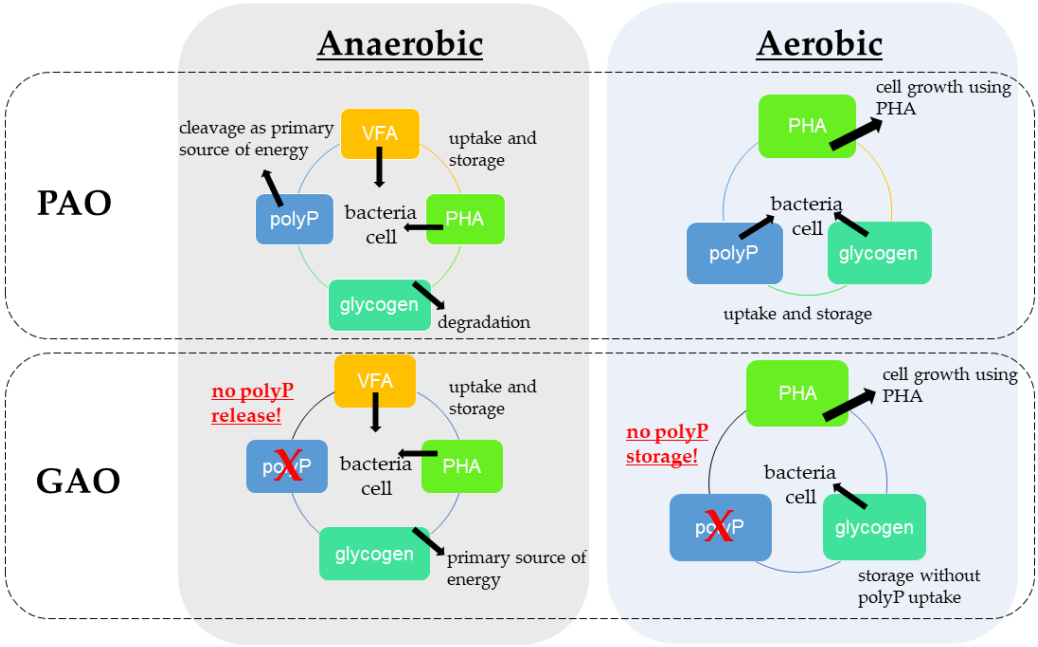

Figure 2.1 Simplified schematic representation of the differences in PAO and GAO metabolism in alternating anaerobic and aerobic conditions.

Along with the denitrification capability, the capacity of *Accumulibacter* to perform both PAO and GAO-like metabolism is of high importance impacting performance in EBPR systems. Zhou et al. (2008) found that *Accumulibacter* were able to use glycolysis as a main source of energy in limited P conditions, while Acevedo et al. (2012) observed a correlation between *Accumulibacter* Type and the level of polyphosphate storage leading to PAO (Type I) or GAO (Type II) metabolism by *Accumulibacter*, when shifted to P-limiting conditions. The study of Welles et al. (2015) concluded that, when phosphate was not limiting, PAO Type I performed typical PAO metabolism, while PAO Type II performed mixed PAO and GAO metabolism. Furthermore, Welles et al. (2017) later showed that *Accumulibacter* clade IIC displayed differing PAO and GAO-like behaviour depending on the availability of the influent P concentration. Still, it remains unclear if a clade-level description of *Accumulibacter* is sufficient to establish the PAO vs GAO metabolic capacity of *Accumulibacter*, as well as if some *Accumulibacter* sub-lineages perform GAO-like metabolism even when influent P levels are not limiting.

2.2 Micropollutants in EBPR process

In addition to the nutrients considered as macro pollutants, the vast number of micropollutants detected in the aquatic environment has been a major concern in recent years. WWTPs can present a valuable barrier to control their introduction to the environment (Nguyen et al., 2021). One of the commonly detected micropollutants, the

non-steroidal anti-inflammatory drug (NSAID) – diclofenac, was included in the watch list of prioritized compounds of the European Union (Barbosa et al., 2016; Loos et al., 2018) and has been considered as a compound with highest acute toxicity of NSAIDs (Nguyen et al., 2021; Vieno and Sillanpää, 2014). Therefore, it is very important to investigate its fate along different processes relevant to WWTPs.

Micropollutants can be the main carbon substrate for cell growth, thus involved in primary metabolism, or they can be transformed by co-metabolism (Nguyen et al., 2021). Diclofenac has proven to be quite recalcitrant in conventional activated sludge systems, with more significant transformation observed only in nitrifying conditions in certain cases (Krkošek et al., 2014; Kruglova et al., 2014; Suarez et al., 2010; G. Wu et al., 2020). However, even in these studies, the transformation efficiencies can be contradictory, leaving the fate of diclofenac in these systems still poorly understood. In addition, diclofenac biotransformation in the EBPR systems is mostly unknown. Zhao et al., (2020) investigated the impact of DCF toxicity to EBPR performance, where 0.01 mg/L of DCF had no significant negative impact on phosphorus removal. Torresi et al., (2019), investigated the degradation of several PhACs, including diclofenac, by PAOs in a moving bed biofilm reactor (MBBR), and concluded that diclofenac was transformed by aerobic PAOs, while denitrifying PAOs and aerobic glycogen accumulating organisms (GAOs) where less able to biodegrade diclofenac. However, the capacity of conventional, non-biofilm EBPR processes to perform DCF biodegradation, the link between the microbial community and DCF biodegradation, as well as the transformation products that can be produced by PAOs remains unknown. This motivated the present thesis to further explore, along with the phosphorus removal, the fate of diclofenac in the EBPR systems.

2.3 Phosphorus as a resource

On the contrary of being a pollutant, phosphorus is an indispensable nutrient for all living organisms, crucial for DNA and RNA formation, cell membranes as well as teeth and bone formation and maintenance (Childers et al., 2011; Yang et al., 2017). The constant increase in the world population and changing habits require higher crop production, therefore higher use of inorganic phosphorus as fertilizer is inevitable (Egle et al., 2016; Günther et al., 2018). This further led to increases in phosphate rock mining as well as the instability and fluctuation of fertilizer prices, initiating extensive analysis and predictions in the area of availability of phosphate rock world reserves. Due to many different scenarios predicting phosphorus scarcity in the near future as well as the limited quality and availability of the natural phosphate rock deposits (Childers et al.,

2011; Cieślik and Konieczka, 2017; Cordell et al., 2009), phosphate rock was classified as a critical raw material by European Commission in 2014 (European Commission, 2017). In addition, available phosphate reserves are unevenly distributed in the world, causing concern for the countries that depend on its importation from several countries. Europe imports around 90% of its phosphorus needs mostly from Morocco and Russia, since there is only one igneous mine in Finland (European Commission, 2017; Geissler et al., 2019).

This phosphorus pollutant vs resource paradigm induced changes in the legislation of some European countries, where Switzerland was the first country to make phosphorus recovery from sewage sludge obligatory (Günther et al., 2018; Liu et al., 2019). Austria, Sweden and Germany have followed the initiative for imposing new regulations, leading to a new Sewage Sludge Ordinance in Germany which will oblige around 500 German wastewater treatment plants (WWTP), that includes treatment of more than 60% of total phosphorus incorporated into sludge, to implement a chosen technology for phosphorus recovery (Günther, et al., 2018; ESPP, 2017). Sewage sludge with a minimum phosphorus content of 20 g/kg total solids (TS) will have to be treated by recovery processes that can extract at least 50 % of phosphorus from the sewage sludge or reduce phosphorus content to less than 20 g/kg TS. In addition, at least 80% of phosphorus contained in sewage sludge incineration ash will have to be recovered (Roskosch, et al, 2018).

Recovering phosphorus from wastewater is certainly not sufficient to substitute entirely its import into Europe, however it might be one of the factors for a significant contribution in the future decrease of the dependence on other countries' phosphate reserves. Wastewater treatment plants can obtain sludge streams containing phosphorus in the range of 1% to 15% in the dry matter residue, depending on the type of treatment implemented at the WWTP (Cieślik and Konieczka, 2017; Egle et al., 2015; He et al., 2016; Yang et al., 2017). Good performance of biological phosphorus removal is very important even at the point of phosphorus recovery, because of the decreased need for chemical addition which could further create problems in solubilisation of P (He et al., 2016; Hu et al., 2019; Liu et al., 2019; Pokhrel et al., 2018; Xie et al., 2011). Organically bound phosphorus can be mainly released by sludge disintegration, bioconversion and microbial metabolism (He et al., 2016). Inorganic phosphorus contains non-apatite inorganic phosphorus (NAIP) which is phosphorus associated to oxides and hydroxides of metals e.g., Fe, Al and Mn; and apatite phosphorus (AP) associated to Ca. NAIP is considered as the most labile form of phosphorus, and in many studies gets dissolved before AP, which is considered as a more stable fraction of P. As for the reported fractions of NAIP and AP in waste activated sludge, NAIP is usually reported as a major

fraction of sewage sludge both from biological phosphorus removal and even higher from chemical phosphorus removal plants. Several studies have reported that the NAIP fraction was 60% to 90% of the inorganic phosphorus, while AP was 4% - 25% (Ali and Kim, 2016; González Medeiros et al., 2005; He et al., 2016; L. Li et al., 2019; Liu et al., 2020; Ping et al., 2020; Pokhrel et al., 2018).

The practice of direct disposal of sewage sludge on land as a fertilizer was abandoned in many countries due to possible health and environmental risks, triggering the research for new ways of sludge disposal as well as recovering added-value products from waste streams. When sludge cannot be disposed on the soil and used directly as fertilizer, different technologies may be applied in the WWTPs to recover phosphorus from the ash, from the liquid phase or from the sludge as indicated in Figure 2.2 (Egle et al., 2016; Quist-Jensen et al., 2018). According to Tan and Lagerkvist (2011) the sludge ash after incineration has a P content higher than 5% by weight, which makes it suitable for P recovery processes. However, phosphorus recovery from the ash is possible mainly for the WWTPs that perform sludge incineration and usually it is performed in a separated facility treating ashes from several WWTPs together. This can make it an expensive and logistically complicated method suitable only for selected plants. Possible streams for P recovery from liquid phase by precipitation are urine from separated toilets, secondary treated effluent and digester supernatant. However, different predictions for recovery efficiencies from these streams that go most frequently around 30% to 50%, as well as the reduced recovery potential when applied to sludge from chemical P removal WWTPs (Egle et al., 2015; Günther et al., 2018; Rahman et al., 2014), makes it suitable for only a limited number of WWTPs. Even if these technologies might achieve high purity products, a lot of phosphorus contained in the sludge is still not being recovered. Almost 90% of phosphorus ends up incorporated into WWTP sludge, making it a promising P recovery resource. Sludge can be used in its raw form or after the anaerobic digester, untreated, thickened or dewatered depending on the selected technology to be applied (Figure 2.2). Many technologies have been investigated for the P dissolution from the sludge (wet-chemical leaching, thermal or chemical hydrolysis, wet-air oxidation etc) but the efficiencies, cost predictions and possibilities for full-scale implementation are still under further study.

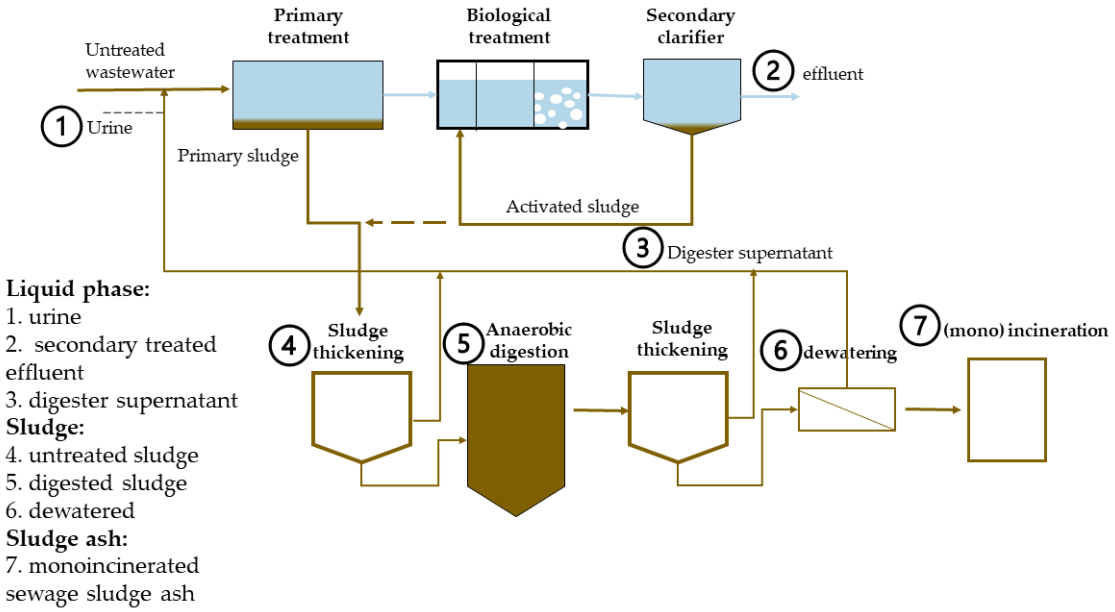

Figure 2.2 Possible access points for P recovery during wastewater and sewage sludge treatment (Adapted from Egle et al., (2015)).

In many studies, maximum solubilisation of phosphorus often requires extreme pH values, but this may be limiting the potential of conventional anaerobic digestion and creates additional costs for chemical addition and further sludge treatment. Chemical acidification to extreme acid pH conditions may also make difficult the recovery of phosphorus after its solubilisation, since processes for phosphorus crystallization usually require alkaline pH. A significant number of experiments performed were batch tests focused on chemical pH adjustment of the sludge and often applied long sludge retention times that makes it difficult to apply on full-scale WWTPs. Furthermore, biological fermentation can provide more than P release, providing added-value products such as volatile fatty acids (VFAs) and biogas production. A study from Latif et al. (2017) provided good results for phosphorus solubilisation (74%) using a single stage continuous anaerobic digestion process at pH 5.5 (known as bioacidification), however it required 12 days SRT and 37°C and the methane production was greatly affected by VFAs accumulation at lower pH.

This thesis focuses on existing mature technologies, trying to fill in the knowledge gaps and to provide a new insight in what could be the benefit of adapting these technologies for the existing new challenges. Therefore, the thesis has three main objectives: i) investigating the metabolism of different *Accumulibacter* lineages and their impact on EBPR performance; ii) evaluate the capacity of an enriched *Accumulibacter* PAO community to biodegrade DCF at environmentally relevant concentrations; iii)

investigate the potential to solubilise P and C from different WRRF sludges in a semi-continuous bioacidification reactor and clarify the individual impact of various operational parameters for full-scale implementation.

3. *ACCUMULIBACTER* DIVERSITY AT THE SUB-CLADE LEVEL IMPACTS ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL PERFORMANCE

3. *ACCUMULIBACTER* DIVERSITY AT THE SUB-CLADE LEVEL IMPACTS ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL PERFORMANCE

SUMMARY: *Accumulibacter* is a well-known group of organisms, typically considered to be polyphosphate accumulating organisms (PAOs), but potentially capable of glycogen accumulating organism (GAO) metabolism under limiting influent phosphate levels. Metabolic features of *Accumulibacter* are typically linked to its phylogenetic identity at the Type or clade level, though it is unclear the extent to which *Accumulibacter* diversity can correlate with its capacity to perform P removal. This paper investigates the fine-scale diversity of *Accumulibacter* and its link with enhanced biological phosphorus removal (EBPR) performance under various operating conditions, to understand the conditions and community structure leading to successful and unsuccessful EBPR operation. For this purpose, the organic carbon feeding rate and total organic carbon concentration were varied during three distinct operational periods, where influent phosphate was never limiting. *Accumulibacter* was always the dominant microbial group (> 80 % of all bacteria according to quantitative fluorescence *in situ* hybridisation - FISH) and low levels of *Competibacter* and other GAOs were consistently observed (< 15 % of all bacteria). Steady state was achieved in each of the three periods, with average phosphorus removal levels of 36 %, 99 % and > 99 %, respectively. Experimentally determined stoichiometric activity supported the expression of a mixed PAO/GAO metabolism in the first steady state period and the typical PAO metabolism in the other two steady state periods. FISH quantification and amplicon sequencing of the polyphosphate kinase (*ppk1*) functional gene indicated that *Accumulibacter* clade IIC was selected in the first steady state period, which shifted to clade IA after decreasing the carbon feeding rate in steady state period 2, and finally shifted back to clade IIC in the third steady state period. Fine-resolution *Ppk*-based phylogenetic analysis revealed three different clusters within *Accumulibacter* clade IIC, where clusters IICii and IICiii were linked to poor EBPR performance in period 1, and cluster IICi was linked to good EBPR performance in period 3. This study shows that the deterioration of EBPR processes through GAO activity at non-limiting P concentrations can be linked to organisms that are typically classified as PAOs, not only to known GAOs such as *Competibacter*. Intra-clade phylogenetic diversity within *Accumulibacter* showed that some clusters actually behave similarly to GAOs even without influent phosphate limitation. This study highlights the need to closely re-examine traditional interpretations regarding the link between the microbial community composition and identity with the performance and metabolism of EBPR systems.

Keywords: Enhanced Biological Phosphorus removal (EBPR), *Candidatus* Accumulibacter phosphatis, intra-clade diversity, PAO metabolism, GAO metabolism

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3.1 Introduction

Phosphorus is both a pollutant in aquatic ecosystems and an indispensable nutrient and resource in agriculture. Enhanced biological phosphorus removal (EBPR) processes are commonly implemented due to the potentially high P removal efficiencies that can be achieved through a chemical-free approach, promoting economic and environmental sustainability, and can be advantageous when implementing complimentary P recovery processes. EBPR relies on polyphosphate accumulating organisms (PAOs) that can take up and store excess amounts of P as polyphosphate, transferring it from the liquid phase to the biomass, achieving P removal requirements and simultaneously creating a valuable P rich sludge, suitable for P recovery (Wang et al., 2016; Yang et al., 2017).

EBPR is achieved by alternating anaerobic and aerobic conditions, where PAO organisms such as *Accumulibacter* take up organic carbon sources such as volatile fatty acids (VFAs) anaerobically and store them as poly-β-hydroxyalkanoates (PHAs), obtaining energy from polyphosphate cleavage and phosphate release from cells. Glycolysis of their stored glycogen provides additional energy and a reducing power source for PHA synthesis. Aerobic PHA oxidation serves as the carbon and energy source for biomass growth, P uptake, polyphosphate storage and glycogen regeneration (Oehmen et al., 2007; Zhou et al., 2008). Glycogen accumulating organisms (GAOs) are the competitors of PAOs, as they can also uptake VFAs in anaerobic conditions but do not perform P uptake, thus do not contribute to P removal (Oehmen et al., 2007). Many studies have focused on investigating the competition between these two organisms, establishing conditions for enrichment of PAOs and inhibition of GAOs, by applying various carbon sources, feeding rates, pH, dissolved oxygen and temperature conditions (Carvalheira et al., 2014a, 2014b, 2014c; Lopez-Vazquez et al., 2009; Tu and Schuler, 2013). For example, Tu and Schuler, (2013) investigated the competition of PAOs and GAOs with different feeding rates, showing that higher acetate feeding rates can enrich GAOs over PAOs and thus, lead to the deterioration of EBPR systems.

Accumulibacter is a well-known PAO that consists of two distinct Types (Type I and Type II), based on the analysis of the 16S rRNA gene and the ppk1 gene (He et al., 2007).

Within these two Types, several clades were identified (IA-I_E and IIA-II_I), reflecting fine differences in the genetic sequence of the ppk1 gene (Camejo et al., 2016). These differences in the specific phylogenetic identity of *Accumulibacter* have been correlated with differences in its metabolic expression. Initially, Carvalho et al. (2007) have detected different cell morphotypes of *Accumulibacter*, where rod morphotypes were able to use nitrate as the electron acceptor, while cocci were not. Oehmen et al. (2010) later found that the rod morphotype in that study corresponded to *Accumulibacter* Type I, and the cocci to *Accumulibacter* Type II. Flowers et al. (2009) achieved a similar conclusion for PAOs belonging to clades IA and IIA, respectively.

More recently, Camejo et al. (2019) and Rubio-Rincón et al. (2019a) investigated the differences in denitrification pathways between the same *Accumulibacter* clade. Both studies investigated *Accumulibacter* clade IC, but intra-clade diversity analysis showed that the IC selected in the study of Camejo et al. (2019) was capable of performing the full pathway for respiratory denitrification (Ca. *Accumulibacter* UW-LDO-IC), while Rubio-Rincón et al. (2019a) enriched a culture of Ca. *Accumulibacter* delftensis IC, which was not capable of using nitrate as the electron acceptor. These studies prove that phylogenetic diversity at the sub-clade level can influence *Accumulibacter* metabolism.

One metabolic aspect of high importance impacting P removal performance in EBPR systems is the capacity of *Accumulibacter* to perform both PAO and GAO-like metabolism. The deterioration of P removal has been observed even in enriched *Accumulibacter* PAO systems, where Zhou et al. (2008) found that *Accumulibacter* were able to use glycolysis as a main source of energy in limited P conditions. Acevedo et al. (2012) observed a correlation between *Accumulibacter* Type and the level of polyphosphate storage leading to PAO (Type I) or GAO (Type II) metabolism by *Accumulibacter*, when shifted to P-limiting conditions. The study of Welles et al. (2015) concluded that, when phosphate was not limiting, PAO Type I performed typical PAO metabolism, while PAO Type II performed mixed PAO and GAO metabolism. However, under phosphate limitation, both Types shifted to a GAO-like metabolism, with a strong competitive advantage of Type II over Type I. Welles et al. (2017) later showed that *Accumulibacter* clade IIC displayed differing PAO and GAO-like behaviour depending on the availability of the influent P concentration. While the metabolic flexibility of *Accumulibacter* is dependent on the clade and environmental conditions (Guedes da Silva et al., 2020), it is unclear if a clade-level description of *Accumulibacter* is sufficient to establish the PAO vs GAO metabolic capacity of *Accumulibacter*. In addition, previous studies have not investigated if some *Accumulibacter* sub-lineages perform GAO-like metabolism even when influent P levels are not limiting.

3. *ACCUMULIBACTER* DIVERSITY AT THE SUB-CLADE LEVEL IMPACTS ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL PERFORMANCE

This study focuses on investigating the metabolism of different *Accumulibacter* lineages and their impact on EBPR performance. An *Accumulibacter* bioreactor was subjected to various conditions in long-term tests that are typically conducive to different ecological profiles. The carbon feeding strategy (feeding rate and loading) was manipulated, while non-limiting influent phosphorus levels were maintained. The feeding strategy represents one of the operating parameters that are known to affect the dominant species of a microbial community. A high or low abundance of resources (such as organic carbon) impacts the microbial profile by enriching for organisms that are either r- or K-strategists, respectively. Therefore, we operated an EBPR reactor with different feeding strategies to select for different PAO cultures. The metabolism was studied along with a fine-scale microbial community analysis of the *Accumulibacter* sludge at the sub-clade level, to identify the link between phylogenetic identity and metabolic function. Three different feeding conditions were investigated for selecting the *Accumulibacter* reactors, where EBPR performance was compared at different organic carbon feeding rates and concentration levels. This study provides needed clarity on factors leading to EBPR failure and upsets, through better understanding of how *Accumulibacter* performance varies as a function of the specific clade and sub-clade within this diverse and important microbial group of organisms.

3.2 Materials and methods

3.2.1 Sequencing batch reactor (SBR) operation

A two-litre reactor was inoculated with sludge from a municipal WWTP Beirolas in Lisbon, Portugal and a gradual COD increase from 50 mg COD/L to 200 mg COD/L was initially applied in the start-up phase in order to select for PAOs. The reactor was operated in sequencing batch mode with 4 cycles per day. The six-hour cycle was comprised of a 2 h anaerobic phase, 3 h aerobic phase and a 1 h settling/decant phase (without stirring). To achieve anaerobic and aerobic conditions, argon and air were continuously bubbled, respectively. Temperature was controlled with a water bath at 20 °C, and pH was controlled between 7 - 7.5 with automatic addition of HCl or NaOH. The sludge retention time (SRT) was 10 days, and the hydraulic retention time (HRT) was 12 h.

3.2.2 Feed composition

The 1 L feed medium was a mixture of two solutions fed separately: A) phosphate and B) carbon and nutrient solutions. The phosphate feeding rate and concentration was constant throughout the entire period of reactor operation. Feeding took place at the beginning of the anaerobic phase for 5 minutes, with a total volume of 750 mL. The solution A contained 124 mg of K_2HPO_4 and 96.8 mg of KH_2PO_4 per litre.

The carbon source was a mixture of acetate (HAc) and propionate (HPr) at a proportion of 75 – 25 % (on a COD basis), respectively, as was suggested to be beneficial for PAOs to outcompete GAOs (Carvalheira et al., 2014b; Lopez-Vazquez et al., 2009). Additionally to carbon source, the composition of solution B consisted of 0.59 g NH_4Cl, 0.95 g $MgSO_4.7H_2O$, 0.44 g $CaCl_2.2H_2O$, 11.7 mg allyl-N thiourea (ATU), 31.7 mg ethylene - diaminetetraacetic (EDTA) and 3.17 mL of a micronutrients per litre, as detailed in Carvalheira et al. (2014a), where 250 mL was added per cycle. The micronutrient solution contained per litre: 1.5 g $FeCl_3.6H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4.5H_2O$, 0.18 g KI, 0.12 g $MnCl_2.4H_2O$, 0.06 g $Na_2MoO.2H_2O$, 0.12 g $ZnSO_4.7H_2O$, 0.15 g $CoCl_2.6H_2O$.

3.2.3 Operation of the SBR in the three steady state periods

As previously mentioned, the phosphate feeding rate and concentration was maintained constant throughout the study. During the first steady state period of reactor operation, COD was fed over 5 minutes after phosphate feeding (fast mode), while afterwards, in steady state periods 2 and 3, it was switched to 50 minutes (slow mode). The period of stabilization after changing the COD feeding rate is referred to as the transition phase. After achieving steady state in each of the three operation periods, reactor performance and the fine-scale PAO community were investigated.

Steady state period 1 – fast COD feeding rate: 200 mg COD/L

In period 1, a fast COD feeding rate strategy was employed at a rate of 40 mg COD/L.min, to achieve 200 mg COD/L in the reactor. These conditions were operated in steady state for 30 days.

Steady state Period 2 – slow COD feeding rate: 200 mg COD/L

In period 2, the reactor was fed with a slow COD feeding rate strategy of 4 mg COD/L.min, to achieve 200 mg COD/L in the reactor. The steady state of period 2 lasted for 100 days.

Steady state period 3 – slow COD feeding rate: 150 mg COD/L

After a shutdown/start-up period, the reactor was re-seeded using sludge from period 2 and a gradual increase of COD concentration was applied during this start-up phase until reaching 150 mg COD/L at steady state. In this period the reactor was also fed with a slow COD feeding rate strategy (3 mg COD/L.min) and steady state was achieved for 150 days.

3.2.4 Sampling and chemical analysis

Routine monitoring of the reactor was performed once per week, where total suspended solids (TSS), volatile suspended solids (VSS), phosphate (PO_4^{3-}-P), ammonium, glycogen (Gly), polyhydroxyalkanoates (PHA) and volatile fatty acids (VFA) were analysed. Samples were collected along the anaerobic and aerobic phases, except TSS and VSS, which were measured at the end of the aerobic phase. Samples were centrifuged for 3 minutes at 10 000 rpm, following separation of the solid and liquid phases with 0.45 μm filters. P, ammonium and VFAs were analysed on the liquid phase, while PHA and glycogen were analysed on lyophilized biomass pellets. P and ammonium analysis were performed with segmented flow analysis (Skalar 5100, Skalar Analytical, The Netherlands). VFA analysis was performed with high performance liquid chromatography (HPLC) with a DAD detector, using 0.01 N sulfuric acid as eluent, at a temperature of 30 °C and a Biorad Aminex HPX-87H 300 x 7.8 MM column. TSS and VSS measurements were performed by standard methods (APHA/AWWA/1995). PHA samples were prepared as described by Lanham et al. (2013) and measured by gas chromatography (GC) using a Bruker 430-GC, FID detector and a Restek column (60 m, 0.53 mm internal diameter, 1 mM df, crossbond). Glycogen samples were prepared using acid digestion of lyophilized biomass at 0.9 M HCl for 3 h, and the supernatant was analysed by HPLC as described in Carvalho et al. (2018). Biomass samples were also collected at the end of the anaerobic phase for microbiological characterisation of the community, as described below.

3.2.5 Calculation of the kinetic and stoichiometric parameters

Stoichiometric and kinetic parameters presented in the results section were calculated via the equations presented below.

P removal was determined by difference between the phosphorus concentration in the beginning ($P_{start,anaerobic}$) of the anaerobic phase (mg P/L) after P feeding and the end of the aerobic phase ($P_{end,aerobic}$), divided by the initial concentration of the anaerobic phase, as shown in Eq. (1):

$$P\ removal\ (\%) = \frac{P_{start,anaerobic} - P_{end,aerobic}}{P_{start,anaeorbic}} \times 100 \quad (1)$$

Active biomass (X) was determined by subtracting the PHA and glycogen from VSS as follows:

$$X\ (g/L) = VSS - PHA - Gly \quad (2)$$

Stoichiometric parameters were determined by phosphorus release (P-mol), glycogen degradation (C-mol) and PHA production (C-mol) in the anaerobic phase per VFAs consumed (C-mol), using the following equations:

$$\frac{P}{VFA}\left(\frac{P-mol}{C-mol}\right) = \frac{P_{end,anaerobic} - P_{start,anaeorbic}}{VFA_{consumed}} \quad (3)$$

$$\frac{Gly}{VFA}\left(\frac{C-mol}{C-mol}\right) = \frac{Gly_{start,anaeorbic} - Gly_{end,anaerobic}}{VFA_{consumed}} \quad (4)$$

$$\frac{PHA}{VFA}\left(\frac{C-mol}{C-mol}\right) = \frac{PHA_{end,anaeorbic} - PHA_{start,anaerobic}}{VFA_{consumed}} \quad (5)$$

The maximum phosphorus release and uptake rates were determined in the first hour of the anaerobic or aerobic phase, respectively, as the majority of the activity was observed in the first hour of each phase. P release or uptake rate calculations are shown below in Eq. (6) and (7), respectively:

$$P\ release\ rate\left(\frac{mgP}{gX.h}\right) = \frac{P_{1h,anaerobic} - P_{start,anaerobic}}{time \times X} \quad (6)$$

$$P\ uptake\ rate\ \left(\frac{mgP}{gX.h}\right) = \frac{P_{start,aerobic} - P_{1h,aerobic}}{time \times X} \quad (7)$$

3.2.6 Microbial community analysis

Fluorescence in situ hybridization (FISH)

Biomass samples from the three different steady-state periods were fixed with 4% paraformaldehyde (PFA) or ethanol as described by Nielsen, (2009). Quantitative FISH (qFISH) was performed according to Amann, (1995), to analyse the microbial composition and abundance of the bacterial populations present in the SBR. Specific fluorescently labelled oligonucleotide probes were applied to the biomass, to allow the visualization of the microbial consortia. Cyanine 5 (Cy5)-labelled EUBmix probe that

targets all Bacteria (mixture of EUB338 (Amann et al., 1990), EUB338-II and EUB338-III (Daims et al., 1999)) was applied together with the cyanine 3-labelled probes ACC-I-444 and ACC-II-444 (Flowers et al., 2009) to target *Candidatus* Accumulibacter Phosphatis Types I and II, respectively, and with the cy3-labelled CPB_654 probe for *Candidatus* Competibater Phosphatis (Simon J. McIlroy et al., 2015). Other oligonucleotide probes and hybridisation details are described in probeBase (Greuter et al., 2016). Hybridised samples were observed using a Zeisss LSM 710 Meta Confocal Laser Scanning Microscope (CLSM), with a magnification of 400x, using an argon laser (488 nm), helium laser (543 nm) and a red diode laser (637 nm). Daime Software (Daims et al., 2006) was used to analyse 20 photos that were taken in several regions of the wells containing the biomass samples. The ratio between the area of the *Accumulibacter* or *Competibacter* population over the Cy5-labelled EUBmix population yields the % biovolume of each organism. The standard error of the mean (SEM) is calculated as the standard deviation divided by the square root of the number of images.

Ppk gene clone library construction and phylogenetic analysis

For this study, there was a need to accurately classify the three communities obtained in this work to the sub-clade level of *Accumulibacter*. *Accumulibacter* Types/clades/clusters are all very close phylogenetically, requiring near-complete and highly accurate gene sequence analysis to detect these subtle differences. Sanger sequencing is the gold standard for this purpose, generating long (up to 1000 bp) and highly accurate (> 99.9 %) sequence reads. However, Sanger is ineffective at sequencing cultures comprised of many different unknown organisms. Cloning is a reliable technique to separate amplicons from diverse organisms prior to Sanger and was thus employed in this work prior to Sanger sequencing. Biomass samples were taken during the three steady-state periods of the process and centrifuged for 3 minutes at 10 000 rpm. Pellets were stored at -20 °C until DNA extraction. Genomic DNA (gDNA) was extracted using the DNeasy UltraClean Microbial Kit (Qiagen, Germany) and quantified with a NanodropTM 1000 spectrophotometer (Thermo Fisher Scientific, Rockford IL). The quality and integrity of the extracted gDNA was assessed in 1 % agarose gel electrophoresis. The gDNA was stored at -20 °C until processing. Polymerase Chain Reaction (PCR) was done to amplify the polyphosphate kinase 1 gene (ppk1) using the general primers ACC-ppk1-254f (TCACCACCGACGGCAAGAC) and ACC-ppk1-1376r (ACGATCATCAGCATCTTGGC) (McMahon et al., 2007). The PCR mixture was achieved using the NZYtaq II 2x Green Master Mix (NZYtech, Portugal) containing dNTPs, 2.5 mM of $MgCl_2$ and 2.5 U of NZYTaq II DNA polymerase, to which were added

400 nM of each forward and reverse primer, 100 ng of template gDNA and nuclease free water to a final volume of 25 μL. Negative controls were made with nuclease free water and no template. The PCR cycling program started with an initial denaturation step for 10 minutes at 95 °C, followed by 30 cycles of denaturation step at 95 °C for 30 seconds, annealing of the primers at 68 °C for 1 minute and extension of the DNA at 72 °C for 2 minutes. In the end of the cycle, a final extension of 12 minutes at 72 °C was performed. The DNA fragments were then purified using the NZYGelpure kit (NZYtech, Portugal), where the correct bands were confirmed through agarose gel electrophoresis and amplicons were quantified using a NanodropTM 1000 spectrophotometer (Thermo Fisher Scientific, Rockford IL). The targeted ppk1 fragments were cloned in a pGEM-T vector using the pGEM-T Easy Vector Systems kit, following the manufacturer's instructions (Promega, USA) using T4 DNA Ligase. Ligations were performed considering 55 ng of insert and 50 ng of vector to a final insert/vector molar ratio of 3:1. Reactions were incubated overnight at 4 °C and transformations of E. coli JM109 cells were made by heat shock of the cells for 50 seconds at 42 °C, followed by 2 minutes on ice. Transformation cultures were plated in LB medium supplemented with IPTG 100 mM, X-Gal 50 mg/mL and ampicillin to a final concentration of 100 μg/mL. For each reactor operation period, 50 white colonies were selected and isolated. Screening of the clones with the correct insert was performed and PCR products were Sanger-sequenced by STABvida (Portugal). Bidirectional FASTA sequences were processed using Geneious Prime software (v2019.1). Consensus sequences were obtained after deNOVO assembly of the forward and reverse sequences. Multiple Sequence Alignment (MSA) was done using the MUSCLE algorithm and phylogenetic trees were constructed using the Neighbour-Joining method with 1000 bootstraps, both included in the Geneious package. MSA was trimmed up to 866 bp using ORF (Opean Reading Frame) Finder to search for the ORFs within the query sequences. Chimeric sequences were identified and removed using Bellerophon (Huber et al., 2004) and BLAST was used as the database for the phylogenetic classification of the clones, based on a pairwise identity of 97 %. The nucleotide sequences were deposited in the GenBank/NCBI database with the accession numbers MW034869-MW034998.

3.3 Results

3.3.1 Reactor performance

The SBR was seeded with WWTP sludge and operated > 300 days using conditions previously proposed as ideal to enrich for PAOs (Carvalheira et al., 2014b). After

inoculation, a gradual increase of the COD concentration was applied until reaching 200 mg COD/L, leading to an increase in anaerobic P release and aerobic P uptake as a response to each COD change. However, the overall P removal efficiency in the start-up period was lower than 40 %.

3.3.2 Steady state period 1

Poor EBPR performance was maintained for more than 3 SRTs after the start-up period, where 200 mg COD/L of VFA were added in the reactor each cycle. Figure 3.1 shows the anaerobic P/VFA ratio and aerobic P removal, with P removal remaining low (≤ 40 %). The experimentally determined stoichiometry for all steady state periods is summarized in Table 3.1, while cycle profiles in the anaerobic and aerobic phase are presented in Figure 3.2. Although VFAs were consumed in the anaerobic phase, the average P/VFA ratio was consistently low (0.29 P-mol/C-mol) as compared to values usually reported in literature for enriched PAO reactors with similar carbon sources (> 0.40 P-mol/C-mol). Glycogen hydrolysis/VFA uptake ratio (Gly/VFA) was 0.79 C-mol/C-mol, supporting the expression of GAO metabolism by the culture (Carvalheira et al., 2014a). The PHA production/VFA uptake ratio was 1.85 C-mol/C-mol with 0.84 and 0.99 C-mol/C-mol of PHB/VFA and PHV/VFA, respectively. Enriched PAO systems fed with a similar fraction of acetate and propionate produce less total PHA with a lower PHV fraction (Carvalheira et al., 2014a), where the higher PHV and overall PHA production can be linked to GAO metabolism. Table 3.2 shows the anaerobic P release and P uptake rates, which were 25.03 and 21.22 mg P/g X.h, respectively, where the P concentration in the effluent was 16.03 ± 2.78 mg P/L, clearly confirming the poor P removal.

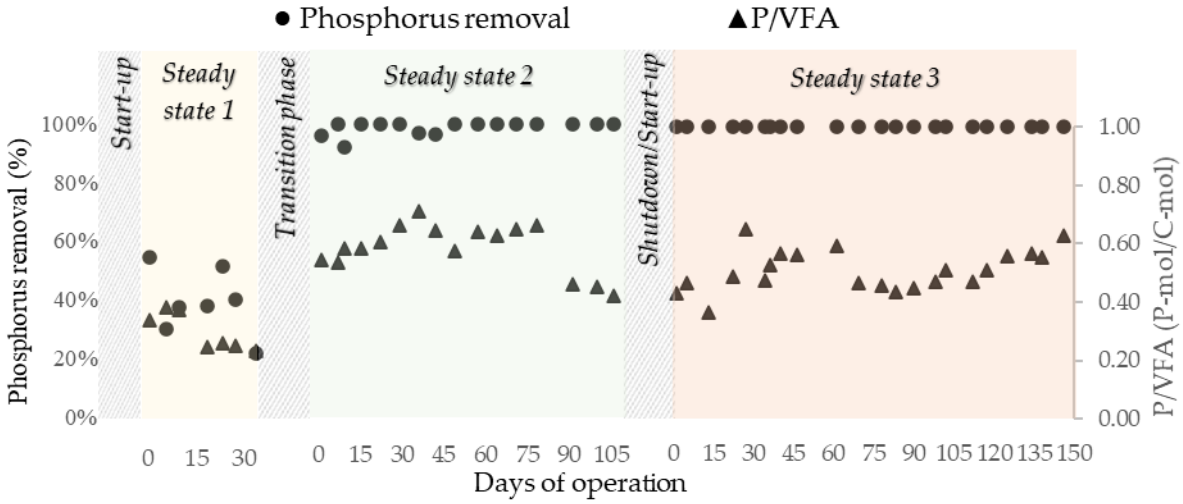

Figure 3.1 Phosphorus removal % and anaerobic P/VFA ratio during three steady state periods of reactor's operation.

3.3.3 Steady state period 2

After steady state period 1, the carbon feeding rate was decreased 10 times, from 40 to 4 mg COD/L.min, but with the same final concentration of COD in the reactor (200 mg COD/L). Immediately after changing the feeding rate, a big increase of P removal was observed, until reaching 99 % P removal (P effluent concentration of 0.21 ± 0.41 mg P/L). The anaerobic P/VFA ratio doubled, with an average of 0.58 P-mol/C-mol, supporting a shift towards PAO metabolism. VFAs were consumed along the extended period of COD addition, and the anaerobic Gly/VFA ratio decreased to 0.51 C-mol/C-mol, indicating a decrease in glycogen use as an energy source, and consistent with PAO metabolism. The anaerobic PHA/VFA ratio also decreased to 1.56 C-mol/C-mol, from which PHB/VFA was 0.94 and PHV/VFA significantly decreased to 0.58 C-mol/C-mol, further supporting a shift from GAO to PAO metabolism. P uptake took place mostly in the first hour of the aerobic phase (Figure 3.2b). The P release and uptake rates were considerably higher in this period (Table 3.2), at 51.46 and 55.63 mg P/g X.h, respectively, as compared to period 1. Each of these indicators confirm a shift in metabolic activity towards PAO metabolism, where good EBPR performance was achieved.

Table 3.1 Metabolic ratios for the three steady state periods: phosphorus release/VFA uptake ratio, glycogen degradation/VFA uptake ratio and PHA, PHB and PHV production/VFA uptake ratio.

Samples	P/VFA (P-mol/C-mol)	Gly/VFA (C-mol/C-mol)	PHA/VFA (C-mol/C-mol)	PHB/VFA (C-mol/C-mol)	PHV/VFA (C-mol/C-mol)
Steady state 1	0.29 ± 0.06	0.79 ± 0.18	1.85 ± 0.17	0.84 ± 0.07	0.99 ± 0.11
Steady state 2	0.58 ± 0.08	0.51 ± 0.16	1.56 ± 0.17	0.94 ± 0.09	0.58 ± 0.07
Steady state 3	0.51 ± 0.07	0.46 ± 0.17	1.54 ± 0.13	0.88 ± 0.08	0.59 ± 0.06

3.3.4 Steady state period 3

In steady state period 3, no P was detected in the effluent, so > 99 % of P removal was achieved with 150 mg COD/L and a feeding rate of 3 mg COD/L.min. The slow feeding rate strategy led to stable P removal performance, even at this lower COD level. The VFAs were consumed immediately, where no accumulation of VFA was detected, and the anaerobic P/VFA ratio was similar to steady-state period 2 at 0.51 P-mol/C-mol, as was the Gly/VFA ratio (0.46 C-mol/C-mol). The anaerobic PHA/VFA ratio was 1.54 C-mol/C-mol, with similar PHB/VFA and PHV/VFA ratios of 0.88 and 0.59 C-mol/C-mol, respectively, compared to steady state period 2. The anaerobic P release rate decreased to 48.48 mg P/g X.h, due to the lower carbon concentration fed to the reactor, but the P uptake rate was kept at an average 55.57 mg P/g X.h for more than 15 SRTs of stable

reactor operation. Overall, good P removal performance with the typical PAO metabolism was maintained in steady state period 3 (Figure 3.2c).

Table 3.2 Anaerobic P release and aerobic P uptake rate for the three steady state periods.

Period	Anaerobic P release		Aerobic P uptake	
	mg P/g X.h	Std. deviation	mg P/g X.h	Std. deviation
Steady state 1	25.03	2.46	21.22	0.79
Steady state 2	51.46	9.27	55.63	10.45
Steady state 3	48.48	8.15	55.57	7.83

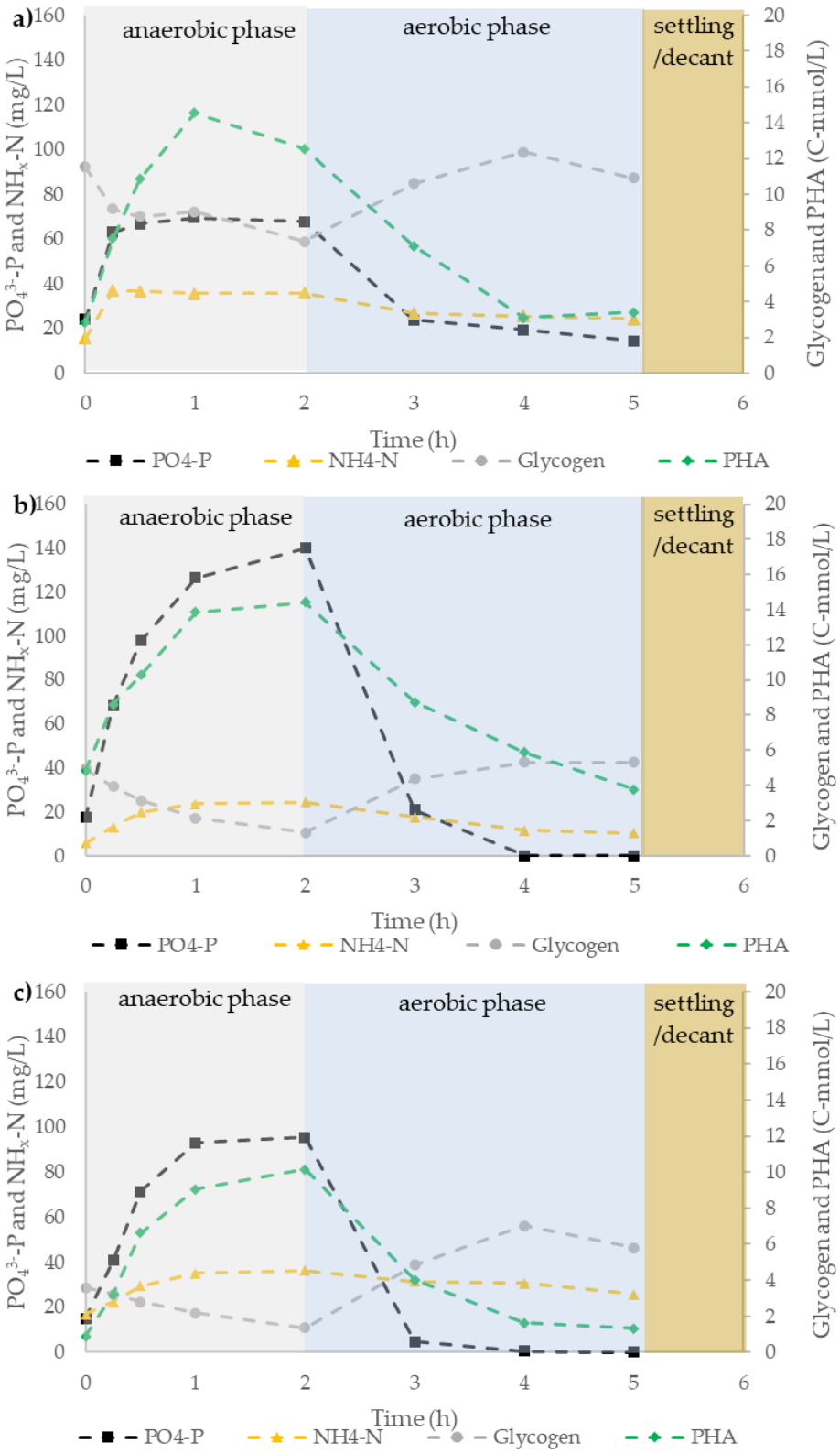

Figure 3.2 Profile of PO_4^{3-}-P, NH_4-N, glycogen and PHA in the reactor during a) steady state period 1; b) steady state period 2 and c) steady state period 3.

3.3.5 Microbial community

Overall PAO and GAO community analysis

Qualitative FISH analyses were performed throughout the reactor operation to follow the microbiological community changes (see Appendix A), and quantitative FISH was performed in the three steady state periods to quantify the microbial groups most relevant for EBPR, i.e., PAOs and GAOs. Figure 3.3 shows quantitative FISH results for *Accumulibacter* Types I and II, which is the finest-level classification possible based on the 16S sequence, and for *Competibacter*. In steady state period 1, *Accumulibacter* Type II was dominant, representing 86.9 % of total bacterial biovolume, while *Accumulibacter* Type I had a very low abundance of 2.3 %. *Competibacter* GAOs were present (13.5 %), but in lower abundance as compared to the total *Accumulibacter* population. After changing the carbon feeding rate from fast to slow, there was a shift in the *Accumulibacter* composition, where Type I increased to 78 % and Type II almost completely disappeared (0.1 %). In this period, a small decrease in *Competibacter* was also observed (7.4 %). A shift in the *Accumulibacter* composition occurred again in steady state period 3. Type I decreased to 10.1 % and Type II proliferated to 84.5 %, similarly to steady state period 1. The *Competibacter* abundance was lowest in this period at 2.0 %. Other GAOs, including *Defluviicoccus* and *Propionivibrio*, were never found at levels > 1 % of all bacteria in any of the steady state periods.

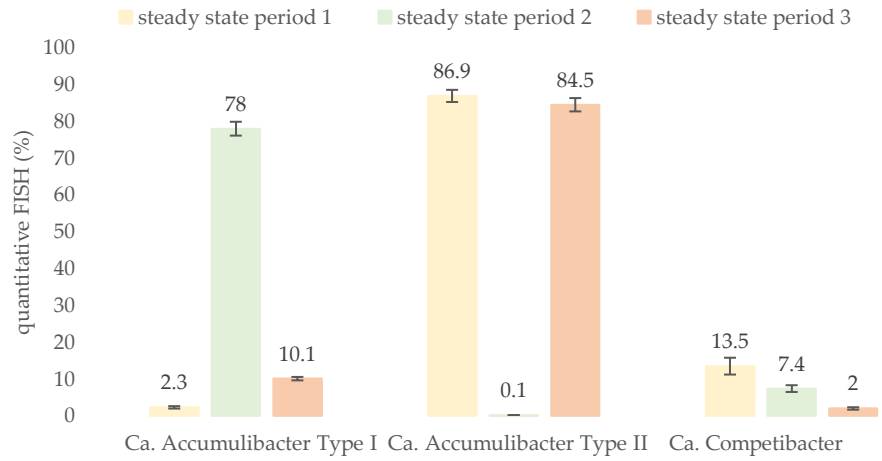

Figure 3.3 FISH quantification results for *Accumulibacter* Types I and II and *Competibacter* in the three steady state periods. The bars represent standard error of the mean (SEM).

Fine-scale PAO community analysis

Regarding the limited capacity of differentiation of 16S-based FISH probes, we investigated the sequence of the gene encoding for the polyphosphate kinase (*ppk1*), in order to further investigate the clade composition of *Accumulibacter* on a finer level. The phylogenetic tree topology (Figure 3.4) supports the FISH quantification results, showing the dominance of *Accumulibacter* Type II in steady state periods 1 and 3, while Type I was dominant in steady state period 2. The phylogenetic output of the Neighbour-joining tree groups the sequences according to their similarity, which leads to a robust differentiation of several clusters, namely one cluster within Type I and four clusters within Type II. The Type I cluster belongs to clade IA, whereas members of clade IIA and IIC were detected from Type II. The sequences for clade IIA are all grouped in the same cluster and the sequences of clade IIC are further sub-divided into three clusters, or sub-clades, IICi, IICii and IICiii. Steady state period 1 was composed of members of clade IIA and members of clade IIC clusters ii and iii. Steady state period 2 shifted to clade IA, while finally in steady state period 3, the community shifted again towards clade IIC, but in this case was mostly composed of members of cluster IICi.

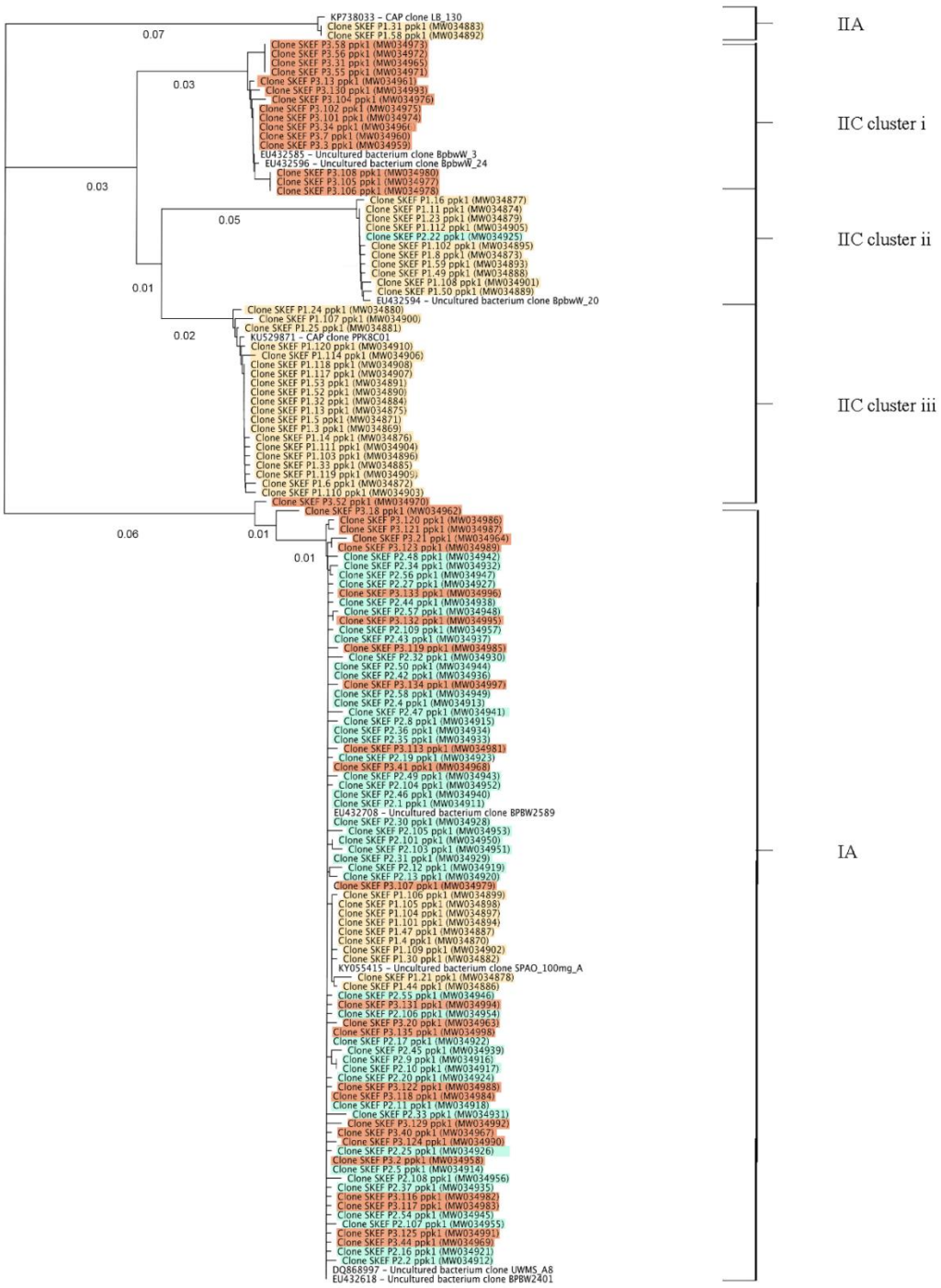

Figure 3.4 Neighbour joining phylogenetic tree showing the identification of *Candidatus* Accumulibacter phosphatis detected in the three steady state periods, based on the *ppk1* marker gene. Bootstrap values are shown on the tree branches with 1000 bootstrap replicates. GenBank accession numbers are shown between the brackets, where P1, P2 and P3 correspond to samples collected during steady state of periods 1 (yellow), 2 (green) and 3 (orange), respectively.

3.4 Discussion

3.4.1 The link between reactor performance and microbial community profile

The mixture of acetate and propionate as carbon substrate, as well as the relatively low COD/P ratio should promote the proliferation of PAOs and good EBPR performance (Carvalheira et al., 2014b; Lu et al., 2006; Oehmen et al., 2007). Carvalheira et al. (2014a, 2014b, 2014c) used similar conditions in their studies, as conditions applied in this study (period 1) and achieved > 95 % of P removal with high *Accumulibacter* and low *Competibacter* abundance (71 - 85 % and 17 - 21 %, respectively). However, in this study, P removal was low throughout steady state period 1 (Figure 3.1) even with a similarly high enrichment of *Accumulibacter* (> 85 %, Figure 3.3) and lower *Competibacter* abundance as compared to Carvalheira et al. (2014a, 2014b, 2014c). The carbon substrate was fully consumed, but low stoichiometric P/VFA and high Gly/VFA ratios suggested that glycogen was an important source of energy for acetate and propionate uptake. The Gly/VFA and PHA/VFA ratios were similar to those reported in the study of Carvalheira et al. (2014a) for a GAO dominated SBR, strongly suggesting the expression of a mixed PAO/GAO metabolism, in steady state period 1. Furthermore, the composition of PHAs produced in the anaerobic phase also indicate GAO metabolism, with a high fraction of PHV and high total PHA/VFA ratio (Zhou et al., 2008). While *Competibacter* was present in the SBR in period 1, its relatively low abundance should not have negatively impacted the P removal performance. The acetate uptake rates for *Accumulibacter* and *Competibacter* are very similar, while propionate uptake rate is significantly higher for *Accumulibacter* (Lopez-Vazquez et al., 2009), indicating that even with some VFA consumption by *Competibacter* present in lower abundance (< 13.5 %), most of the VFAs (85 %, or approximately 170 mg COD/L) were consumed by *Accumulibacter*. Indeed, in period 3 where only 150 mg COD/L was fed to the SBR, complete P removal was still achieved, supporting the fact that the VFA level in the feed during period 1 should have been sufficient to achieve a low P concentration in the effluent. Thus, the *Accumulibacter* population observed during period 1 displayed ineffective P removal capacity.

The carbon feeding rate was previously shown to influence the PAO/GAO competition, where *Accumulibacter* displayed a competitive advantage over *Defluviicoccus* GAOs with a slow carbon feeding strategy (Tu and Schuler, 2013). Their study suggested that GAOs correspond to r-strategists, while PAOs correspond to K strategists. In this study, the decreased carbon feeding rate in steady state period 2 immediately caused an improvement in EBPR performance, though without a corresponding shift in the PAO vs GAO global abundances. Instead, the *Accumulibacter*

population shifted from clade IIC to clade IA during these periods. Moreover, in steady-state period 3, the P removal performance and characteristic PAO metabolism was maintained by the SBR, despite the lower organic carbon source addition and a further shift in the *Accumulibacter* population from clade IA back to clade IIC. This result prompted further phylogenetic characterization of the bacterial community, which indicated three distinct clade IIC clusters: i, ii and iii (Figure 3.4). With a fast carbon feeding rate strategy, clade IIA and IIC clusters ii and iii were present inside the bioreactor, which exhibited a mixed PAO/GAO metabolism. It should be noted that clade IIC has been previously shown to be capable of expressing GAO metabolism under P-limiting conditions (Welles et al., 2017), suggesting that the poor EBPR performance in this period could be attributed to the high abundance of the clade IIC clusters. In steady state period 3, the phylogenetically distant cluster i from clade IIC was detected. In contrast to clusters ii and iii from clade IIC in period 1 that exhibited GAO metabolism, this cluster was linked with good EBPR performance and the typical PAO metabolism. Thus, it is clear that metabolic differences exhibiting a high impact on EBPR performance can be linked within the previously identified *Accumulibacter* clades, highlighting that knowledge of intra-clade diversity is just as important as inter-clade diversity for this key group of organisms. Furthermore, this study also shows that the characteristic GAO metabolism can also be employed by some members of the *Accumulibacter* even when the influent P concentration is not limiting, implying that *Accumulibacter* clade IIC clusters ii and iii are effectively GAOs and are undesirable organisms in EBPR plants.

3.4.2 *Accumulibacter* diversity and its link with metabolism

Over the past 10 years, EBPR studies have more frequently linked the process performance with not only the abundance of the total *Accumulibacter* group or other groups of PAOs or GAOs, but also the dominant Type or clade of these organisms. Table 3.3 provides an overview of the results of literature studies in this regard for *Accumulibacter*, focussing on metabolic aspects including denitrification capacity, PAO or GAO metabolic shifts, as well as the toxicity of nitrite and free nitrous acid (FNA). Several inconsistencies can be found in studies examining links between metabolism and Type I and Type II *Accumulibacter*, but other studies have also noted inconsistencies even at the clade level. Rubio-Rincón et al. (2019a) selected *Accumulibacter* clade IC, which was not able to perform anoxic P removal using nitrate, but the study of Camejo et al. (2019) showed that anoxic P uptake did proceed with nitrate for *Accumulibacter* clade IC. Thus, in addition to this study, whereby clusters within *Accumulibacter* clade IIC led to distinct PAO or GAO metabolism, intra-clade diversity can also lead to other important

metabolic differences within EBPR systems. The present study emphasizes the complexity of EBPR microbial ecology, where poor EBPR performance cannot be solely attributed to GAOs, but also to intra-clade phylogenetic diversity within *Accumulibacter*, a group of organisms that is typically regarded as a PAO. While *Accumulibacter* is known to be capable of GAO metabolism under P-limiting conditions, the fact that that there's differences at the sub-clade level that impacts performance even when P is not limiting, suggests for the first time that some *Accumulibacter* are effectively GAOs. Indeed, *Accumulibacter* should be considered as a diverse group of organisms rather than a single organism with a unique phenotype. This has a significant impact on future research in EBPR, whereby such a consideration is not always implied during studies investigating the link between the microbial community composition, its metabolism and the EBPR process performance.

Table 3.3 The link between the phylogenetic diversity of *Accumulibacter* and observed metabolic correlations.

Study reference	Type/ Clade	Denitrification capacity		Metabolism		Toxicity tolerance
		NO_3	NO_2	PAO	GAO	NO_2/FNA
(Acevedo et al., 2017, 2012; Lanham et al., 2011; Rubio-Rincón et al., 2017; Tian et al., 2013, 2017; Welles et al., 2016, 2015)	I	✓✖	✓	✓	✓✖	-
(Acevedo et al., 2012; Meng et al., 2020; Tian et al., 2013, 2017; Wang et al., 2015; Welles et al., 2016, 2015)	II	✓✖	✓	✓	✓	high
(Fan et al., 2020a, 2020b; Flowers et al., 2013, 2009; Gao et al., 2019; Slater et al., 2010; this study)	IA	✓	✓	✓	-	-
(Camejo et al., 2019, 2016; Gao et al., 2019; F. J. Rubio-Rincón et al., 2019a; Saad et al., 2016)	IC	✓✖	✓	✓	-	-
(Flowers et al., 2013, 2009)	IIA	✖	-	✓	-	-
(N. S. Fan et al., 2020; Z. Fan et al., 2020b; Slater et al., 2010; Welles et al., 2017; Zeng et al., 2017a, 2017b, 2016a, 2016c; this study)	IIC	✓✖	✓	✓	✓✖	low
(Zeng et al., 2017a, 2017b, 2016a, 2016b)	IID	-	✓	✓	-	high
(B. Wang et al., 2020; Zeng et al., 2017b, 2016b, 2016c)	IIF	✓	-	✓	-	low

✓ - positive association of the denitrification capacity or inherent metabolism with the specific *Accumulibacter* Type/clade

✖ - negative association of the denitrification capacity and inherent metabolism with the specific *Accumulibacter* Type/clade

✓✕ - contrasting results reported for denitrification capacity and inherent metabolism of the specific *Accumulibacter* Type/clade

3.5 Conclusions

This study shows that intra-clade diversity within *Accumulibacter* can influence the EBPR performance and lead to poor P removal, even without limiting levels of P in the wastewater influent. Thus, some members of the *Accumulibacter*, a group of organisms widely recognised as important PAOs, effectively behave like GAOs in EBPR systems. Future studies should focus on the link between EBPR performance and finer-scale characterisation of the *Accumulibacter* group, since recharacterization of the *Accumulibacter* group beyond the clade level may be necessary to understand the link between intra-clade diversities of *Accumulibacter* and EBPR performance. GAO-like activity should not be linked solely to *Competibacter* and other GAOs, but also specific clades of organisms currently recognised as PAOs.

3. *ACCUMULIBACTER* DIVERSITY AT THE SUB-CLADE LEVEL IMPACTS ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL PERFORMANCE

4. DICLOFENAC BIOTRANSFORMATION IN THE ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL PROCESS

4. DICLOFENAC BIOTRANSFORMATION IN THE ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL PROCESS

SUMMARY: Diclofenac is a pharmaceutical active compound frequently detected in wastewater and water bodies, and often reported to be persistent and difficult to biodegrade. While many previous studies have focussed on assessing diclofenac biodegradation in nitrification and denitrification processes, this study focusses on diclofenac biodegradation in the enhanced biological phosphorus removal (EBPR) process, where the efficiency of this process for diclofenac biodegradation as well as the metabolites generated are not well understood. An enrichment of *Accumulibacter* polyphosphate accumulating organisms (PAOs) was operated in an SBR for over 300 d, and acclimatized to 20 μg/L of diclofenac, which is in a similar range to that observed in domestic wastewater influents. The diclofenac biotransformation was monitored in four steady state periods and linked to the microbial community and metabolic behaviour in each period. Nitrification was observed in two of the four periods despite the addition of a nitrification inhibitor, and these periods were positively correlated with increased diclofenac biodegradation. Interestingly, in two periods with excellent phosphorus removal (> 99 %) and no nitrification, different levels of diclofenac biotransformation were observed. Period 2, enriched in *Accumulibacter* Type II achieved more significant diclofenac biotransformation (3.4 μg/g X), while period 4, enriched in *Accumulibacter* Type I achieved lower diclofenac biotransformation (0.4 μg/g X). In total, 23 transformation products were identified, with lower toxicity than the parent compound, enabling the elucidation of multiple metabolic pathways for diclofenac biotransformation. This study showed that PAOs can contribute to diclofenac biotransformation, yielding less toxic transformation products, and can complement the biodegradation carried out by other organisms in activated sludge, particularly nitrifiers.

Keywords: Pharmaceutical active compounds (PhACs), micropollutants, biological nutrient removal, biotransformation, *Candidatus* Accumulibacter phosphatis

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4. DICLOFENAC BIOTRANSFORMATION IN THE ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL PROCESS

4.1 Introduction

The vast number of pharmaceutical compounds detected in the aquatic environment has been a major concern in recent years. Pharmaceutical active compounds (PhACs) are synthesized to produce therapeutic effects on humans and animals. The growing demand for pharmaceutical compounds and the advances in medicine lead to the constant increase of PhACs, in addition to the thousands of PhACs already in use (Tiwari et al., 2017). After human or animal consumption, either the parent compounds or their transformation products (TPs) are excreted and end up in wastewater treatment plants (WWTPs) or in the aquatic environment. Although these compounds are usually detected in low concentrations, negative effects of long term exposure of aquatic organisms to low concentrations of PhACs has been widely reported (Turk Sekulic et al., 2019).

Although WWTPs are the source of PhACs discharge, they can also present a valuable barrier to control their introduction to the environment (Nguyen et al., 2021). The monitoring of PhACs as well as their transformation products and metabolic pathways presents a difficult task, due to the low concentration of the compounds and lack of available data. The monitoring of some PhACs is included in frameworks of several developed countries such as Australia, the European Union and the United States (Nguyen et al., 2021). The European Union has created a watch list of prioritized compounds, which includes the non-steroidal anti-inflammatory drug (NSAID) diclofenac (Barbosa et al., 2016; Loos et al., 2018).

Diclofenac is a commonly known pain killer, used worldwide and usually detected in higher concentrations in wastewaters (up to 20 μg/L) as compared to many PhACs (Petrović et al., 2014; Salgado et al., 2011; Sathishkumar et al., 2020). It has been considered as a compound with highest acute toxicity of NSAIDs and has shown to bioconcentrate in mussels and fish (Nguyen et al., 2021; Vieno and Sillanpää, 2014). Also, it was reported that DCF concentrations of 1μg/L can pose harmful effects on living species (Haap et al., 2008). Therefore, investigating its fate along different processes relevant to WWTPs is of great importance to successfully mitigate its environmental impact.

Biotransformation of multiple PhACs is an important degradation process within WWTPs, and can occur via primary metabolism, where PhACs are used as the main carbon substrate for cell growth, or by co-metabolism, where they are transformed in tandem with other substrates (Nguyen et al., 2021). Diclofenac has proven to be more resistant to biodegradation in conventional activated sludge processes and was classified as hardly biodegradable in anoxic conditions (Suarez et al., 2010), although

biodegradation was observed in certain situations under aerobic nitrifying conditions (Krkošek et al., 2014; Kruglova et al., 2014; Suarez et al., 2010; G. Wu et al., 2020). Furthermore, the identification and understanding of transformation products as well as their toxicity is very important, as some TPs can present the same or higher toxicity as the parent compound (Grabarczyk et al., 2020; Nguyen et al., 2021). Sorption of DCF to sludge has been found to be negligible in most studies (Deng et al., 2016; Terzic et al., 2018; Topuz et al., 2014; Zorita et al., 2009), though it can still be a significant mechanism of DCF removal in some situations (Lonappan et al., 2016b; Torresi et al., 2019).

While DCF in nitrification and denitrification processes has been frequently studied, less information is available regarding the impact of the enhanced biological phosphorus removal (EBPR) process on DCF biotransformation, where polyphosphate accumulating organisms (PAOs) grow under alternating anaerobic and aerobic conditions (Kolakovic et al., 2021). However, combinations of anaerobic and aerobic conditions can be of interest in the biodegradation of difficult to degrade PhACs (Alvarino et al., 2018; Nguyen et al., 2021). Some studies have investigated the removal of other PhACs by PAOs (Muz et al., 2014; Ogunlaja and Parker, 2018), while Zhao et al., (2020) investigated the impact of DCF toxicity to EBPR performance, where 0.01 mg/L of DCF had no significant negative impact on phosphorus removal. To the best of our knowledge, the only existing study of DCF biodegradation in EBPR was by Torresi et al., (2019), who investigated the degradation of several PhACs, including diclofenac, by PAOs in a moving bed biofilm reactor (MBBR). In their study, 8 – 72 % of DCF was transformed by aerobic PAOs, with denitrifying PAOs and aerobic glycogen accumulating organisms (GAOs) suggested to be less able to biodegrade DCF. However, much remains unknown regarding the impact of EBPR on DCF biodegradation, such as the capacity of conventional, non-biofilm EBPR processes to perform DCF biodegradation, the link between the microbial community and DCF biodegradation, as well as the transformation products that can be produced by PAOs. This motivated the research of the present chapter, in order to understand the role of PAOs in DCF biotransformation. The most widely known PAO in EBPR is *Accumulibacter*, where recent studies have shown that the specific *Accumulibacter* Type, clade or sub-clade can have an impact on the metabolic activity observed (Camejo et al., 2019; Kolakovic et al., 2021; F. J. Rubio-Rincón et al., 2019a). Thus, this chapter also examines the impact of *Accumulibacter* Type on DCF biodegradation efficiency.

The aim of this chapter is to evaluate the capacity of an enriched *Accumulibacter* PAO community to biodegrade DCF at environmentally relevant concentrations. A long-term bioreactor was subjected to several conditions leading to different ecological profiles, where the most abundant microbial community was identified and linked to

phosphorus removal and DCF transformation performance. The mechanisms of DCF biological removal were also evaluated, including the biotransformation products and their toxicity, as well as adsorption of DCF to the sludge. To the best of our knowledge, this is the first study assessing DCF biotransformation in a conventional EBPR reactor, identifying the transformation products, and comparing the DCF biotransformation by two distinct *Accumulibacter* PAO populations.

4.2 Materials and methods

4.2.1 Chemicals and reagents

HPLC-grade acetonitrile, methanol (p.a.) and formic acid were purchased from Panreac (Portugal). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). The diclofenac standard and meclofenamic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions (1 mg/mL) of each compound were prepared in methanol and stored at +4 °C. Working solutions were prepared by dilution of the stock solution in methanol. Prior to use, the mobile phase solutions were filtered through 0.2 μm SPARTAN filters (Whatman, Dassel, Germany). The laboratory water used in the study was provided by the Milli-Q water system (Millipore, CA, U.S.A.).

4.2.2 Lab-scale sequencing batch reactor (SBR) operation

A two-litre SBR reactor was operated with a 6 h cycle, 2 h anaerobic period and 3 h, 2.5 h or 2.75 h of aerobic period. After the aerobic phase, a settling/decant period for supernatant removal was performed. The hydraulic retention time (HRT) was 12 h and solids retention time (SRT) was 10 days. The temperature was controlled with a water bath at 20 ± 1 °C and pH at 7.0 - 7.5, by automatic addition of 0.1 M HCl or NaOH. Argon was bubbled to ensure anaerobic conditions, and dissolved oxygen was maintained at 8 ± 0.5 mg O_2/L with continuous aeration.

The reactor was fed with 1 L of feed solution, while 1 L of reactor was withdrawn after each cycle. The food solution contained 750 mL of solution A (phosphate feed) and 250 mL of solution B (carbon feed). Phosphate feed was fed during the first 5 min of the anaerobic phase to achieve a concentration of 16 mg P/L in the reactor, followed by the slow carbon feeding during 50 min to achieve a final COD concentration in the reactor of 150 or 100 mg COD/L. The carbon medium was composed of a micronutrient and

macronutrient solution, where acetate (HAc) and propionate (HPr) at a proportion of 75 - 25 % on a COD basis were the carbon sources. Allyl-N thiourea (ATU) was added in the carbon media to inhibit the nitrification. DCF was added with the carbon over the 50 min to achieve a 10 μg/L concentration in the reactor (20 μg/L in the feed) if fully consumed in the previous cycle, which is similar to the reported environmental concentrations found in previous studies (Salgado et al., 2011; Sathishkumar et al., 2020). The detailed phosphate and carbon media composition used in SBR operation was described in Kolakovic et al., (2021). The differences in carbon feed and the operating conditions in four periods are described in Table 4.1.

Table 4.1 Operating conditions in the SBR reactor in 4 steady-state periods.

Period	Duration	Concentrations in the feed			Anaerobic phase	Aerobic phase
	days	Total VFAs (mg COD/L)	ATU (mg ATU/L)	DCF (μg/L)	(h)	(h)
1	42	300	3	20	2	3
2	85	300	3	20	2	2.5
3	78	200	6 – 24	20	2	2.75
4	79	300	80	20	2	2.75

4.2.3 Sampling and analysis

i) Sampling and chemical analysis

Routine monitoring of the reactor was performed once a week. Total phosphates (P_{tot}), total suspended solids (TSS) and volatile suspended solids (VSS) were measured at the end of the aerobic phase. Phosphate (PO_4^{3-}-P), ammonium, diclofenac (DCF), volatile fatty acids (VFA), glycogen (Gly) and polyhydroxyalkanoates (PHA) were measured along the aerobic and anaerobic phases. Active biomass (X) was calculated as the VSS – PHA – Gly at the end of the aerobic phase. PO_4^{3-}-P, ammonium, DCF and VFAs were measured in the liquid phase (following centrifugation and filtration with 0.45 μm glass fibre membranes), P_{tot} in the total sample, DCF adsorbed to biomass was also measured (section *iv*), while Gly and PHAs were measured in lyophilized biomass pellets. Biomass samples were collected and stabilized for microbiological analysis as described in section *v*.

TSS and VSS were analysed by standard methods (APHA/AWWA/1995). For P_{tot} determination, acid digestion of 0.5 mL of sample suspension was performed with 5 mL of 0.3 M H_2SO_4 and 73 mg/L of $K_2S_2O_8$ during 1 h, further analysed with segmented flow analysis (Skalar 5100, Skalar Analytical, The Netherlands). Soluble phosphate and ammonium were also analysed by segmented flow analysis. VFAs were determined using high performance liquid chromatography (HPLC) with a DAD detector, using 0.01 N sulfuric acid as eluent, at a temperature of 30 °C and a Biorad Aminex HPX-87H 300 x 7.8 MM column. Glycogen samples were analysed after the acid digestion (0.9 M HCl for 3 h) of lyophilized biomass pellet, by HPLC with a VARIAN Metacarb 87H column and a Merck Differencial Refractometer RI-71 detector. The elution rate of the eluent (0.01 N sulfuric acid) was 0.5 mL/min at a 30 °C temperature. Biomass pellets for PHA analysis were prepared as described by Lanham et al. (2013) and measured by gas chromatography (GC) using a Bruker 430-GC with FID detector and a Restek column (60 m, 0.53 mm internal diameter, 1 mM df, crossbond). Diclofenac samples from the liquid phase were analysed by HPLC-DAD, after a solid phase extraction (SPE) procedure described in section *ii*. Identification of diclofenac transformation products was performed by LC-MS/MS as described in section *iii*. Biomass samples collected to determine the DCF adsorbed, were redissolved by Ultrasonic solvent extraction (see section *iv*) and analysed by HPLC-DAD.

ii) Diclofenac analysis in the liquid phase

DCF samples were concentrated using solid phase extraction Oasis MCX cartridges (60 mg, Waters, Germany). Samples were adjusted to pH 2.8 with sulfuric acid and spiked with meclofenamic acid as an internal standard. Each cartridge was previously conditioned with 1×2 mL heptane, 1×2 mL acetone, 3×2 mL methanol and 4×2 mL of MiliQ water at pH 2.8. After the conditioning step, samples were passed through the SPE cartridges at a flow rate of 10 - 20 mL/min and vacuum pressure of −5 psi, and further dried under a gentle nitrogen stream. Samples were eluted from the cartridges using 4x1 mL of acetone. The extracts were evaporated to 100 μL by a gentle nitrogen stream, then 300 μL of methanol was added and once again evaporated with nitrogen as described in Bouju et al. (2016). The final eluent was diluted with HPLC mobile phase and concentrated samples were further analysed using HPLC-DAD or HPLC-MS/MS.

The analysis of DCF was performed using an HPLC system (Thermo Scientific™, Dionex™, USA) coupled with a pump and controller, an in-line degasser, an autosampler and a photodiode array detector (DAD, Thermo Scientific™, Dionex™, USA). The column used was a Nucleodur C18 Pyramide column, 150x4mm, 3 μm

particle size, Merck, and diode array detector (DAD) from 200 to 400 nm. Two eluents were used for the mobile phase: water with 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B). The following linear gradient elution was applied: from 2 % to 40 % of B for 5 min, 40 % to 67 % B for 15 min, and finally 67 % to 98 % B for 5 min at 1.0 mL/min running for 15 min. The column temperature was 40 °C and the sample temperature was 25 °C. The volume of sample injected was 50 μl.

iii) Identification of diclofenac transformation products

To identify diclofenac transformation products generated from the SBR reactor, HPLC-MS/MS was used to complement the previous analytical techniques applied. The HPLC-MS/MS analysis of diclofenac commercial standards available was carried out using a Waters Acquity™ UPLC system (Milford, MA, U.S.A.) coupled to a triple quadrupole (TQ) mass spectrometer Acquity™ (Acquity, Waters) outfitted with electrospray ionization source (ESI). Capillary and cone were set respectively at 3.0 kV and 30 k, the source temperature was kept at 120 °C and the desolvation temperature at 350 °C. The compounds were ionized in negative mode and spectra were recorded in the range m/z 100 – 450. A reverse-phase chromatography column Mediterranea Sea 18, 2.2 μm, 10 x 0.21 (TeknoKroma, U.S.A.) and a pre-column were kept at 30 °C. The mobile phase consisted of water with 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B) at a flow rate of 0.30 mL/min. All solvents were filtered through a 0.22 μm PVDF membrane (Millipore, U.S.A.) prior to analysis. The system was run with the following gradient programme: 0 - 5 min from 60 % to 99 % B; 5 - 15 min at 99 % B, and finally a return to the initial conditions. The injection volume was 20 μL. MS spectra were analysed with a MassLynx™ software data acquisition system.

iv) Diclofenac extraction from biomass samples

Ultrasonic solvent extraction was used prior to SPE, to dissolute diclofenac adsorbed to the biomass as described in Salgado et al. (2010). Briefly, sludge samples were collected and centrifuged for 5 minutes at 10 000 rpm, supernatant was removed, and 2 g of biomass was washed with 2 mL of methanol mixed in an ultrasonic bath for 5 minutes (35 kHz). The slurry was then centrifuged for 1 minute at 10 000 rpm and the supernatant was collected in a separate vial. An additional washing step and mixing of the slurry in an ultrasonic bath were performed, followed by repeated centrifugation and the supernatant collection. The extracts were combined and evaporated under a gentle nitrogen stream to a volume of approximately 1 mL. The concentrated extract was

then diluted with 150 mL of MiliQ water and passed through SPE as previously described. Finally, the samples were analysed by HPLC-DAD for DCF and HPLC-MS/MS for determination of the transformation products.

v) Microbiological analysis

Biomass samples were collected for microbiological characterisation and fixed with 4 % paraformaldehyde (PFA) or ethanol as described by Nielsen (2009), then analysed by fluorescence *in situ* hybridization (FISH). The oligonucleotide probes applied to the biomass were: Fluorescein isothiocyanate (FITC)-labelled EUBmix probe that targets all Bacteria (mixture of EUB338 (Amann et al., 1990), EUB338-II and EUB338-III (Daims et al., 1999)) applied together with the following cyanine 3-labelled probes: PAO mix (a mixture of PAO651, PAO462 and PAO846) for *Candidatus* Accumulibacter phosphatis (Crocetti et al., 2000); ACC-I-444 and ACC-II-444 (Flowers et al., 2009) to target *Candidatus* Accumulibacter Phosphatis Types I and II, respectively; CPB_654 probe for *Candidatus* Competibater Phosphatis (Simon J. McIlroy et al., 2015); Prop207 was applied for *Candidatus* Propionivibrio aalborgensis (Albertsen et al., 2016); and SuperDFmix, a mixture of TFOmix (TFO_DF218 + TFO_DF618 (Wong et al., 2004), DFmix (DF988 + DF1020 (Meyer et al., 2006)) and DF198 (Nittami et al., 2009)) to target *Defluviicoccus vanus* clusters I, II and III, respectively. Other oligonucleotide probes and hybridisation details are described in probeBase (Greuter et al., 2016). Hybridised samples were observed using a Zeiss Imager D2 epifluorescence microscope, with a magnification of 1000x. Semiquantification of each of the specific probes against EUBmix was performed by visual inspection of a minimum 10 independent fields by an expert operator.

4.2.4 Prediction of the DCF transformation products toxicity

The proprieties of the DCF and the transformation products were predicted using the EPI (version 4.1) software (Syracuse Research Corporation (SRC) (U.S. Environmental Protection Agency version)), which is based on quantitative structure-activity relationships (QSARs). To predict the toxicity of the DCF and the transformation products, the ECOSAR™ application (2000-2012 U.S. Environmental Protection Agency) was used. The predicted results use QSAR equations based on the logKow and the toxicity of each compound when exposed to fish (96 h), daphnia (48 h) and green algae (96 h) to evaluate acute effects. Chronic effect concentrations for DCF and transformation products were also obtained with the ECOSAR software. For freshwater fish equations,

the model includes the blue gill sun fish (*Lepomis macrochirus*), the common carp (*Cyprinus carpio*), the fathead minnow (*Pimephales promelas*), the guppy (*Poecilia reticulate*), the rainbow trout (*Oncorhynchus mykiss*), the red killifish (*Oryzias latipes*) or the zebrafish (*Brachydanio rerio*). For freshwater invertebrates, species that the model includes are *Daphnia magna* or *Daphnia pulex*. For freshwater algae, species frequently included are *Desmodesmus subspicatus* or *Pseudokirchneriella subcapitata*. ECOSAR generated an LC50 value in mg/L for each product based on the molecular structure. The dataset of actual and predicted values includes metabolites from a broad range of chemical classes, differing greatly in their physical-chemical characteristics and modes of action.

4.3 Results and discussion

4.3.1 Diclofenac biotransformation and adsorption

Table 4.2 summarizes the main reactor parameters in four steady state periods with different DCF transformation efficiencies and Figure 4.1 represents cycle profiles of the main parameters. Glycogen and PHA profiles in these four periods can be observed in Figure B1 in the Appendix B. In period 1, the average P removal was > 98 %, with high ammonium consumption and nitrate production (Table 4.2), indicating that nitrifying activity accompanied aerobic P uptake. Diclofenac biotransformation showed a higher degree of variance as compared to P removal during the steady state periods, as shown by the μg of DCF transformed per g of active biomass (X). In the SBR, 7.4 μg/g X of DCF was transformed, mostly in the aerobic phase (Figure 4.1). This is consistent with the studies compared in Nguyen et al. (2021), where anaerobic and anoxic conditions proved to be less effective than aerobic conditions for micropollutant transformation. In period 2, P removal was 100 % and the nitrification activity decreased, as no nitrate and nitrite production were observed. The DCF biotransformation decreased as well with an average of 3.4 μg/g X of DCF being biotransformed. The P/VFA, PHA/VFA and Gly/VFA ratios remained similar in periods 1 and 2 with values reflective of a typical PAO metabolism (Carvalheira et al., 2014a; Kolakovic et al., 2021). In period 3, the EBPR performance deteriorated with less than 30 % of P removal. Although ATU concentration was increased several times in the reactor during this period, high ammonium consumption and NO_3 production was observed simultaneously with the highest average DCF biotransformation (11.1 μg/g X) recorded during the study. The anaerobic P/VFA ratio also decreased to 0.3, suggesting decreased PAO metabolic activity (Kolakovic et al., 2021). However, phosphorus removal recovered in the final

period 4 when a significantly higher level of ATU was added to inhibit nitrification (Table 4.1). During period 4, an initial low level of aerobic ammonium consumption was observed, which was followed by ammonium release. This may have been due to the decay of nitrifying biomass, supporting the decreased nitrifying activity as well. In this period, very low DCF transformation was observed as compared to previous periods (0.4 μg/g X). Nevertheless, it should be pointed out that 400 ng still represents a significant transformation level for a micropollutant in many WWTPs. The P/VFA ratio in this period was the highest during the reactor operation, reaching 0.73 P-mol/C-mol, confirming the high level of PAO activity. Comparing the results in the four periods highlights that increased diclofenac biotransformation accompanied increased nitrification in the EBPR reactor. This is consistent with other studies that achieved higher DCF degradation with nitrifying biomass (Krkošek et al., 2014; Kruglova et al., 2014; Suarez et al., 2010; G. Wu et al., 2020) as compared to heterotrophic organisms. However, since the objective of this study was to determine the potential role of PAOs in DCF biotransformation, periods 2 and 4 were investigated further, as nitrifying activity was negligible during these operational times.

Table 4.2 Removal efficiencies and metabolic ratios for four SBR operation periods: P removal, ammonium consumption and NO_3 production, DCF biotransformation, phosphorus release/VFA uptake ratio, PHA production/VFA uptake, glycogen degradation/VFA uptake ratio and adsorption to sludge.

	Conditions	P removal	Nitrogen		DCF biotransformation	P/VFA	PHA/VFA	Gly/VFA	Adsorption to sludge
		(%)	NH_x-N (mg/L)	NO_3 (mg/L)	(μg/g X)	(P-mol/C-mol)	(C-mol/C-mol)	(C-mol/C-mol)	(%)
1	150 mg/L COD, aerobic phase 3h	98.1 ± 3.7	14.4 ± 2.1	10.2 ± 0.0	7.4 ± 3.0	0.45 ± 0.05	1.63 ± 0.07	0.36 ± 0.13	-
2	150 mg/L COD, aerobic phase 2.5h	100 ± 0.0	5.7 ± 2.4	0.0 ± 0.0	3.4 ± 1.5	0.50 ± 0.06	1.48 ± 0.09	0.49 ± 0.21	0.1
3	100 mg/L COD, aerobic phase 2.75h	28.7 ± 9.7	11.7 ± 5.4	6.3 ± 3.8	11.1 ± 6.4	0.28 ± 0.13	1.47 ± 0.28	0.45 ± 0.14	-
4	150 mg/L COD, aerobic phase 2.75h	99.8 ± 0.4	0.0*	0.0 ± 0.0	0.4 ± 0.5	0.73 ± 0.11	1.37 ± 0.07	0.34 ± 0.17	5

*Initial aerobic consumption of 2.5 mg NH_3/L was observed, with subsequent ammonium release.

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In both periods 2 and 4, the level of DCF adsorption to the EBPR biomass was determined, and related to the total amount of DCF degradation, as determined by a mass balance (see Text B1 in the Appendix B for details). Adsorption was shown to be negligible in both operational periods (< 5 % of total DCF transformation). This is also in accordance with several studies where very low or no DCF adsorption to biomass was observed (Deng et al., 2016; Terzic et al., 2018; Topuz et al., 2014). Furthermore, the microbial community was analysed, and the results presented in Table 4.3 correlated well with the reactor performance observed in the four periods. *Accumulibacter* PAOs were the most abundant bacteria detected in all periods except in period 3, which corresponded to the period where low P removal was achieved. *Accumulibacter* Type II was dominant in periods 1 and 2 but decreased in period 3 and the most abundant PAO shifted to *Accumulibacter* Type I in period 4. Interestingly, DCF transformation in period 2 was significantly different than period 4, possibly indicating that DCF metabolism is influenced by different *Accumulibacter* Types. Higher DCF transformation was observed in the *Accumulibacter* Type II-dominated reactor, during period 2. Other metabolic differences between *Accumulibacter* clades and sub-clades have been found in recent studies as well (Camejo et al., 2019; Kolakovic et al., 2021; F. J. Rubio-Rincón et al., 2019a). The possibility of different DCF transformation by different Types, clades or sub-clades of *Accumulibacter* should be further investigated. In period 3, *Competibacter* GAOs dominated the SBR, while *Competibacter* was present, but not abundant, in periods 2 or 4 (Table 4.3). *Propionivibrio* and *Defluviicoccus* were practically non-existent in all of the operational periods, as shown in Table 4.3. Table B2 in the Appendix B shows FISH images of each group of PAOs and GAOs observed during this study. Increased nitrification activity led to the highest DCF transformation with decreased PAO activity and proliferation of GAO community. However, it is unclear if the presence of GAOs impacted DCF biotransformation, since similar transformations were achieved for both nitrification periods. In the study of Torresi et al. (2019), PAOs exhibited promising potential for DCF transformation (up to 80 %) in the MBBR reactor, while GAOs where unable to transform any DCF. However, that study did not analyse the microbial community, so the link between specific bacteria and DCF transformation was not possible. Furthermore, MBBR reactors are difficult to compare to conventional EBPR systems, as MBBR operation promotes much longer SRTs and microbial composition of biofilms can vary substantially as compared to conventional EBPR processes.

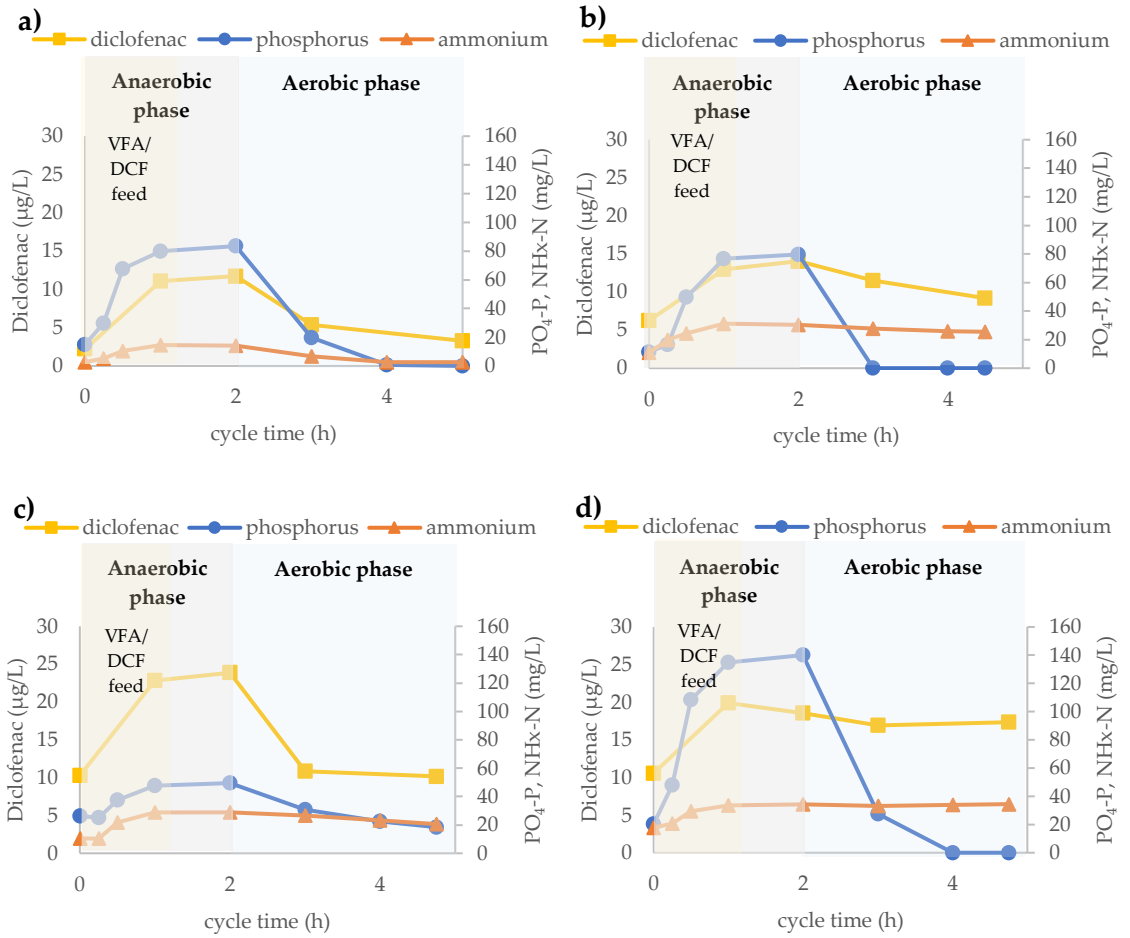

Figure 4.1 Profile of diclofenac and PO_4-P in the reactor during a) steady state period 1; b) steady state period 2; c) steady state period 3 and d) steady state period 4.

Table 4.3 FISH results for *Accumulibacter* Types I and II, *Competibacter*, *Propionivibrio* and *Defluvvicocus* in the four operation periods.

Period	**PAOmix** (*Accumulibacter*)	**Acc-I-444** (PAO Type I)	**Acc-II-444** (PAO Type II)	**CPB_654** (*Competibacter*)	**Prop207** (*Propionivibrio*)	**SuperDFmix** (*Defluviicoccus - rhodospirillaceae*)
1	+++	++	+++	++	+-	-
2	+++	+	+++	+-	+-	-
3	++	++	+	+++	+-	+-
4	+++	+++	+	+	-	+-

(-) non-existent; (+-) almost non-existent; (+) present; (++) abundant; (+++) dominant.

4.3.2 Diclofenac biotransformation pathway by PAOs

Since nitrification was completely inhibited in period 4, the DCF transformation products (TPs) were further investigated for this period. According to the chromatographic retention time, mass-to-charge ratio (m/z), the TPs in the fourth reactor operation period were identified by LC-MS/MS (ESI-) (Table B1, Appendix B). With the chemical structures detected during the biotransformation, it was possible to propose four metabolic pathways for the biotransformation of DCF by the enriched *Accumulibacter* PAO Type I culture (Figure 4.2). A total of 23 different TPs were identified and aligned in 4 possible pathways. Although most of the DCF biotransformation was observed in the aerobic phase, due to cyclic SBR operation, where anaerobic and aerobic phases were alternated, it was impossible to determine the exact phase where each TP was formed.

First of the DCF metabolic pathways corresponds to the formation of TPs 1, 2 and 3. TP 1 results from the cleavage of the amine atom and aromatic ring and subsequent autoxidation. TP 2 was further formed from TP 1 by hydroxylation of the aromatic ring and was also obtained with *Rhodococcus ruber* IEGM346 (Ivshina et al., 2019). A second metabolic pathway started with DCF dechlorination and hydroxylation of the first aromatic ring to form TP 4 and TP 5. After this, TPs 4 and 5 were hydroxylated step wisely into TPs 6 - 9, and then to TP 10, before being further oxidised by dehydrogenation to TP 13. The existence of electron donors and H^+ during the anaerobic process is one of the possible drivers for hydroxylation reactions (Schink et al., 2000; Sierra-García et al., 2014). From TP 4 to TP 10, the number of hydroxyl groups added to the aromatic ring varied from 1 to 5. In TPs 11, 12 and 13 the hydroxyl groups are converted to oxygen double bonds to the ring, forming quinone amines. The addition of oxygen by hydroxylation to the ring induces a structural rearrangement that leads to the loss of two hydrogens to form the double bond to oxygen, with loss of the double bond in the aromatic ring (Moreira et al., 2018). The formation of quinone amine species from the hydroxyl groups was observed in studies with the bacterial strain *Labrys portucalensis* F11 (Moreira et al., 2018), with *Rhodococcus ruber* IEGM346 (Ivshina et al., 2019) and *Pseudoxanthomonas sp*. DIN-3 (Lu et al., 2019).

The mono-hydroxylation of either of the aromatic rings of DCF can result in 4 isomers, from which it was possible to detect TP 14 (4'-hydroxydiclofenac) and TP 15 (5'-hydroxydiclofenac) in the third degradation pathway. These TPs are the primary products of transformation of DCF formed in activated sludge processes and frequently detected in DCF degradation studies (Nguyen et al., 2021; G. Wu et al., 2020). These TPs were also observed by other authors investigating DCF degradation by Actinobacteria

of the genus *Rhodococcus* (Tyumina et al., 2019), by the with fungi *Trametes* versicolor (Marco-Urrea et al., 2010), and by the white rot fungus *Phanerochaete sordida* YK-624 (Hata et al., 2010). It has been suggested that this hydroxylation is catalyzed via the cytochrome P450 system (Hata et al., 2010) or by mono- and dioxygenases (Moreira et al., 2018). Further on, from TP 14 and TP 15 to TP 21, hydroxylation and oxidation by dehydrogenation were the mechanisms involved in formation of subsequent TPs. Under aerobic conditions, the microorganisms use molecular oxygen as a co-substrate to introduce one, two or more oxygen atoms via oxygenase that usually contains metals present in the synthetic media. The metal-bound O_2 can be reduced to the peroxide and converted into a metal-bound oxygen atom that performs the attack on the molecular structure (Widdel and Musat, 2016). The degradation through hydroxylation may result from a chemical species present in the media acting as a reducing agent and helping the introduction of the hydroxyl groups in the double bond of the aromatic ring (Schink et al., 2000). Many bacteria and fungi produce enzymes that hydroxylate molecules, corresponding in most of the cases, to a first step in the degradation of aromatic compounds by mono- or dioxygenases (Moreira et al., 2018). One of the enzymes mostly reported as being responsible for this process is the cytochrome P450 (Domaradzka et al., 2015; Hata et al., 2010; Moreira et al., 2018). TPs 13, 16, 17 and 18 were also observed in biomass samples, meaning that they were adsorbed and desorbed in different phases.

A fourth pathway can be proposed based on the structures of TP 22 and TP 23, which possibly resulted from the degradation of TP 4, with the removal of two of the chlorines and hydroxylation of the aromatic rings. The elimination of the chlorine group could have resulted from the cleavage of the amine atom and aromatic ring and subsequent autoxidation, as reported for *Pseudoxanthomonas sp*. DIN-3 (Lu et al., 2019). Although some similar TPs to those in this study were previously detected with nitrifying and heterotrophic sludge systems (G. Wu et al., 2020), none of the TPs identified in this study had previously been found in EBPR processes, since this is the first study to analyse the TPs formed in these systems.

In the recent review of Nguyen et al. (2021), six transformation mechanisms of DCF were proposed: hydroxylation of aromatic rings, amidation/de-amidation, sulphate conjugation of phenolic hydroxyl groups, reductive dechlorination, oxidative ring-opening and oxidation by dehydrogenation of phenolic moieties. In our study, the hydroxylation and oxidation by dehydrogenation were the two main biotransformation pathways observed for DCF.

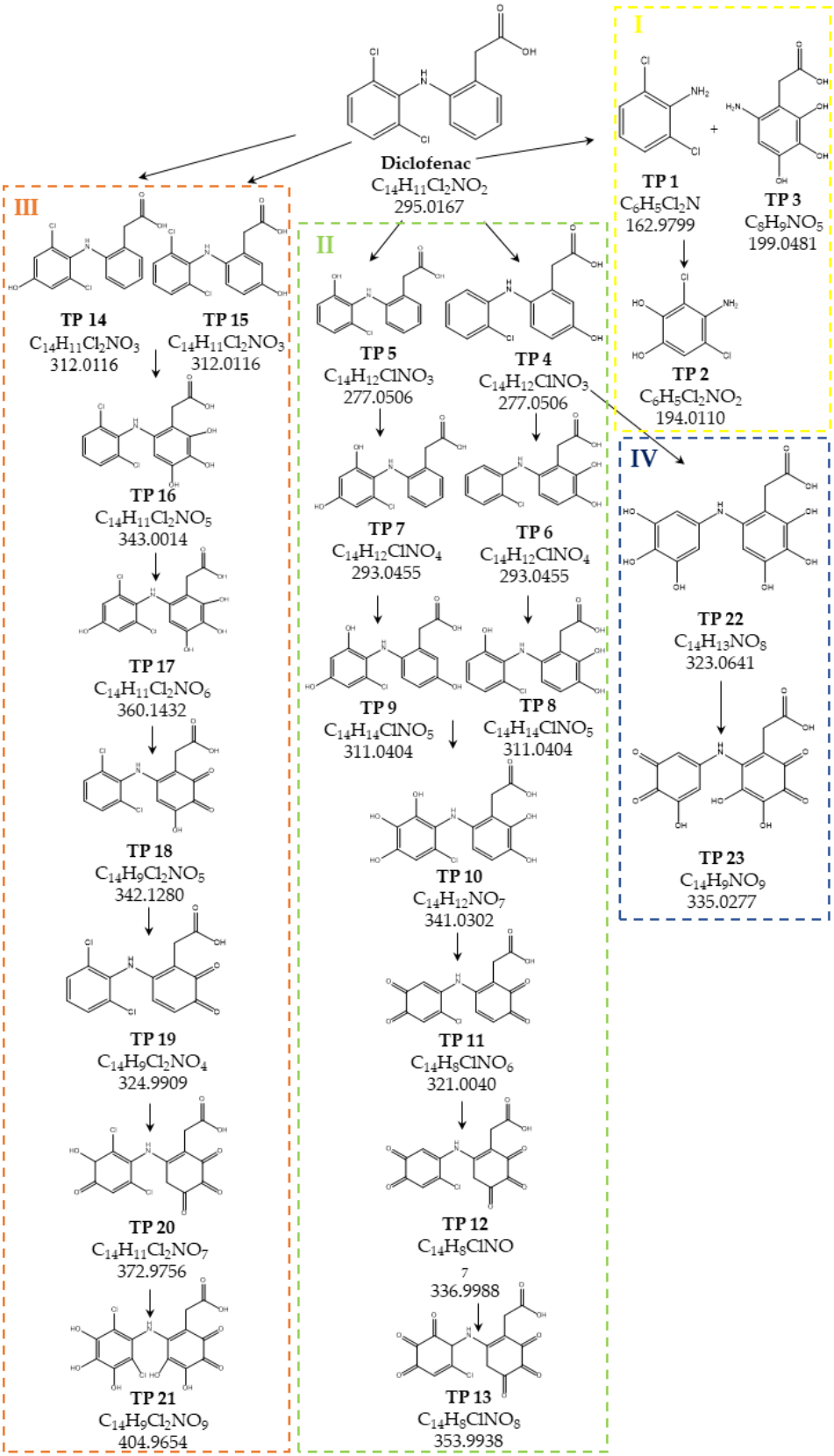

Figure 4.2 Proposed DCF degradation pathways in period 4 of the SBR reactor operation. Transformation products were identified by exact mass spectral data.

4.3.3 Ecotoxicological effects of diclofenac transformation products

The ecotoxicity of the different DCF TPs was predicted using the EPI suite software based on the ECOSAR model to predict the concentration that causes the death of 50 % of the tested organisms (LC50). In the toxicity assessments done using this method, the data of acute and chronic toxicity based on fish (96 h), daphnia (48 h) and green algae (96 h) were predicted to obtain the LC50 for DCF and the different TPs identified above. Logarithmic values of LC50 for chronic effect (acute effects not shown) were calculated for each organism and presented in Figure 4.3.

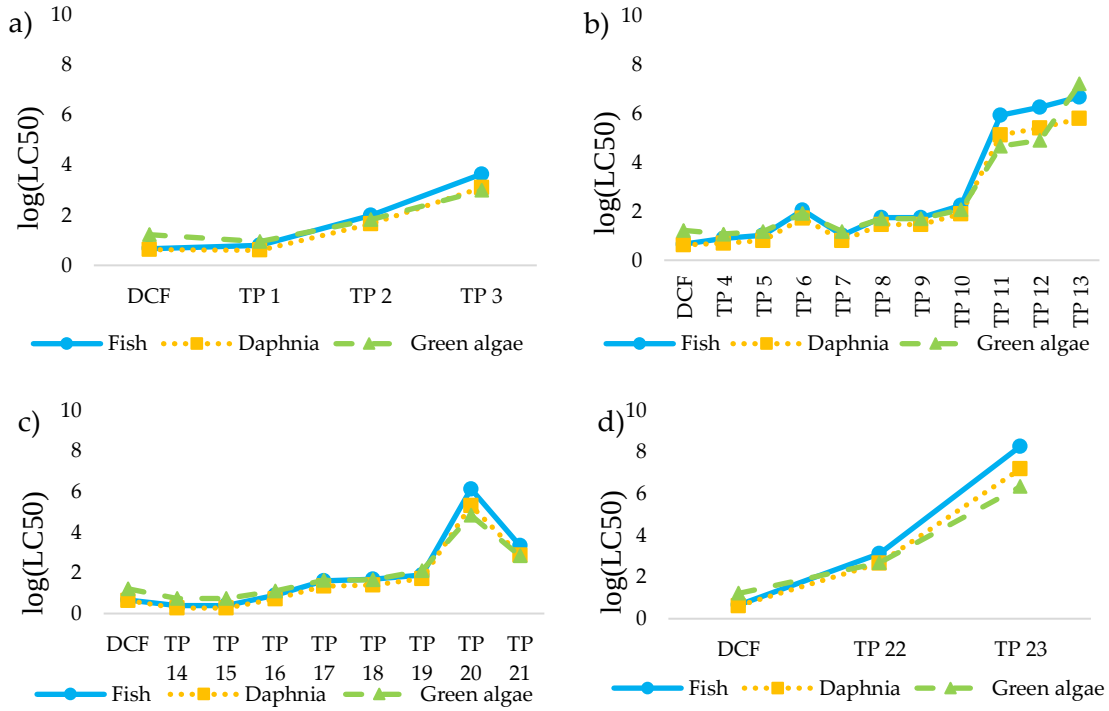

Figure 4.3 Ecotoxicity of the DCF transformation products predicted after fish, daphnia and green algae exposure (chronic ecotoxicity in logarithmic values of LC50) for: a) degradation pathway I; b) degradation pathway II; c) degradation pathway III and d) degradation pathway IV.

The ecotoxicity of the DCF parent compound proved to be equivalent or higher than the identified transformation products. The lethal concentration gradually increases (e.g., the toxicity decreases) with the progressive transformation of the chemical structures along the metabolic pathways suggested above. The LC50 for each compound is consistent for the fish, daphnia and green algae considered in this study. Cleuvers (2004) also did not find significant differences between the DCF toxicity using daphnia and the green algae *D. subspicatus*. The predicted logarithmic values of LC50 values in this study for DCF were 0.7 for fish, 0.6 for daphnia and 1.2 for green algae

(Figure 4.3). Logarithmic values of LC50 less than 1 can be classified as "toxic to aquatic organisms", LC50 between 1 and 2 can be considered as "harmful to aquatic organisms" and LC50 > 2 are not classified (Cleuvers, 2004; Licht et al., 2004). According to Lonappan et al., (2016), long term exposure of aquatic organisms to DCF, which promotes the accumulation of DCF in the organism, can lead to mortality by damaging renal and gastrointestinal tissues in fish. Studies with the green algae *Scenedesmus vacuolatus* demonstrated that DCF has a high toxicity when compared with the transformation products found in water, although some have similar toxicity to the parent compound. The toxicity of the DCF transformation products for the green algae was reported to be 10 times lower than that of DCF (Schulze et al., 2010). In our study, only commonly detected TPs 14 and 15 (4-hydroxydiclofenac and 5-hydroxydiclofenac) presented slightly higher toxicity according to the EPI suite software.

4.3.4 Implications of the study

Diclofenac is considered as one of the most recalcitrant micropollutants and has been typically reported to be only partially biodegraded by mainly nitrifying biomass in WWTPs (Kruglova et al., 2014; G. Wu et al., 2020). However, this study has demonstrated that *Accumulibacter* PAOs can also contribute towards diclofenac biotransformation in conventional activated sludge processes, where enrichment of Type I vs Type II was correlated with the concentration of diclofenac biodegraded. Furthermore, the four metabolic pathways proposed in this study during assessment of the transformation products revealed the formation of less toxic by-products in each pathway. This suggests that although *Accumulibacter* PAOs did not perform diclofenac biotransformation as efficiently as compared to nitrifiers, they could still contribute towards its overall degradation by forming TPs that were largely less dangerous for the environment than the parent compound. This opens a new line of investigation where biological processes could be interesting for diclofenac removal, as previous studies have reported higher toxicity of transformation products formed in e.g. physical/chemical processes (Diniz et al., 2015; Schmitt-Jansen et al., 2007). Schmitt-Jansen et al. (2007) and Schulze et al. (2010) have detected DCF products with six to ten times higher acute toxicity compared to the parent compound, while Diniz et al. (2015) showed higher ecotoxicity impacts associated with DCF TPs in photo-transformation processes. Osorio et al. (2016) investigated the toxicity of DCF and transformation products in nitrifying activated sludge and found only one TP to be slightly more toxic than the parent compound. However, some TPs of DCF had increased toxicity by synergetic effects when a mixture of TPs and other micropollutants were present in the aquatic environment (Osorio et al., 2016; Zind et al.,

2021). Thus, future research should focus on the link between toxicity of metabolic pathways and the transformation products as well as establishing operational strategies that would be viable in conventional biological WWTPs to detoxify DCF among other persistent micropollutants, potentially employing a wider diversity of organisms capable of performing biodegradation (e.g., nitrifiers and PAOs).

4.4 Conclusions

This study shows that *Accumulibacter*, a known PAO responsible for phosphorus removal in EBPR processes, can simultaneously contribute to diclofenac biotransformation into products that were less toxic than the parent compound. Adsorption of diclofenac to biomass was negligible, while four biotransformation metabolic pathways were identified with hydroxylation and oxidation by dehydrogenation as the main transformation mechanisms. Two distinct *Accumulibacter* populations showed different transformation capacities, where Type II was capable of biotransforming diclofenac more efficiently than Type I. Nitrifying activity was shown to be linked to substantial DCF biotransformation, which is consistent with previous reports, but PAO activity showed the potential to contribute towards detoxifying diclofenac and its transformation products.

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5. PHOSPHORUS AND CARBON SOLUBILISATION STRATEGIES FOR WASTEWATER SLUDGE VALORISATION

5. PHOSPHORUS AND CARBON SOLUBILISATION STRATEGIES FOR WASTEWATER SLUDGE VALORISATION

SUMMARY: Phosphorus and carbon solubilisation from wastewater sludges is a difficult task due to specific sludge characteristics and low biodegradability of sludges. This work evaluates operational strategies to solubilise phosphorus and/or carbon from different municipal sludges in a bioacidification reactor operated at low sludge retention time (SRT) compared to conventional digestors, with the objective of valorising sludge and recovering resources onsite of a water resource recovery facility (WRRF). The individual impact of three municipal sludges, three organic loading rates (OLR), two SRTs, two temperatures and thermal hydrolysis pre-treatment was assessed through a well-designed experimental methodology. Bioacidification provided a maximum 79 % of PO_4^{3-}-P/P_{tot} and 29 % of COD_{sol}/COD_{tot} solubilisation ratios, which was an increase of approximately 60 % and 26 %, respectively, compared to a non bioacidified sludge. Similar performance was achieved for the three different sludges and OLRs used in this study under SRT of 4 days and 35 °C. Kinetic tests elucidated that lower SRT (2 days) and temperature (20 °C) negatively impacted P release due to biological fermentation decrease. Iron and polyphosphate release was impacted by present microbial community, while calcium release was more dependent on pH. Organic phosphorus, nitrogen and carbon solubilisation was limited by low sludge hydrolysis and increased only after a thermal-alkaline sludge hydrolysis pre-treatment (pH 9, 80 °C for 1 h) was combined with the bioacidification. The results achieved in kinetic tests were confirmed in a pilot reactor, demonstrating the high potential of the bioacidification process to valorise sludge, and providing recommendations for its implementation onsite based on the individual requirements of each WRRF.

Keywords: phosphorus release, VFA production, metal solubilisation, bioacidification, wastewater sludge valorisation.

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Other portions of the work will be submitted in addition to another modelling work that is in progress.

5. PHOSPHORUS AND CARBON SOLUBILISATION STRATEGIES FOR WASTEWATER SLUDGE VALORISATION

5.1 Introduction

Phosphorus is an indispensable nutrient for all living organisms (Egle et al., 2016). It is mined from phosphate rock which is classified as a critical raw material of high economic importance, since on Earth there is no other substitute for mineral fertilizers. Furthermore, some regions are increasingly dependent on P imports due to the uneven distribution of P rock deposits worldwide (Childers et al., 2011; Cieślik and Konieczka, 2017). Water resource recovery facilities (WRRFs) can play an important role in the recovery and supply of P as biofertilizer, as 15 – 20 % of phosphate rock world demand is being lost as human waste through sewage waters (Wang et al., 2016). In WRRFs, P can be removed efficiently by enhanced biological P removal (EBPR), chemical P precipitation processes, or by combination of both. Potential recovery can be implemented in the treatment line to recover P from liquid phase, sludge, or sludge ash. Recovery yields may reach 30 % in the liquid phase and vary significantly in the sludge phase or sludge ash due to the large amount of P 'entrapped' in the sludge (Cornel and Schaum, 2009; Egle et al., 2016).

In recent years, some regions have been implementing policies that obligate P recovery from sludge (Günther et al., 2018). To comply with these regulations, WRRFs need to expand their process schemes with technologies that provide high P recovery yields with minimal operating and/or capital costs. Two emerging process schemes include: i) mono-incineration technologies or ii) processes for P dissolution from sludge (i.e., wet-chemical leaching, thermal or chemical hydrolysis, anaerobic digestion etc) combined with crystallization technologies. Although mono-incineration can reach more than 90 % efficiency, its implementation onsite is only feasible for large WRRFs or centralized facilities due to the high investment costs (Cieślik and Konieczka, 2017; Cornel and Schaum, 2009). Therefore, other process schemes have been investigated for P recovery onsite, with possible small adaptions in WRRF process line. Biological fermentation processes have been investigated due to simultaneous P release and generation of added-value products, with less chemical addition when compared to some chemical processes.

Biological acidification process (hereafter referred to as bioacidification process) can be implemented in WRRF sludge line as an additional digestion step. It can simultaneously treat different sludge types or mixtures, solubilise P and carbon (C) as phosphates (PO_4^{3-}-P) and volatile fatty acids (VFAs), respectively, and produce environmentally friendly fuel hydrogen (Chen et al., 2018). PO_4^{3-}-P can be precipitated for fertilizer production, while VFAs can be recirculated to improve the performance of the EBPR process and biogas or biopolymers production (i.e., polyhydroxyalkanoates - PHAs). However, the implementation of the bioacidification process at WRRFs requires

investigation of the optimal operational conditions for C and P solubilisation, since the performance of this process is highly affected by the different contents in P, readily biodegradable COD (rbCOD) and metals found in various types of municipal sludges. Inorganic P is composed of polyphosphates that are hydrolysed under anaerobic conditions (Cieślik and Konieczka, 2017; Wang et al., 2016) and precipitates associated to oxides and hydroxides of metals that are generally difficult to release in neutral pH digestion (He et al., 2016; Y. Li et al., 2019; Ping et al., 2020; Pokhrel et al., 2018). Organic P integrated in biomass cells can be released by sludge disintegration, bioconversion or microbial metabolism, but it is often reported as difficult to release during fermentation processes (He et al., 2016; Latif et al., 2017; Pokhrel et al., 2018).

Most of the available bioacidification kinetic studies that investigate the simultaneous release of P and C from municipal sludge focus on pH and batch operation (He et al., 2016; Liu et al., 2020; Pokhrel et al., 2018; Zou et al., 2018), neglecting the effect of continuous acclimatization of microorganisms on the overall process performance. Furthermore, operational strategies for sludge fermentation as a sole substrate are less studied compared to co-fermentation of sludge with other organic substrates or chemical additions (Guo et al., 2017; Yang et al., 2019; Zou et al., 2017). In studies with co-fermentation of organic substrates with distinct characteristics (i.e., fats, fruit and vegetable waste), the hydrolysis of the sludge can be affected due to the preference of the microorganisms for readily biodegradable organic substrates, that typically also have low P content (Y. Wu et al., 2020a). The reported efficiencies for sludge fermentation as a sole substrate are also contradictory due to different sludge types and operational conditions applied. For instance, 75 % of P was released from waste activated sludge (WAS) in the acidic fermentation at pH 5.5, SRT 12 days and 37 °C (Latif et al., 2017) while a maximum of 54 % P release from WAS was achieved under batch fermentation at pH 4 (Pokhrel et al., 2018). At neutral pH and 30 °C, only 35 % of P was released from the food processing WAS produced in WRRF with anaerobic/anoxic/aerobic (A^2O) configuration, with insignificant increase of P release (4 % increase) after applying thermal hydrolysis pre-treatment (Liu et al., 2020). Other studies report better P release or VFA production under batch fermentation at pH 11 - 12 (Chen et al., 2019, 2007; He et al., 2016). Several parameters such as temperature, SRT and OLR also affect the performance of the fermentation process, however the effect of these parameters on overall P release is not sufficiently investigated. Reduced sludge hydrolysis is reported for lower temperatures and shorter SRTs (Chen et al., 2007; Pham et al., 2014; Pokhrel et al., 2018; Wu et al., 2009; Y. Wu et al., 2020a; Yuan et al., 2011) and, therefore, most studies apply longer SRTs and high temperatures.

5. PHOSPHORUS AND CARBON SOLUBILISATION STRATEGIES FOR WASTEWATER SLUDGE VALORISATION

The assessment of the optimum conditions for P and C solubilisation is constrained by the different sludge characteristics and operational conditions tested in previous studies. The aim of this study is to investigate the potential to solubilise P and C from different WRRF sludges in a semi-continuous bioacidification reactor and clarify the individual impact of various operational parameters for full-scale implementation. The reproducibility of the bioacidification process efficiency was investigated using three municipal sewage sludges with different characteristics and sources, as well as three organic loading rates. The importance of fermentation on the release of different P fractions was assessed through two short SRTs and two temperatures applied. Different thermal hydrolysis pre-treatments were assessed to investigate the potential increase of efficiency compared to sole bioacidification. To the best of our knowledge, this is the first study that integrates the individual impact of several process parameters on different sludges for simultaneous P and C solubilisation by semi-continuous bioacidification at low SRT, providing a reliable process for full-scale implementation.

5.2 Materials and methods

5.2.1 Sources of municipal sewage sludge and inoculum

Three types of sewage sludge were used for the kinetic tests: i) primary sludge and ii) waste activated sludge (non-EBPR WAS) collected after the thickening and flotation processes of the Frielas WRRF (Portugal), respectively, and iii) waste activated sludge (EBPR WAS) collected after the thickening process of the Schonebeck WRRF (Germany). The Frielas WWRF has an aerated activated sludge process designed to remove biochemical oxygen demand (BOD) and N with $FeCl_3$ dosage for odour control after the primary treatment. The Schonebeck WRRF has an A^2O activated sludge process designed to simultaneously remove BOD, N and P with $FeClSO_4$ solution dosed at the raw influent and $FeCl_3$ dosed in the secondary clarifier to remove P chemically, whenever the PO_4^{3-}-P concentrations in the effluent exceed 1 mg P/L. Table 5.1 summarises the characteristics of the three sludge types. The inoculum was collected from a bioacidification pilot reactor fed with non-EBPR WAS from Frielas WRRF and operated in a semi-continuous mode at SRT 4 days, pH 5 and 32 °C.

5.2.2 Bioacidification pilot reactor

A 150 L bioacidification pilot reactor was operated in semi-continuous mode for 137 days through 4 phases. It was inoculated with Frielas non-EBPR WAS and operated

at HRT 4 days, pH 5 and temperature of ± 32 °C. The OLR was gradually increased in phases I and II (2 - 8 g COD/L.days), when high and stable effluent ratios of soluble P per total P (PO_4^{3-}-P/P_{tot}) were achieved. In phase III, the thermal sludge pre-treatment at 80 °C was included, while thermal alkaline sludge pre-treatment at 80 °C and pH 9 was included in phase IV. The pilot system comprised of the bioacidification reactor and a continuous stirred feed tank that was used for sludge storage prior to reactor feeding. The sampling and chemical analysis were performed as explained in the following sections.

5.2.3 Bioacidification bench scale system setup and routine sampling

A bench scale system composed of a 10 L continuous stirred vessel (feed tank) and a 5 L anaerobic reactor was set up to perform the bioacidification tests described in section 2.3. The start-up and operation of each test had the following steps: i) reactor was seeded with 5 L of inoculum, ii) nitrogen gas was bubbled for 30 min to ensure anaerobic conditions prior sludge feeding and iii) reactor was operated in semi-continuous mode for minimum two sludge retention times (SRT). Note that sludge was not recirculated in this system and, therefore, SRT was equal to hydraulic retention time (HRT). The sludges were diluted to achieve the same organic loading rate (OLR) in COD basis. Temperature and pH were controlled in the anaerobic reactor by water bath and automatic addition of 1 M HCl or NaOH, respectively.

Routine sampling of the anaerobic reactor and feed tank was performed to characterize the total and soluble components. The parameters measured in the total sample were: total solids (TS), volatile solids (VS), total nitrogen (N_{tot}), total phosphorus (P_{tot}), total chemical oxygen demand (COD_{tot}), total iron (Fe_{tot}) calcium (Ca_{tot}) and magnesium (Mg_{tot}). For parameters measured in soluble sample, total samples were centrifuged and filtered with 0.2µm filters to measure: ammonia (NH_x-N), phosphates (PO_4^{3-}-P), soluble COD (COD_{sol}), volatile fatty acids (VFA), soluble iron (Fe_{sol}), soluble calcium (Ca_{sol}), and magnesium (Mg_{sol}). Microbiological samples were regularly collected for DNA sequencing analysis. Temperature and pH were continuously measured by probes and registered in the pc acquisition unit.

5.2.4 Bioacidification kinetic tests

The kinetic tests were designed to provide optimal strategies for the implementation of bioacidification in municipal WRRFs. Operational conditions are summarised in Table 5.2.

5. PHOSPHORUS AND CARBON SOLUBILISATION STRATEGIES FOR WASTEWATER SLUDGE VALORISATION

i) Impact of different municipal sewage sludges: primary, non-EBPR and EBPR WAS. Two kinetic tests (i-A and i-B) were performed to evaluate whether the solubilisation of P and the production of VFAs in the bioacidification reactor are affected by different rbCOD, P_{tot}, N_{tot} and metals in the sludge (see Table 5.1). In both tests, the anaerobic reactor was first fed with non-EBPR WAS for two SRTs (period 1). After this period, the feed was changed to primary sludge in test i-A and EBPR WAS in test i-B (period 2).

ii) Impact of different sewage sludge loading rates. Two kinetic tests (ii-A and ii-B) were performed to evaluate whether the solubilisation of P and C in the bioacidification reactor are affected by the increase in sludge loading rates. Compared to the previous tests, the OLR was increased from 2.6 to 8 g COD/L.days in test ii-A and the anaerobic reactor was operated at SRT of 4 days and fed with non-EBPR WAS (period 1) and with EBPR WAS (period 2). In test ii-B, the anaerobic reactor was first fed with EBPR WAS and operated at similar conditions as the test ii-A, with SRT of 4 days and OLR of ~10 g COD/L.days. The period 1 served as the stabilisation period for the reactor. After this period, the OLR was increased to 20 g COD/L.days by reducing the SRT to 2 days (period 2).

iii) *Impact of temperature.* A kinetic test (iii) was executed to evaluate whether the performance of the bioacidification reactor is significantly affected by the reduction of temperature. Test (iii) is an extension of the test ii-B, where the anaerobic reactor was fed with the same EBPR WAS and the temperature was decreased from 30 °C to 20 °C. The temperature of the feed tank was also controlled at 20 °C.

iv) Impact of thermal hydrolysis pre-treatment. The kinetic test (iv) was performed to evaluate the effect of a thermal hydrolysis pre-treatment on the solubilisation of P and C from sludge. The non-EBPR WAS was previously pre-treated with thermal hydrolysis at 120 °C for 1 h under different pH conditions (non-adjusted pH - 6.7, 4.7, and 9.0) in order to select the best pH condition for the simultaneous hydrolysis of C and P. The pH of the sludge was adjusted prior to the thermal treatment.

Test iv was performed with the pH of the non-EBPR and EBPR WAS adjusted to 9 and pre-treated by thermal hydrolysis at 80 °C for 1 h. The hydrolysed non-EBPR WAS was fed to the bioacidification reactor (period 1) and after this period, the feed was changed to the hydrolysed EBPR WAS (period 2). The conditions for this last test were chosen due to the economic feasibility of the process, since it was reported that only extreme temperatures higher than 150 °C can achieve significant C solubilisation (Bougrier et al.,

2008). This would significantly difficult full-scale implementation, so alkaline pH was applied to compensate for low temperature thermolysis.

5.2.5 Chemical and microbial analysis

TS and VS measurements were performed using standard methods (APHA, 1995), while COD_{tot}, COD_{sol} and N_{tot} were analysed using Hach analysis kits. PO_4^{3-}-P and ammonia were analysed by segmented flow analysis (Skalar 5100, Skalar Analytical, The Netherlands). P_{tot} was analysed through the same equipment after acid digestion of the 0.5 mL of sample suspension with 73 mg/L of $K_2S_2O_8$ and 5 mL of 0.3 M H_2SO_4 for 1 h at 100 °C. The P_{tot} concentration was determined by the PO_4^{3-}-P concentration measured after the acid sample digestion. VFAs were analysed by high performance liquid chromatography (HPLC) using a VWR Hitachi Chromaster with Diode Array Detector, Infrared detector and a Biorad Aminex HPX-87H 300x7.8 MM column. The eluent was sulphuric acid (0.005 M) at a flow rate of 0.6 mL/min and the operating temperature was 60 °C. Fe_{sol}, Ca_{sol} and Mg_{sol} were analysed from the filtered sample, which was acidified with nitric acid (5 %, v/v). Fe_{tot}, Ca_{tot} and Mg_{tot} were analysed from the total sample, first digested with nitric acid (5 %, v/v) at 120 °C for 2 h and filtered by 0.2 μm filters. Both metal contents were analysed by Horiba Jobin Yvon ULTIMA sequential ICP, monochromator with a Czerny Turner spectrometer.

The samples collected for microbial characterisation were centrifuged and sent to DNASense (https://dnasense.com/) for DNA sequencing. Briefly, DNA was extracted with 16s rRNA gene amplicon sequencing, targeting the bacterial V1-3 region using MiSeq Illumina Technology (Caporaso et al., 2012). Raw data was processed using research standard UPARSE workflow and data was analysed by Rstudio ampvis2 package developed by Aalborg University (Albertsen et al., 2015). The obtained operational taxonomic units (OTUs) were classified using MiDAS database (Simon Jon McIlroy et al., 2015).

5.3 Results

5.3.1 Impact of different municipal sewage sludges

This experiment was designed to evaluate the benefits of treating the activated sludge separately from the primary sludge due to the different compositions in rbCOD, P_{tot}, N_{tot} and metals. Table 5.1 shows that the EBPR WAS has the highest P and Fe_{tot} content while the primary sludge has the lowest Fe_{tot} content because in Frielas WRRF,

$FeCl_3$ is dosed after the primary treatment. The impact of these sludges on P and C solubilisation was tested in a bioacidification reactor with conditions selected to favour sludge hydrolysis and fermentation (Bouzas et al., 2007; Ucisik and Henze, 2008).

Table 5.1 Sewage sludge characteristics.

Parameters	Sludge type/Water resource recovery facility/collection point:		
	Primary sludge Frielas WRRF/ thickening of primary sludge	**non-EBPR WAS** Frielas WRRF/ flotation of activated sludge	**EBPR WAS** Schonebeck WRRF/ thickening of activated sludge
VS/TS (g S/g S)	0.8	0.8 ± 0.1	0.7 ± 0.1
COD_{tot}/VS (g COD/g VS)	1.4	1.5 ± 0.3	1.5 ± 0.2
P_{tot}/TS (mg P/g TS)	**9.6**	**19.5 ± 2.2**	**46.6 ± 2.5**
N_{tot}/TS (mg N/g TS)	**53.3**	**89.7 ± 5.3**	**76.5 ± 8.6**
COD_{sol}/COD_{tot} (%)	4.4	2.9 ± 1.9	2.9 ± 1.2
COD_{VFA}/COD_{sol} (%)	79.0	57.0 ± 19.7	54.1 ± 12.4
PO_4^{3-}-P/P_{tot} (%)	18.1	22.8 ± 9.9	30.4 ± 1.9
NH_x-N/ N_{tot} (%)	8.6	14.7 ± 7.6	10.2 ± 7.8
Fe_{tot}/TS (mg Fe/g TS)	**11.1**	**16.5 ± 5.8**	**21.4 ± 1.6**
Ca_{tot}/TS (mg Ca/g TS)	25.7	19.6 ± 2.2	24.3 ± 2.6
Mg_{tot}/TS (mg Mg/g TS)	**3.8**	**5.2 ± 1.0**	**9.3 ± 1.0**
Fe_{sol}/Fe_{tot} (%)	1.6	0.8 ± 0.6	0.3 ± 0.2
Ca_{sol}/Ca_{tot} (%)	20.0	20.9 ± 7.0	11.4 ± 3.7
Mg_{sol}/Mg_{tot} (%)	33.6	37.2 ± 9.0	29.2 ± 2.7

Figure 5.1a and b show the effluent PO_4^{3-}-P and P_{tot} concentrations and the PO_4^{3-}-P/P_{tot} solubilisation ratios obtained in tests i-A and i-B. The maximum effluent PO_4^{3-}-P concentrations achieved were 91 mg P/L, 149 mg P/L and 263 mg P/L with the primary sludge, non-EBPR WAS and EBPR WAS, respectively. The PO_4^{3-}-P/P_{tot} solubilisation ratios were maintained mostly stable in test i-A (Figure 5.1a) when the non-EBPR WAS was switched to primary sludge, and slightly decreased in test i-B (Figure 5.1b), when the non-EBPR WAS was switched to EBPR WAS. Despite these differences, the average PO_4^{3-}-P/P_{tot} ratio achieved in all sludge types was higher than 60 % (Table 5.2), with the highest ratio achieved with the non-EBPR WAS (73 %).

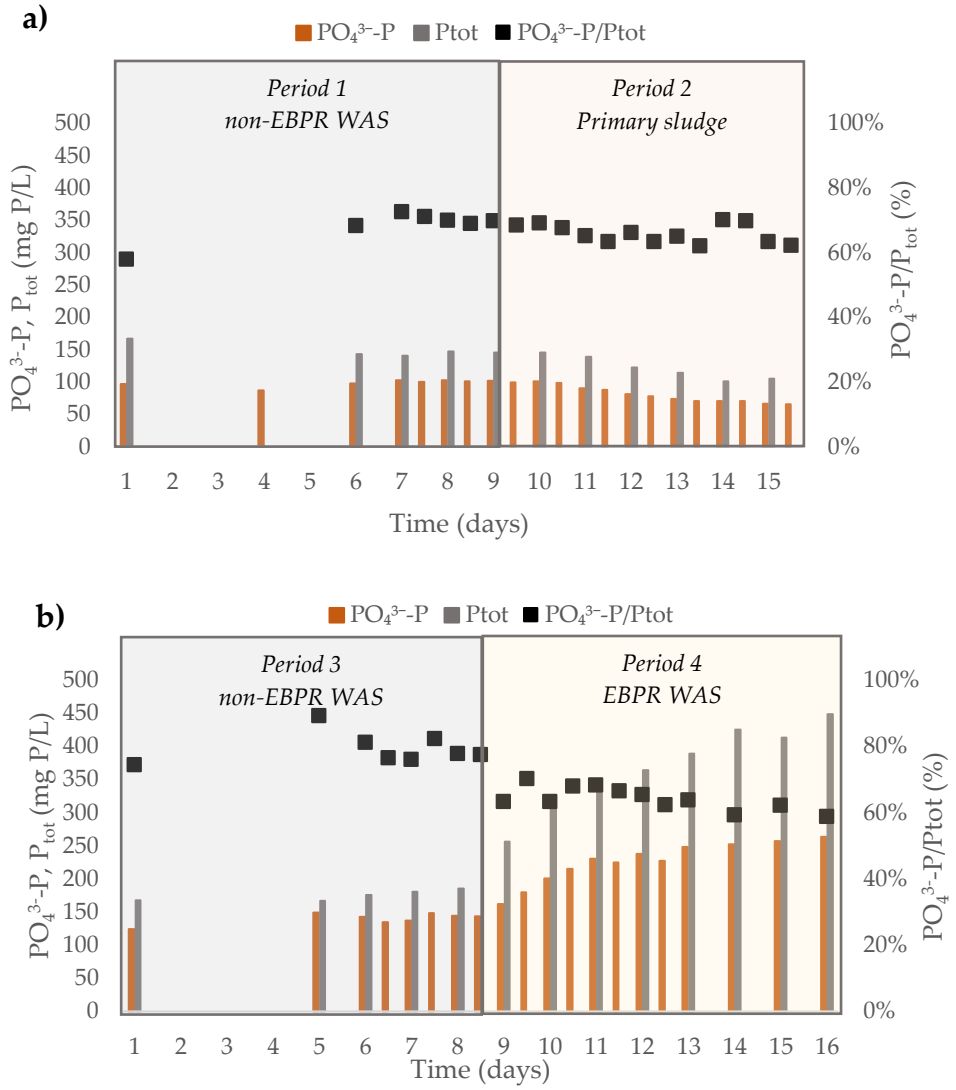


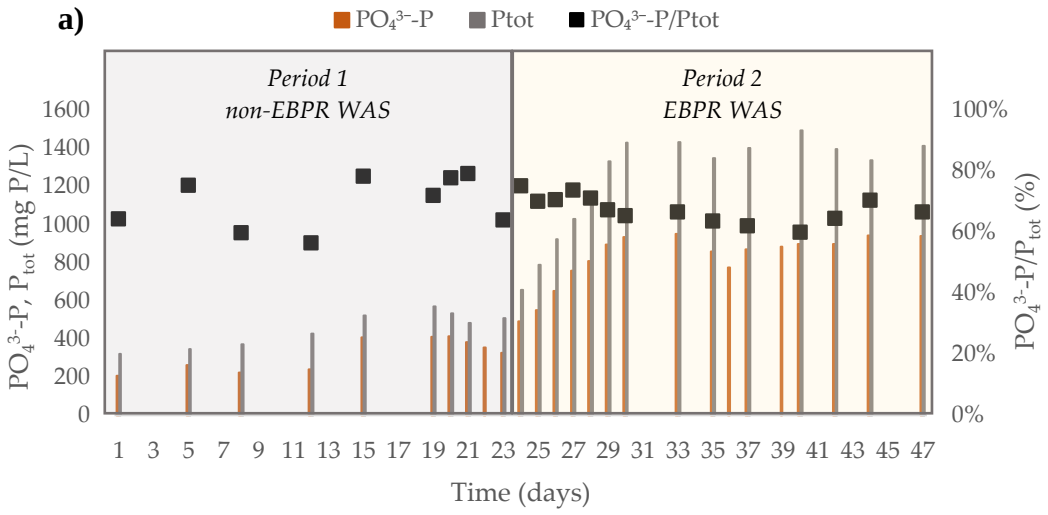
Figure 5.1 PO_4^{3-}-P and P_{tot} concentrations in the bioacidification reactor and PO_4^{3-}-P/P_{tot} solubilisation ratio. a) test i-A: non-EBPR WAS and primary sludge; b) test i-B: non-EBPR and EBPR WAS.

The Fe, Ca and Mg solubilisation ratios in the bioacidification reactor for each sludge type are presented in Table 5.2, where high values were obtained compared to the raw sludge in feed tank (Table 5.1), especially for Fe that was not dissolved prior to the bioacidification. Almost 60 % of Fe and 70 – 80 % of Ca and Mg from the primary and non-EBPR WAS were solubilized, suggesting that the applied conditions were able to dissolve metal complexes. On the other hand, the lowest solubilisation was obtained with the EBPR WAS, leading to 27 %, 45 % and 73 % of Fe_{sol}, Ca_{sol} and Mg_{sol}, respectively, possibly due to long SRT and neutral pH applied at this WRRF that can cause sludge aging and formation of more stable precipitates (Lu et al., 2016). Additionally, EBPR WAS had the highest metal content per TSS, as presented in Table 5.1 with raw sludge characteristics.

The low COD_{sol}/COD_{tot} ratios (Table 5.2) and the high COD_{VFA}/COD_{sol} ratios (77 – 91 %, data not shown) obtained for each sludge type suggest that the limiting step for C solubilisation was the hydrolysis of particulate organic matter and not the fermentation. The highest VFA production was achieved with the primary sludge (on average 229 mg COD_{VFA}/g VS), where acetate (46 %) and propionate (24 %) were the VFAs most produced, while the lowest VFA production was achieved with the EBPR WAS (on average 117 mg COD_{VFA}/g VS).

5.3.2 Impact of organic loading rate

In order to achieve higher process productivity without causing the washout of acidogenic bacteria present in the inoculum, two strategies were investigated to increase the OLR of the bioacidification: increase sludge concentration (test ii-A) and decrease SRT at the maximum sludge concentration reached (test ii-B). Figure 5.2a and 2b show the effluent PO_4^{3-}-P, P_{tot} concentrations and PO_4^{3-}-P solubilisation ratios obtained with the activated sludges in these tests. The maximum PO_4^{3-}-P concentrations achieved in test ii-A were 415 mg P/L and 952 mg P/L with non-EBPR and EBPR WAS, respectively. When SRT was decreased from 4 to 2 days (test ii-B), the maximum PO_4^{3-}-P concentration achieved with EBPR WAS was 903 mg P/L. The PO_4^{3-}-P/P_{tot} solubilisation ratios in test ii-A were maintained similar to the previous test, reaching an average of 70 % and 66 % for non-EBPR and EBPR WAS, respectively. These results demonstrate the stability of the bioacidification process for the treatment of high sludge concentration (22 - 32 g TS/L). Additionally, the PO_4^{3-}-P/P_{tot} solubilisation ratio in test ii-B (60 %) was not significantly affected by the SRT of two days.



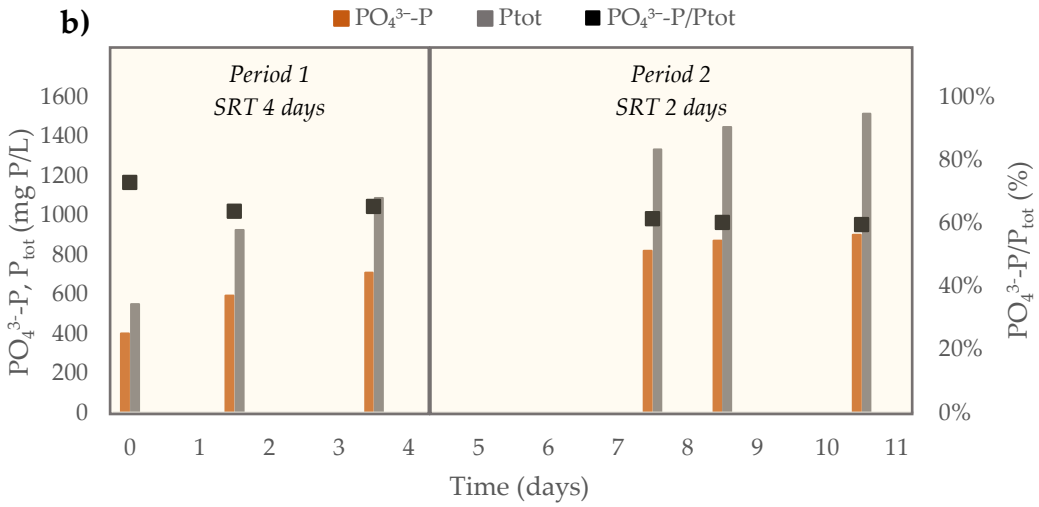


Figure 5.2 PO_4^{3-}-P and P_{tot} concentrations in the bioacidification reactor and PO_4^{3-}-P/P_{tot} solubilisation ratios: a) test ii-A: non-EBPR (period 1) and EBPR WAS (period 2); b) test ii-B: EBPR WAS SRT 4 days (period 1) and SRT 2 days (period 2).

Table 5.2 compares solubilisation ratios in the bioacidfication reactor for all tests. In test ii-A, solubilisation of Fe and Ca was also higher with non-EBPR WAS. However, Ca solubilisation ratio with EBPR WAS reached 73 %, which is a 28 % increase compared to the previous test. This difference is explained by the lower pH tested in ii-A (pH 4.5) compared to i-B (pH 5.0), which probably induced metal dissolution (Cao et al., 2019; Chen et al., 2007; Zou et al., 2018). The strategy to reduce the SRT with EBPR WAS in test ii-B resulted in decreased Fe (16 %) solubilisation ratio, as lower fermentation and the washout of acidogenic and iron reducing bacteria led to less iron reduction (described in section 5.3.5 Microbial community). Additionally, it was recently reported that SRT decrease can negatively affect P release from inorganic precipitates (Y. Wu et al., 2020a).

The COD_{sol}/COD_{tot} ratios in test ii-A were 12 – 13 % for both sludge substrates. Fermentation efficiency with EBPR WAS was not affected by lower SRT leading to similar VFA production as previous tests (127 mg COD_{VFA}/g VS and 131.4 mg COD_{VFA}/g VS in test ii-A and ii-B, respectively). The highest VFA production was achieved with non-EBPR WAS (on average 194 mg COD_{VFA}/g VS). Similarly to other studies (Chen et al., 2007; Ucisik and Henze, 2008), acetate (33 %) and propionate (19 %) were the most produced VFAs.

5.3.3 Impact of temperature

Implementing bioacidification process in countries with colder climate can be a challenge as energy for reaching high temperatures significantly increases operational costs. When temperature was decreased from 30 °C (test ii-B) to 20 °C (test iii), an overall deterioration of the system was observed (Table 5.2). Consequently, the average PO_4^{3-}-

P/P_{tot} solubilisation ratio dropped almost immediately from 60 % to 52 %. Compared to other tests performed at ≥ 30 °C, the COD_{sol}/COD_{tot} and $NH_x\text{-}N/N_{tot}$ ratios decreased by more than 5 % and 15 %, respectively. Lower hydrolysis and sludge disintegration significantly decreased VFA production (61 mg COD_{VFA}/g VS) and consequently the release of organic P. In addition, the solubilisation of Mg, Fe and Ca also decreased, contributing to an overall P release reduction.

5.3.4 Impact of thermal hydrolysis pre-treatment

Previous bioacidification tests demonstrated that sludge hydrolysis was the limiting-step for the VFA production and organic P solubilisation. To overcome this kinetic limitation thermal hydrolysis batch pre-treatments were performed at 120 °C for 1 h under different pH conditions (see Figure C2 in Appendix C). These batch tests elucidated that C solubilisation (26 %) was higher under thermal alkaline conditions (pH 9), while PO_4^{3-}-P concentration (P solubilisation 35 %) was higher under thermal acidic conditions (pH 4.7). Although C solubilisation increased in the thermal hydrolysis pre-treatments, P solubilisation was lower compared to the bioacidification reactor.

The solubilisation ratios obtained in the combined thermal alkaline hydrolysis pre-treatment and bioacidification process (test iv) are shown in Figure 5.3 and compared with those obtained in bioacidification test ii-A. In test iv, the maximum COD_{sol}/COD_{tot} (29 %) and $PO_4^{3-}\text{-}P/P_{tot}$ (79 %) solubilisation ratios were achieved with the non-EBPR activated sludge, which was an increase of 16 % and 7 %, respectively, compared to those obtained in the bioacidification ii-A. Figure 5.3 also shows that the COD_{sol}/COD_{tot}, NH_x-N, $PO_4^{3-}\text{-}P/P_{tot}$ solubilisation ratios increased simultaneously in the feed tank when the thermal alkaline pre-treatment was applied, while the solubilisation of Ca and Mg decreased. Fe release was not observed in the feed tank after sludge pre-treatment. Therefore, bioacidification proved to be crucial for Fe release as no Fe_{sol} was detected in feed tank with or without thermal hydrolysis. Although these results suggest that the thermal alkaline pre-treatment increased the hydrolysis of organic matter and, therefore, solubilisation of organic N and P, the low solubilisation of metals suggest that part of the released P in the feed tank may have been recaptured by precipitates (He et al., 2016). Thermal hydrolysis under alkaline conditions may have induced the formation of iron precipitates such as vivianite, thus recapturing P that was previously solubilised. Also, an increase of sludge surface with higher hydrolysis enhances the adsorption and affects net Fe release (Cao et al., 2019; Y. Wu et al., 2020a).

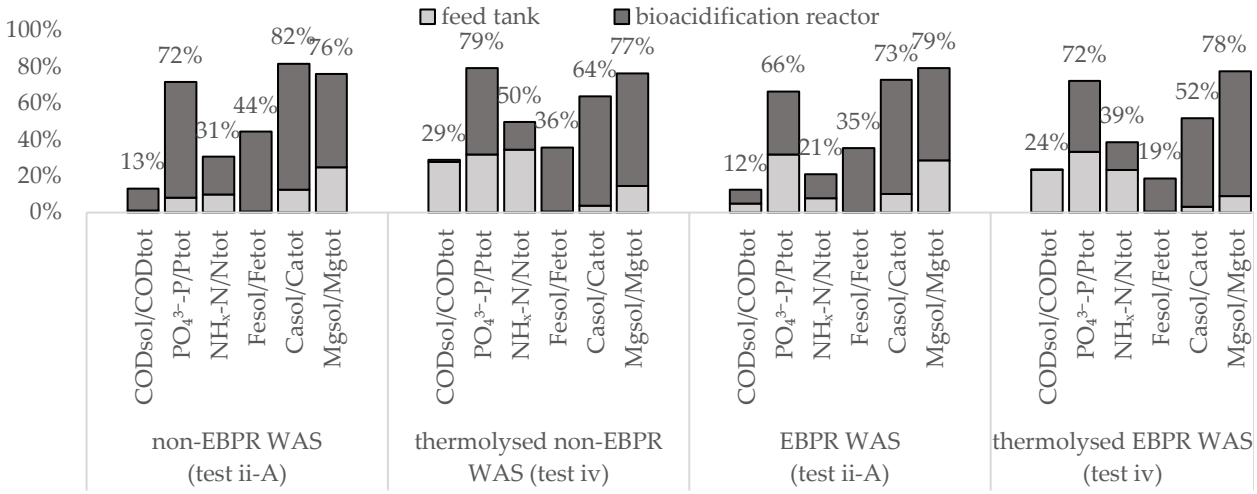


Figure 5.3 Compared COD_{sol}/COD_{tot}, PO_4^{3-}-P/P_{tot}, NH_x-N/N_{tot}, Fe_{sol}/Fe_{tot}, Ca_{sol}/Ca_{tot} and Mg_{sol}/Mg_{tot} solubilisation ratios for tests ii-A and iv in feed tank (with or without thermolysis pre-treatment) and bioacidification reactor.

Table 5.2 shows that the average VFA production achieved in the thermal alkaline hydrolysis pre-treatment and bioacidification process (test iv) was 392 mg COD_{VFA}/g VS and 303 mg CODVFA/g VS with non-EBPR and EBPR WAS, respectively, which is about 2 - 2.8 times higher than that obtained in the tests without thermal pre-treatment. These results suggest that combining thermal alkaline hydrolysis pre-treatment with bioacidification is needed to simultaneously increase VFA production and organic P solubilisation. Figure C3 in Appendix C shows a comparison of the VFA composition and COD_{sol} obtained in tests iv and ii-A. Acetate and propionate remained the main contributors of total VFAs, accounting for an average of 42 % and 19 %, respectively.

Table 5.2 Operational conditions and average effluent ratios obtained in this study and in the literature using similar conditions.

Studies	Sludge type	pre-treatment	SRT (days)	Temp (°C)	pH	OLR (g COD/L.days)	PO_4^{3-}-P /P_{tot} (%)	NHx-N/ N_{tot} (%)	COD_{sol}/COD_{tot} (%)	COD_{VFA}/VS^* (mg COD_{VFA}/g VS)	Fe_{sol}/Fe_{tot} (%)	Ca_{sol}/Ca_{tot} (%)	Mg_{sol}/Mg_{tot} (%)
test i-A	Primary sludge	-	4.0	32	5	2.4	65 ± 3	27 ± 2	13 ± 1	229 ± 17	55 ± 3	79 ± 7	72 ± 5
test i-A and i-B	non-EBPR WAS**	-	4.1	31	5	2.7	73 ± 10	28 ± 4	13 ± 1	197 ± 17	60 ± 3	77 ± 2	77 ± 1
test i-B	EBPR WAS	-	4.7	32	5	2.6	62 ± 2	29 ± 3	10 ± 1	117 ± 6	27 ± 6	45 ± 6	73 ± 3
test ii-A	non-EBPR WAS	-	4.2	33	4.5	8.0	70 ± 8	31 ± 1	13 ± 1	194 ± 13	44 ± 2	82 ± 0	76 ± 1
test ii-A	EBPR WAS	-	4.0	33	4.5	8.5	66 ± 4	21 ± 5	12 ± 2	131 ± 26	35 ± 7	73 ± 6	79 ± 4
test ii -B***	EBPR WAS	-	2.0	30	5	19.7	60 ± 1	18 ± 1	8 ± 0	127 ± 7	16 ± 1	41 ± 4	71 ± 3
test iii	EBPR WAS	-	2.1	20	5	22.8	52 ± 1	9 ± 0	6 ± 0	61 ± 3	9 ± 2	30 ± 3	64 ± 3
test iv	non-EBPR WAS	pH 9, 80°C, 1h	4.3	34	5	7.9	79 ± 3	50 ± 4	29 ± 2	392 ± 36	36 ± 6	64 ± 11	76 ± 5
test iv	EBPR WAS	pH 9, 80°C, 1h	4.2	34	5	7.6	72 ± 3	39 ± 3	24 ± 1	303 ± 22	19 ± 7	52 ± 4	77 ± 5
Latif et al. (2017)	WAS	-	12	37	5.5	1.9	74 ± 5	-	-	-	-	54 ± 5	88 ± 2
Pokhrel et al. (2018)	Non-EBPR WAS	-	19 (batch)	35	4	-	54	-	-	-	-	-	-
Zou et al. (2018)	Nitrifying granular sludge (lab)	-	10 (batch)	35	4	-	81	-	30	188	< 10	~73	~57
Liu et al. (2020)	EBPR WAS	150°C, 0.5h	7 (batch)	30	6.5	-	39	27	-	-	-	-	-
He et al. (2016)	EBPR WAS	-	12 (batch)	35	4-5	-	30-40						
Ucisik and Henze (2008)	Primary and WAS	-	5	37	5-7	-	-	-	8-21 (primary) 2-6 (WAS)	168 – 270 (primary) 62 (WAS)	-	-	-
Zou and Li (2016)	P gran/floc sludge (lab)	70°C, 1h	7 (batch)	35	~6	-	73-80	-	-	135-207 (without pre-treatment) 355 (with pre-treatment)	-	-	-

* in this study, the calculation was performed by mg COD_{VFA}/g VS, while in studies from literature the values are in mg COD_{VFA}/g VSS.

**Average ratios from period 1 with non-EBPR WAS in tests i-A and i-B.

***Results from stabilisation period 1 not shown in the Table.

5.3.5 Microbial community

Figure 5.4 shows the microbial characterisation performed through DNA sequencing analysis of the inoculum and samples from each test. Phylum *Firmicutes* was the most abundant group in the inoculum, counting to more than 80 % of total bacteria, followed by *Proteobacteria* (5 %). The *Firmicutes* are fermenting bacteria often found in anaerobic digestors and responsible for hydrolysis of organic matter and acidogenesis (Petriglieri et al., 2018; Ping et al., 2020). Most abundant groups on genus level (see Figure C4 in Appendix C) were *Intestinibacter* (43 %), *Clostridium sensu stricto* (19 %) and *Peptoclostridium* (5 %) capable of acetate, CO_2 and hydrogen production (Chen et al., 2018). Detected *Clostridium sensu stricto* and *Arcobacter* can perform iron reduction as well (Zou et al., 2018). From the group of phosphate accumulating organisms (PAOs), low presence of *Tetrasphaera* (1 %) known as putative PAOs was detected (Marques et al., 2017).

Samples from test ii-A day 30, test ii-B day 11 and test iii day 16 correspond to periods when bioacidification reactor was fed with EBPR WAS. Compared to the inoculum, the percentage of the phylum *Firmicutes* reduced to 23 %, 9 % and 7 % in samples from tests ii-A, ii-B and iii, respectively. On the other hand, the phylum Actinobacteria increased to 36 %, 55 % and 54 % respectively, showing that the microbial community of the inoculum was diluted with the microbial community of the EBPR WAS. This sludge was highly enriched in *Actinobacteria* phylum (64 % of total bacteria), in which the *Candidatus Microthrix* (47 %) and *Tetrasphaera* (5 %) were the most abundant genus (Figure C4). *Candidatus Microthrix* is a filamentous bacterium often detected at WRRFs, possibly capable of using polyphosphates as energy supply for anaerobic long chain fatty acids uptake (Mcilroy et al., 2013). Interestingly, less than 1 % of *Accumulibacter* PAOs was detected in EBPR WAS. However, several studies previously reported that *Accumulibacter* PAOs are underestimated by sequencing analysis (Carvalho et al., 2021; F. J. Rubio-Rincón et al., 2019a; Francisco J. Rubio-Rincón et al., 2019; Valverde-Pérez et al., 2016). The results also demonstrate that the growth of the acidogenic bacteria was sustained under OLR 8.5 g COD/L.days, SRT 4 days, 33 °C, pH 4.5 (sample ii-A days 30), while the reduction of the SRT and temperature (samples ii-B day 11 and iii day 16) led to the washout of acidogenic and iron reducing bacteria.

Samples from test iv day 9 and day 18 correspond to the test with hydrolysed non-EBPR and EBPR WAS, respectively. The reactor was still highly enriched in acidogenic bacteria when thermal sludge pre-treatment was applied, with slight decrease of

Firmicutes to 74 % and 72 %, respectively. The *Peptoclostridium* (43 – 53 %), *Intestinibacter* (6 – 7 %) and *Clostridium* (5 – 14 %) were the most abundant genus.

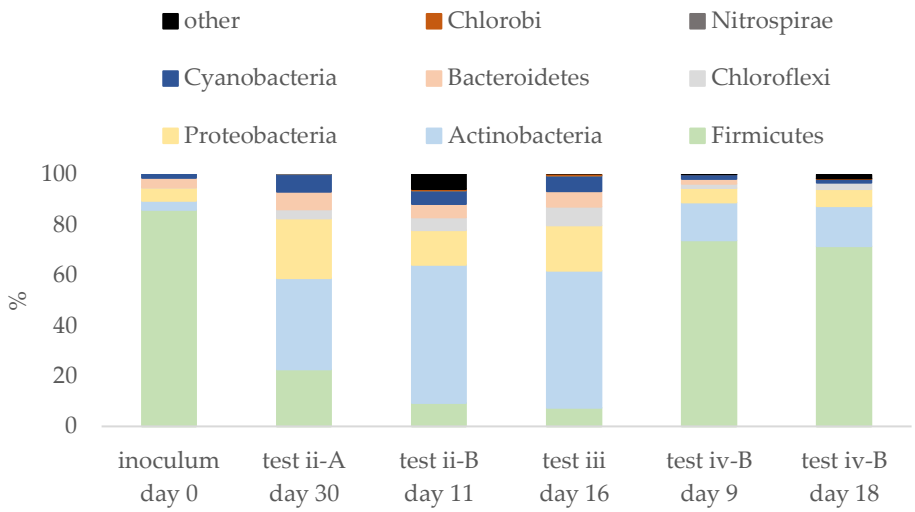


Figure 5.4 Compared microbial community DNA sequencing results with most abundant microorganisms on phylum level. For further details on each test, readers are referred to Table 5.2.

5.3.6 Pilot reactor operation

Figure 5.5 presents effluent concentrations and effluent ratios in the four periods of pilot reactor operation. A gradual increase of soluble phosphorus was observed in the first period of the reactor start-up phase reaching an average of 68 % PO_4^{3-}-P/P_{tot} ratio and 20 % of NH_x-N/N_{tot} ratio (Table C1, Appendix C). When OLR was increased in the second phase, the phosphorus solubilisation was not affected and remained high. An addition of the thermal sludge pre-treatment has increased NHx-N release (35 %) while phosphorus release remained on average 67 %. Finally, when thermal alkaline pre-treatment was added, the average PO_4^{3-}-P/P_{tot} ratio was 78 % while average NH_x-N/N_{tot} ratio was 45 % (Table C1, Appendix C). This is consistent with the results achieved in kinetic tests, where thermal alkaline pre-treatment has increased phosphorus solubilisation by a maximum of 10 %. Furthermore, the metal release was also higher when thermal alkaline pre-treatment was applied compared to solely thermal pre-treatment but remained lower compared to kinetic test ii-A operated at lower pH (4.5).

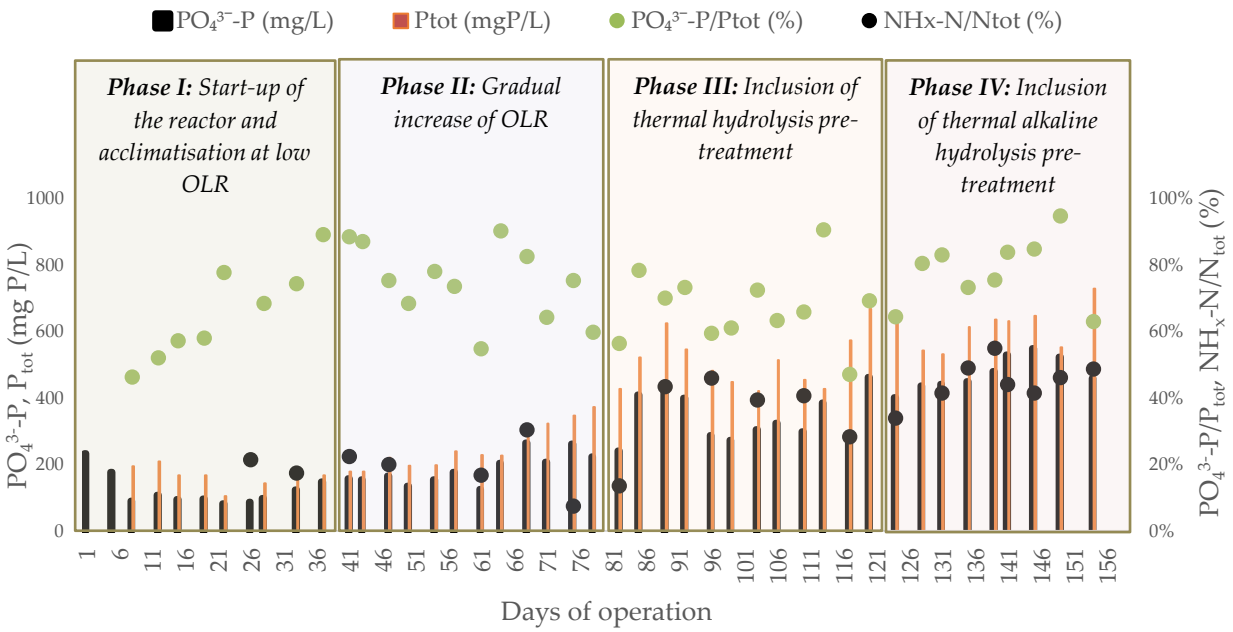


Figure 5.5 PO_4^{3-}-P and P_{tot} concentrations and PO_4^{3-}-P/P_{tot} and NH_x-N/N_{tot} solubilisation ratios in the pilot bioacidification reactor operated with Frielas non-EBPR WAS.

The production of volatile fatty acids was also highly increased with the addition of sludge pre-treatments due to higher sludge hydrolysis and COD solubilisation (Figure 5.6). The highest average VFA production was in phase IV reaching 423 mg COD_{VFA}/g VS (Table C1, Appendix C).

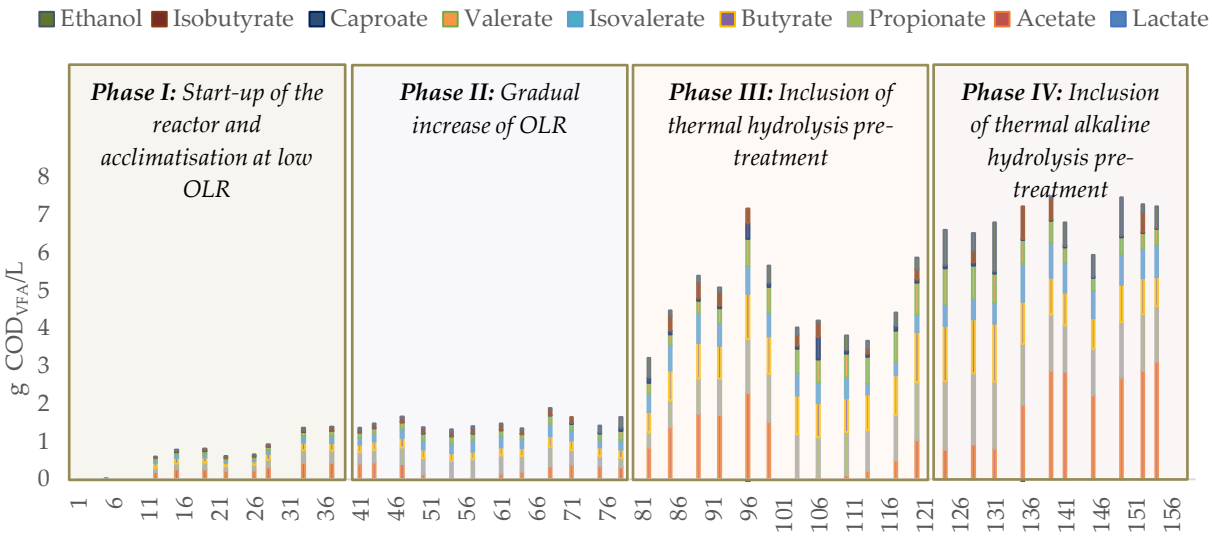


Figure 5.6 VFA production in the four phases of pilot bioacidification reactor operation.

5.4 Discussion

5.4.1 Phosphorus and metal solubilisation

This study demonstrated that the bioacidification process has a high potential to solubilise P from different municipal sludges when operated in semi-continuous mode at short SRTs (2 – 4 days), 30 °C and pH 4.5 - 5. Similar P solubilisation efficiency was observed for all sludges, with highest PO_4^{3-}-P concentrations with EBPR WAS, due to high P content. Lowest metal solubilisation was achieved also with EBPR WAS, leading to the lowest PO_4^{3-}-P/P_{tot} solubilisation ratios. This could be explained by long SRT causing sludge aging as previously mentioned, however, it should be also noted that, in the period of sludge collection, increased Fe dosage was performed in this WRRF leading to the highest Fe content in this sludge (Table 5.1). Although primary sludge can contain newly formed precipitates easier to dissolve due to lower sludge age (Ucisik and Henze, 2008), it also has low P content and dilutes bioacidification influent.

Bioacidification was required to achieve high total PO_4^{3-}-P/P_{tot} effluent ratios (60 – 73 %), since pre-treating the sludge with thermal hydrolysis for 1 h at 120 °C and pH 4.7 only solubilised 35 % of P, while combination of both reached 70 – 79 % PO_4^{3-}-P/P_{tot} effluent ratios. Semi-continuous pilot scale reactor operation further confirmed the results achieved in the kinetic tests, demonstrating that the process could be feasible for larger scale implementation. Table 5.2 shows that high P solubilisation ratios were obtained in this study compared to other literature studies. Similar PO_4^{3-}-P/P_{tot} effluent ratios were achieved only by Latif et al. (2017), Zou and Li (2016) and Zou et al. (2018). However, Latif et al. (2017) operated the bioacidification reactor at high SRTs (12 days), while Zou and Li (2016) and Zou et al. (2018) did not use sludge from full-scale WRRFs. The authors used granular sludge from an acclimatized laboratory SBRs to remove P and N, which had a higher abundance of *Accumulibacter* PAOs compared to full-scale WRRFs.

Bioacidification kinetic tests with different types of raw and thermolyzed sludge, OLRs, SRTs and temperatures were needed to elucidate the optimal conditions for the release of the following P fractions: polyphosphates, Fe-P, Ca-P and organic P.

Polyphosphates were the easiest fraction to release in all applied conditions, as high solubilisation of Mg was observed in all tests (Table 5.2) and hydrolysis of polyphosphate produces Mg^{2+} (Oehmen et al., 2007). These results suggest that EBPR WRRFs with good biological performance are the preferable target plants for recovering P, as PAOs can increase P content of sludge 4 – 8 % based on dry biomass (Yang et al.,

2017). In anaerobic conditions, PAOs consume VFAs and store them as PHAs using hydrolysis of intracellular polyphosphates and glycogen as sources of energy and reducing power, respectively (Kolakovic et al., 2021). In prolonged anaerobic conditions, PAOs perform maintenance by using polyphosphates and glycogen as sources of energy, and decay when these polymers are exhausted (Santos et al., 2020). Brdjanovic et al. (1997) observed that the anaerobic and aerobic maintenance rates of PAOs are dependent on the temperature within the range of 5 °C – 30 °C. At higher temperatures, PAOs decay rates are higher compared to typical temperatures of activated sludge processes (~18 – 20 °C), thus, causing faster polyphosphate release (Brdjanovic et al., 1997; Wang et al., 2016).

Fe was more solubilised in tests with good bioacidification performance. Figure 5.3 clearly shows that Fe was not released prior to the bioacidification reactor. The fermentation step is important for Fe (III) reduction while acidic conditions are crucial to avoid undesired precipitates formation at this step (Cao et al., 2019; Y. Wu et al., 2020a). The bioacidification kinetic tests operated with lower temperature (20 °C) and SRT (2 days) showed a simultaneous deterioration in the VFA production and washout of Fe reducing bacteria, which was also accompanied with the decreased Fe and P solubilisation. These results suggest that operational conditions that favour the hydrolysis and production of VFAs should be applied to increase the release of inorganic P from Fe-P complexes. The presence of humic substances and VFAs provide electron donors for ferric iron reduction (Cao et al., 2019; R. hong Li et al., 2019; Y. Wu et al., 2020b). Some studies hypothesized that even fermenting bacteria, sulfate-reducing bacteria, or methanogens could also reduce ferric iron under these conditions (Wilfert et al., 2015).

Ca was more solubilised when pH 4.5 was applied instead of 5. Ca dissolution was mostly impacted by acidic pH, as calcium phosphates dissolve in acids (Wang and Nancollas, 2008). The presence of organic acids, hydroxyl ions and CO_2 produced in fermentation may decrease pH and chelate the cations thus release P from Ca-P (He et al., 2016; Pokhrel et al., 2018). The results of this study suggest that a pH lower than 4.5 may be necessary to solubilise sludges rich in Ca-P complexes and that alkaline pre-treatment should be avoided or replaced by neutral/acid thermal hydrolysis pre-treatment.

The hydrolysis of organic matter was the limiting step in all bioacidification kinetic tests. Other studies also observed low organic P release in the anaerobic fermentation in the pH range of 5 - 8 or temperatures up to 35 °C (He et al., 2016; Pokhrel et al., 2018). Based on the sludge characteristics and limiting fraction for P release,

alkaline sludge thermolysis should be applied if the limiting step is organic P release. Therefore, in this study, combining a thermal alkaline hydrolysis batch pre-treatment with the bioacidification process was investigated to increase the release of organic P. However, this study showed that the increase in P solubilisation was around 7 – 10 % when the thermal alkaline hydrolysis batch pre-treatment was performed at 80 °C for 1 h and, therefore, the gain vs. cost increase of adding a pre-treatment step should be considered prior to implementation. Future research is needed to address the cost of potentially more extreme pre-treatment (at higher pH and temperatures) for organic P rich sludges vs. potential gain based on the application of the filtrate obtained in bioacidification process.

5.4.2 Carbon solubilisation and VFA production

The fermentation yields obtained in this study (120 - 392 mg COD_{VFA}/g VS) are in higher range of values reported in literature (see Table 5.2). The final period of pilot reactor operation was able to reach VFA production of 423 mg COD_{VFA}/g VS. The hydrolysis was the limiting step as sewage sludge is a difficult substrate to degrade and it was reported that only 30 – 50 % of COD_{tot} or VS can be degraded after 30 days (Parkin and Owen, 1986). The objective of this study was to increase VFA production without compromising high P solubilisation. The highest yield achieved with primary sludge is due to higher content of readily biodegradable COD compared to activated sludges that usually have less available organic matter due to accumulation of suspended inert matter and cellular residues with high SRTs used in activated sludge processes (Ucisik and Henze, 2008). Thus, it also supports the benefit for direct treatment of primary sludge in the conventional digestor. However, to increase sludge hydrolysis and VFA production, thermal pre-treatment could be inevitable both for primary sludge, as for activated sludges. Temperature was also confirmed to be important factor since 20 °C had negative impact on hydrolysis as previously reported (Pham et al., 2014; Wu et al., 2009; Yuan et al., 2011). The decrease of temperature does not completely inhibit, but slows down the microorganism's activity, thus reducing the fermentation rate as well (Yuan et al., 2011). Thus, other process parameters such as longer SRT or pre-treatment of the sludge should be used to compensate when low temperature bioacidification is considered.

5.4.3 Implications of the study for wastewater sludge valorisation

To achieve simultaneous P and C recovery, bioacidification conditions should be assessed based on the downstream process requirements and operational costs. Although similar P solubilisation ratios were achieved with all sludges tested, lower and higher effluent PO_4^{3-}-P concentrations were obtained in the bioacidification of primary and EBPR WAS sludges due its low and high P content, respectively. These results suggest that high PO_4^{3-}-P productivities can be obtained by treating only P-rich activated sludge in the bioacidification process. P rich sludge treatment in conventional digestors can lead to struvite precipitation under neutral or alkaline pH, causing pipes and other equipment incrustation and increased maintenance costs (Cornel and Schaum, 2009). Therefore, treating separately P rich activated sludge in the bioacidification process, while sending primary sludge directly to the conventional anaerobic digestor, can be a feasible option simultaneously reducing the problem of incrustation. The effluent from bioacidification can be separated with screw press into P and VFA rich filtrate and bioacidified sludge. The filtrate can be further used to produce other value-added products, while dewatered bioacidified sludge can be afterwards mixed with primary sludge and sent to the conventional anaerobic digestor.

This study demonstrated that there is no need of increasing the operational costs (e. g. adding thermal pre-treatment) if the bioacidified effluent characteristics are satisfactory for resource recovery processes. The high PO_4^{3-}-P obtained (maximum 560 - 980 mg P/L) can be used in precipitation/crystallization processes, which have high efficiencies leading to a possible total P recovery from sludge of 60 – 80 %. Furthermore, higher PO_4^{3-}-P concentration in filtrate can lead to lower process costs of crystallization (Egle et al., 2016; Liu et al., 2020). In the study of Liu et al. (2020), the highest yield of struvite production (187 mg/g SS) was linked to the highest P_{sol} (967 mg P/L), although other process parameters and dissolved compounds must be taken into consideration. Remaining VFAs could be mixed back with the sludge from bioacidification and sent to the conventional digestor for biogas production, as two-stage anaerobic digestion has proved to have advantages in biogas yield (Baldi et al., 2019).

The high VFAs (0.30 - 0.42 g COD_{VFA}/g VS) produced by fermentation of sludge could also potentially provide substrate for PHA production, biopolymers that are produced by microorganisms and are environmentally friendly substitution for conventional petrol-based plastics (X. Wang et al., 2020). Finally, produced VFAs could also be recirculated to the water line, as many WRRFs have limited carbon source for biological nutrient removal and need to add methanol or acetate as external C. It was reported that VFA yield of 0.26 g COD_{VFA}/g VSS was sufficient to meet more than 94 %

of the total organic carbon source demand for nutrient removal in full-scale study (Liu et al., 2018). Following these possible applications, optimal conditions for bioacidification can be adapted based on the efficiency and economic feasibility by further investigating proposed process schemes for waste valorisation.

5.5 Conclusions

This study demonstrated the potential of using the semi-continuous bioacidification process at SRT of 4 days to solubilise P and C from sewage sludge and provided scientific and technical knowledge to select conditions based on the sludge characteristics. Bioacidification was always required to achieve high total PO_4^{3-}-P/P_{tot} effluent ratios (60 – 73 %), since sole thermal hydrolysis for 1 h at 120 °C and pH 4.7 solubilised only 35 % of P. The combination of both thermal alkaline pre-treatment and bioacidification has reached maximum PO_4^{3-}-P/P_{tot} (70 – 79 %) and COD_{sol}/COD_{tot} (24 -29 %) effluent ratios. Similar efficiencies were achieved in kinetic tests using three different sludges and in the pilot reactor, where polyphosphates were easily hydrolysed due to the anaerobic and endogenous metabolism of PAOs. High fermentation induced metal release, while thermal-alkaline batch pre-treatment led to some metal precipitation, harder to dissolve in bioacidification. Therefore, sludge pre-treatment should be applied only for higher solubilisation of C or if the limiting fraction for P release is organic P. Future work should focus on full-scale implementation of the bioacidification process and subsequent downstream processes requirements needed to achieve a viable technology process scheme for wastewater sludge valorisation.

5. PHOSPHORUS AND CARBON SOLUBILISATION STRATEGIES FOR WASTEWATER SLUDGE VALORISATION

6. GENERAL CONCLUSIONS AND FUTURE WORK

6.1 General conclusions

This work aimed to contribute towards better understanding of the important aspects of modern water resource recovery facilities. The results achieved in the scope of this thesis provide new insight into EBPR processes, which is a widely researched topic, however, this thesis has proved there is still much to explore. In addition to the clarification of possible causes for biological phosphorus removal instabilities, the thesis work indicates a possible new role for EBPR processes - detoxication of recalcitrant micropollutants. Furthermore, the thesis results also provide scientific and technical knowledge for resource recovery, which is the main objective of all the modern WRRFs.

Instabilities in the EBPR processes are causing significant problems in wastewater treatment operation, causing the implementation of the mixed biological/chemical phosphorus removal or full chemical phosphorus removal. Other than being more costly and environmentally challenging, chemical phosphorus removal can provoke problems in the processes of phosphorus recovery, by introducing the metal complexes that are more difficult to control. Therefore, good EBPR process operation can resolve simultaneously two problems – phosphorus as a pollutant and phosphorus as a resource. The investigation of the *Accumulibacter* diversity at the sub-clade level has shown that controlling PAOs over GAOs growth is not enough for successful phosphorus removal. In fact, highly enriched *Accumulibacter* systems with sufficient carbon and phosphate source can still perform poor phosphorus removal. Furthermore, *Accumulibacter* Types and sub-clades have shown inconsistent metabolic expression when submitted to different operational conditions. Dump feeding of the organic carbon source was the main reason for poor phosphorus removal, where specific *Accumulibacter* IIc clusters achieved less than 40 % of phosphorus removal. Although, full-scale WRRFs usually operate under continuous operation, where carbon source from the influent is introduced continuously, some industrial or other discharges with higher organic content can provoke instabilities in the EBPR operation and cause *Accumulibacter* to switch to mixed PAO/GAO metabolism. To avoid these inconsistencies in phosphorus removal, such discharges should be diluted, or added at a slow flow rate, when being introduced to the wastewater treatment line. Furthermore, the role of *Accumulibacter* in EBPR should be reassessed, since recharacterization of the *Accumulibacter* group beyond the clade level may be necessary to understand the link between intra-clade diversities of *Accumulibacter* and EBPR performance.

Phosphorus removal is the primary task of EBPR processes, but if conventional processes could be optimised to tackle other problems in the area of wastewater treatment, such as micropollutant removal, it would decrease the need of configuration

changes and introduction of advanced technologies. Diclofenac has been shown to be quite recalcitrant in conventional processes, however a lot of contradictory findings exist. This thesis shows that EBPR processes can potentially contribute towards diclofenac biotransformation. Although *Accumulibacter* PAOs did not perform diclofenac biotransformation as efficiently as compared to nitrifiers, the proposed metabolic pathways in an enriched EBPR process revealed the formation of less toxic by-products. Additionally, different diclofenac biotransformation was observed for two different *Accumulibacter* Types, confirming the need to closely reexamine and further investigate the metabolism of these interesting bacteria. Some advanced physical/chemical processes have proved to form more toxic transformation products, so the results from this thesis could open a new line of investigation where biological processes could employ a wider diversity of organisms capable of performing biodegradation of more resistant micropollutants.

Due to the increasing concern for the scarcity of crucial resources, the possibility for biological phosphorus recovery was addressed in this thesis. Biological acidification is potentially readily implementable in conventional wastewater treatment plants, but its link with phosphorus recovery is not yet fully understood. The consistency and resilience of the process is very important for full scale application, and this thesis has highlighted the high resilience of the bioacidification process, by alternating sludge sources and crucial operational parameters. Furthermore, by implementing the guidelines obtained in the kinetic tests into the pilot scale reactor on real WRRF sites for more than 130 days, the thesis results provide additional incentives to successfully apply this process at full-scale WRRFs. Good EBPR performance was shown to be crucial as well for biological phosphorus recovery, as a higher fraction of polyphosphate was present in the sludge, which facilitates the release of phosphorus that is entrapped in the sludge cells. Other than high phosphorus release and VFA production achieved in this study, the benefit of this process could be observed through other aspects, such as extracting phosphorus prior to conventional digestors, thus avoiding pipe maintenance costs, low chemical requirements due to the presence of acidogenic bacteria and avoidance of other hazardous streams during the treatment. The thesis provided suggestions for process implementation taking into consideration influent sludge characteristics and the downstream process requirements. Linking the scientific and industrial areas was crucial throughout the scope of this thesis in order to enable real application of the results achieved.

6.2 Future work

The advances achieved in this work have raised further questions and a lot of possibilities for future research. Some of the future needs of the area are discussed below:

- Although fluorescence *in situ* hybridisation and high throughput sequencing techniques are widely applied for determination of the microbial community present in EBPR systems, it is important to perform fine-scale community analysis, in order to understand the link between intra-clade diversities of *Accumulibacter* and EBPR performance. Future studies should seek answers concerning the different metabolic expression even from phylogenetically similar bacteria, as these could help avoid process upsets in real WRRFs. Recharacterization of the *Accumulibacter* group beyond the clade level may be necessary as GAO-like activity should not be linked solely to *Competibacter* and other GAOs.
- Micropollutant transformations cannot be solely linked with complete removal of the parent compound, as some of the products formed during the degradation process can be even more hazardous. Thus, future research should focus on the toxicity of metabolic pathways and the transformation products. Furthermore, aditional studies on biotransformation capacity of different *Accumulibater* Types, clades and sub-clades are needed, as there might be specific groups of PAOs capable of more efficient diclofenac biotransformation compared to others.
- Investigating different operational parameters within different wastewater treatment process configurations is necessary as well, as potentially small process adaptations could help establish operational strategies viable for conventional biological WWTPs to detoxify DCF among other persistent micropollutants, potentially employing a wider diversity of organisms capable of performing biodegradation (e.g., nitrifiers and PAOs).
- Operational strategies for increasing phosphorus release from WWTP sludges were discussed, however, results obtained did not provide full process schemes for resource recovery. In addition to optimization of process

parameters, economical assessment as well as the comparison between different downstream options should be performed prior to full-scale application. This would enable each WRRF to choose its application, as well as the effluent requirements based on the specific on-site possibilities. The economical assessment and comparison of the possibilities for producing different types of phosphorus fertilizer, as well as the several options for VFA application, are crucial for decision making about process implementation.

- Phosphorus recovery through struvite is widely investigated, however other pathways should be compared as they could be more beneficial for specific WWTPs using for example iron salts in the treatment processes, which could have potential for simultaneous phosphorus and iron recovery through vivianite formation. Therefore, process implementation should include the monetary comparison, applicability as well as the environmental risks of all the potential products from the process.
- Modelling can be a strong decision-making tool for predicting the process outcomes based on the influent characteristics, thus enabling process adaptations based on sludge characteristics and downstream requirements. Therefore, an effort should be made to update existing activated sludge models in order to include these process adaptations and facilitate the economic and technical comparison between different treatment schemes.

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APPENDIX A

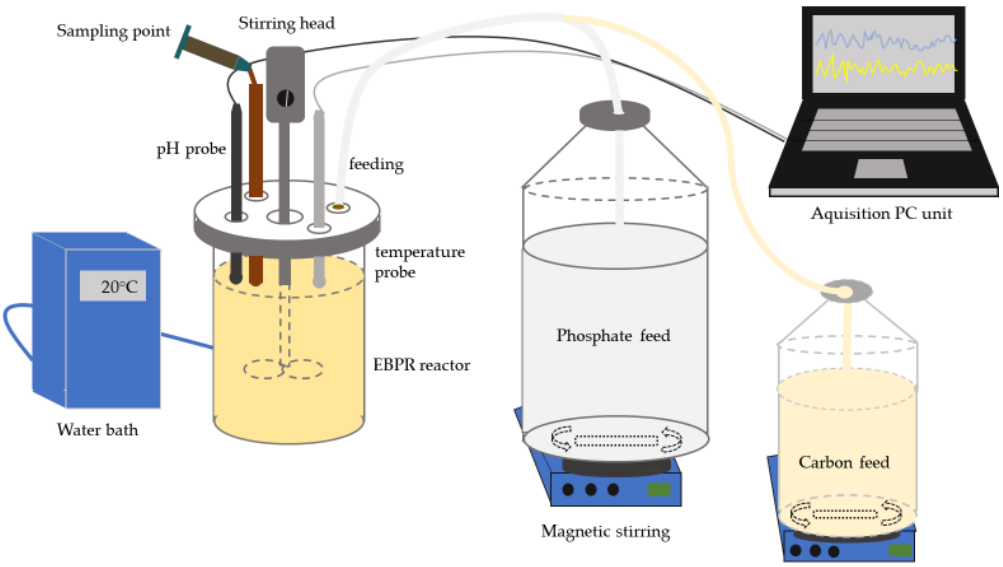


Figure A1 Scheme for setup of the experiments.

Table A1 Qualitative FISH analysis along the reactor operation.

days of operation *	**PAOmix** (PAO651+PAO462+PAO846)	**CPB_654** (*Competibacter*)	**Acc-I-444** (PAOs Type I)	**Acc-II-444** (PAOs Type II)	**Prop207** (*Propionivibrio*)	**SuperDFmix** (*Defluviicoccus - rhodospirillaceae*)
19	+++	+	+	+++	-	+-
9	+++	++	+++	+	-	+-
22	+++	+	+++	+	-	+-
57	+++	+	+++	+-	+-	+-
71	+++	+	+++	+-	-	+-
91	+++	++	+++	+-	-	+-
1	+++	++	++	+++	+-	+-
22	+++	+	++	+++	+-	-
46	+++	+-	+	+++	+-	-
69	+++	+-	+	+++	+-	-
98	+++	+-	+	+++	+-	-
117	+++	+	+	+++	+-	+-
138	+++	+-	++	+++	-	+-

(-) non-existent; (+-) almost non-existent; (+) present; (++) abundant; (+++) dominant.

*days of operation in steady state periods 1 (yellow), 2 (green) and 3 (orange).

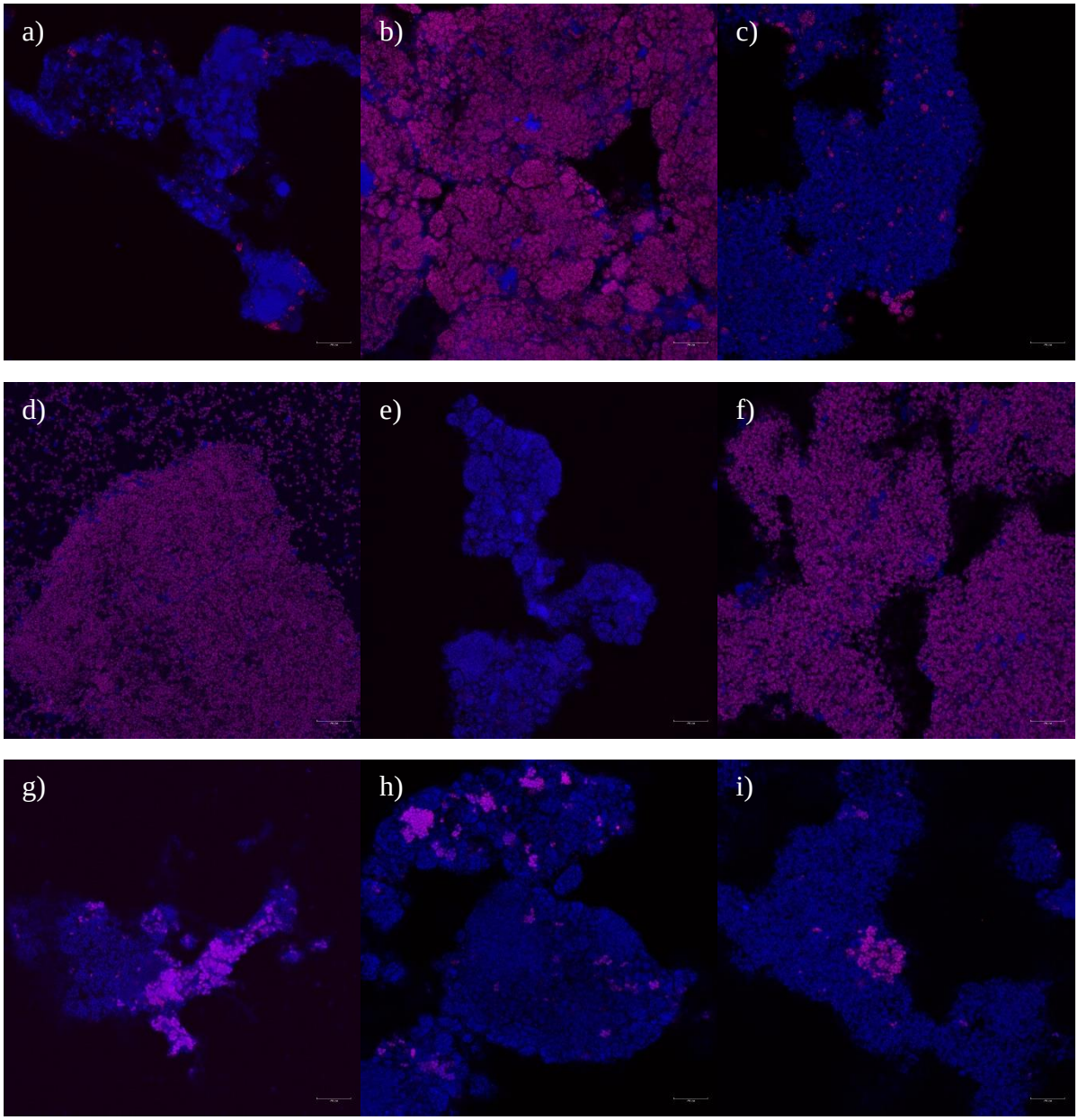
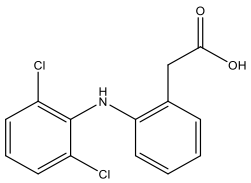
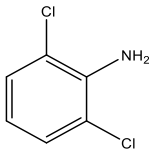
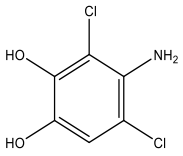
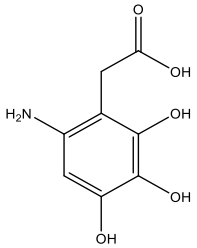
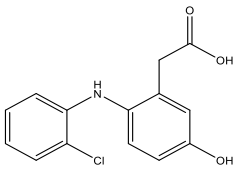
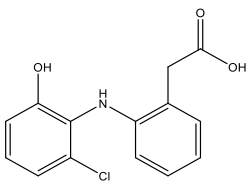
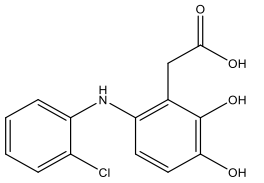
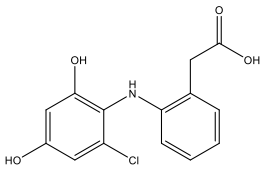
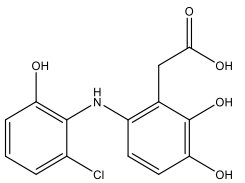
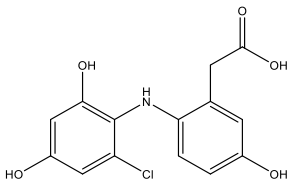


Figure A2 Quantitative FISH images of: a) *Accumulibacter* Type I, period 1; b) *Accumulibacter* Type I, period 2; c) *Accumulibacter* Type I, period 3; d) *Accumulibacter* Type II, period 1; e) *Accumulibacter* Type II, period 2; f) *Accumulibacter* Type II, period 3; g) *Competibacter*, period 1; h) *Competibacter*, period 2; i) *Competibacter*, period 3. All bacteria are in blue, while specific populations are in magenta (bar is 20 μm).

APPENDIX B

Table B1 Identification of diclofenac metabolites generated by biodegradation though LC-MS/MS (ESI-)

Compound	Structure	Formula/ exact mass measured m/z	t_r (min)	*m/z* (% Relative abundance)	Transformation product number
Diclofenac		$C_{14}H_{11}Cl_2NO_2$ 295.0167	3.630	294 (60%) 277 (100%) 250 (15%) 161 (20%) 137 (20%)	-
2,6-dichloroaniline		$C_6H_5Cl_2N$ 162.9799	1.187	161 (60%) 151 (90%) 115 (100%)	TP 1
4-amino-3,5-dichlorobenzene-1,2-diol		$C_6H_5Cl_2NO_2$ 194.0110	1.955	193 (90%) 163 (20%) 145 (20%)	TP 2
2-(6-amino-2,3,4-trihydroxyphenyl)acetic acid		$C_8H_9NO_5$ 199.0481	1.204	198 (100%) 137 (50%)	TP 3
2-(2-((2-chlorophenyl)amino)-5-hydroxyphenyl)acetic acid	 	$C_{14}H_{12}ClNO_3$ 277.0506	4.206	277 (100%) 263 (15%) 161 (20%) 137 (20%)	TP 4 TP 5

2-(2-((2-chloro-6-hydroxyphenyl)amino)phenyl)acetic acid					
2-(6-((2-chlorophenyl)amino)-2,3-dihydroxyphenyl)acetic acid 2-(2-((2-chloro4,6-dihydroxyphenyl)amino) phenyl)acetic acid	 	$C_{14}H_{12}ClNO_4$ 293.0455	2.513	293 (100%) 199 (30%) 163 (40%) 137 (50%)	TP 6 TP 7
2-(6-((2-chloro-6-hydroxyphenyl)amino)-2,3-dihydroxyphenyl)acetic acid 2-(2-((2-chloro-4,6-dihydroxyphenyl)amino)-5-hydroxyphenyl)acetic acid	 	$C_{14}H_{14}ClNO_5$ 311.0404	5.758	311 (100%) 263 (20%) 207 (60%) 163 (50%)	TP 8 TP 9

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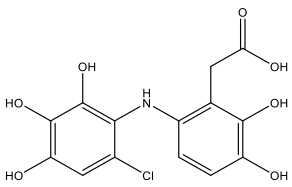
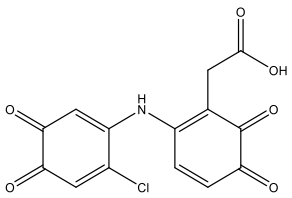
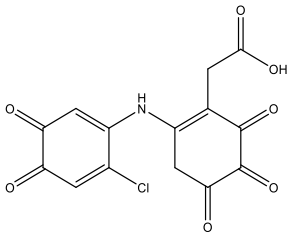
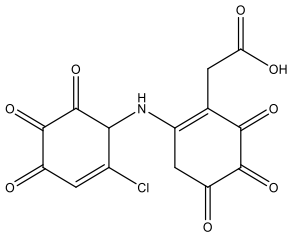
2-(6-((6-chloro-2,3,4-trihydroxyphenyl)amino)-2,3-dihydroxyphenyl)acetic acid		$C_{14}H_{12}NO_7$ 341.0302	5.863	340 (20%) 291 (100%) 277 (30%) 137 (40%)	TP 10
2-(2-((6-chloro-3,4-dioxocyclohexa-1,5-dien-1-yl)amino)-5,6-dioxocyclohexa-1,3-dien-1-yl)acetic acid		$C_{14}H_8ClNO_6$ 321.0040	5.060	321 (100%) 263 (20%) 161 (50%)	TP 11
2-(2-((6-chloro-3,4-dioxocyclohexa-1,5-dien-1-yl)amino)-4,5,6-trioxocyclohex-1-en-1-yl)acetic acid		$C_{14}H_8ClNO_7$ 337.9989	5.811	337 (30%) 311 (100%) 163 (30%) 137 (40%)	TP 12
2-(2-((2-chloro-4,5,6-trioxocyclohex-2-en-1-yl)amino)-4,5,6-trioxocyclohex-1-en-1-yl)acetic acid		$C_{14}H_8ClNO_8$ 353.9938	6.596	352 (20%) 351 (100%)	TP 13

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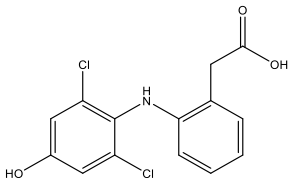
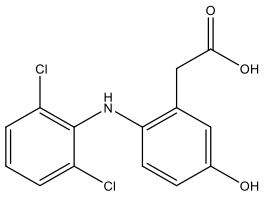
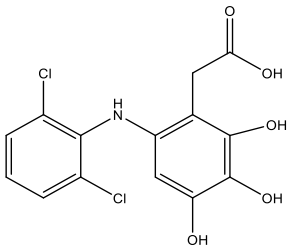
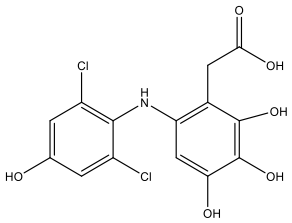
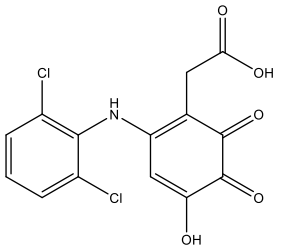
2-(2-((2,6-dichloro-4-hydroxyphenyl)amino)phenyl)acetic acid or 4′-Hydroxydiclofenac 2-(2-((2,6-dichlorophenyl)amino)-5-hydroxyphenyl)acetic acid or 5-Hydroxydiclofenac	 	$C_{14}H_{11}Cl_2NO_3$ 312.0116	6.370	311 (90%) 291 (100%) 277 (50%)	TP 14 TP 15
2-(6-((2,6-dichlorophenyl)amino)-2,3,4-trihydroxyphenyl)acetic acid		$C_{14}H_{11}Cl_2NO_5$ 344	1.396	343 (40%) 317 (70%) 245 (90%) 131 (100%)	TP 16
2-(6-((2,6-dichloro-4-hydroxyphenyl)amino)-2,3,4-trihydroxyphenyl)acetic acid		$C_{14}H_{11}Cl_2NO_6$ 360	2.373	359 (100%) 293 (60%) 245 (30%)	TP 17
2-(2-((2,6-dichlorophenyl)amino)-4-hydroxy-5,6-dioxocyclohexa-1,3-dien-1-yl)acetic acid		DCF 20 $C_{14}H_9Cl_2NO_5$ 342.1280	14.606	341 (10%) 271 (100%) 219 (40%)	TP 18

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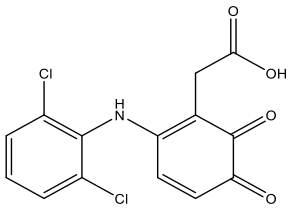
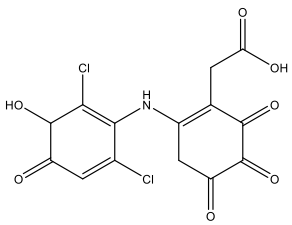
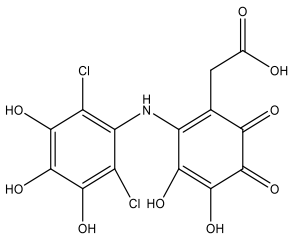
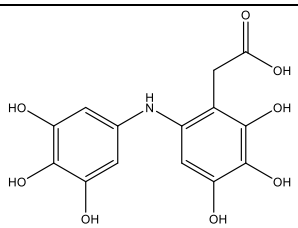
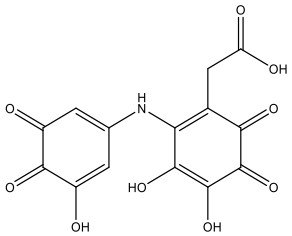
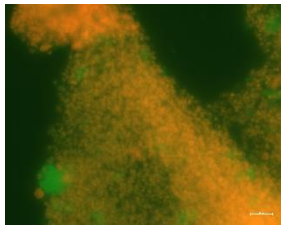
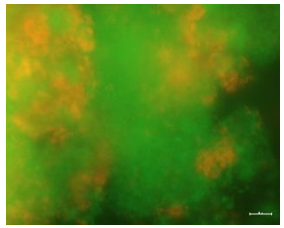
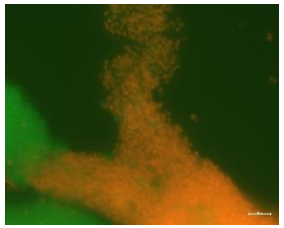
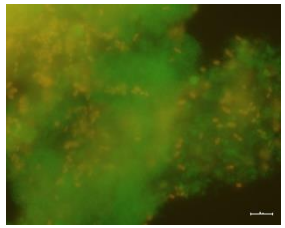
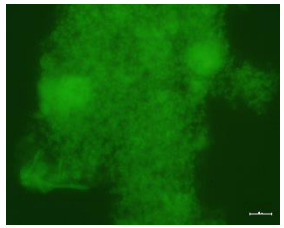
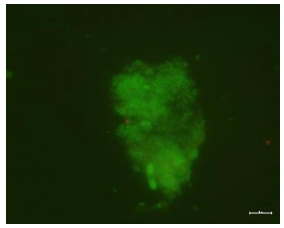
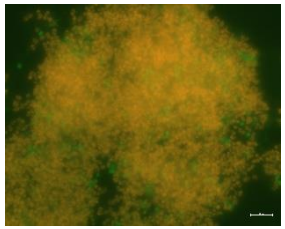
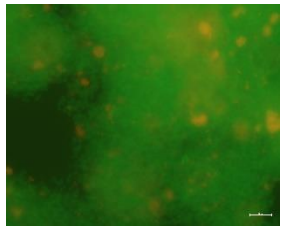
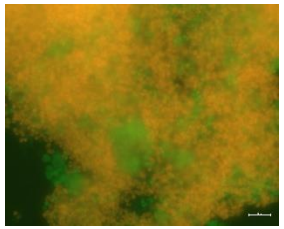
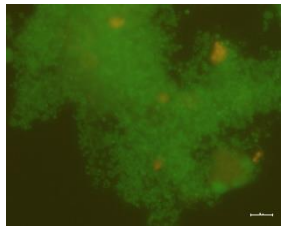
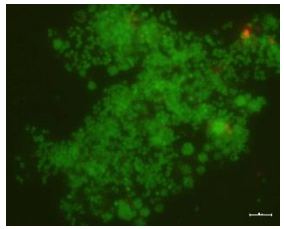
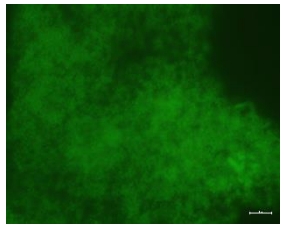
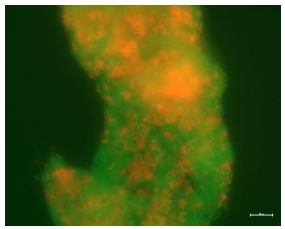
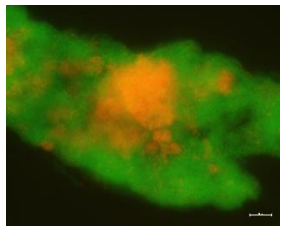
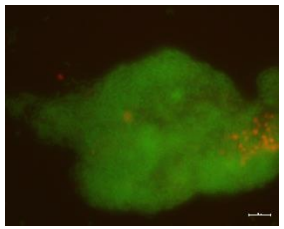
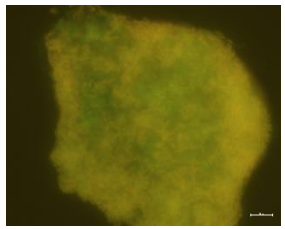
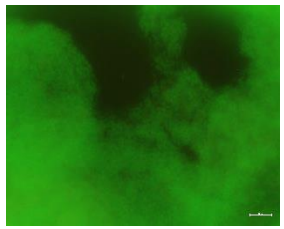
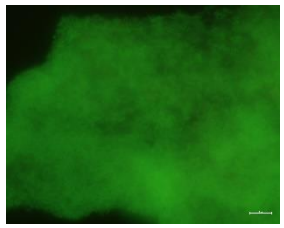
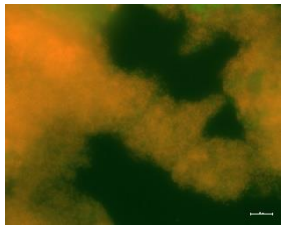
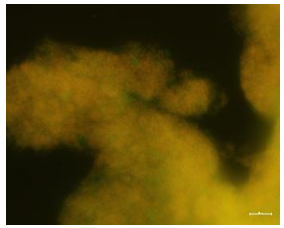
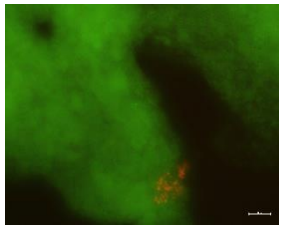
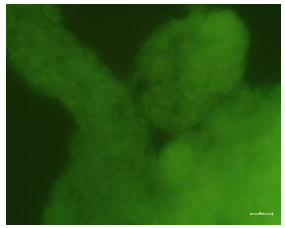
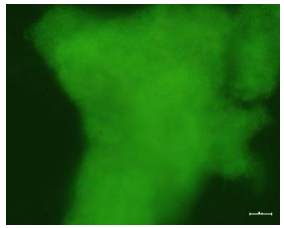
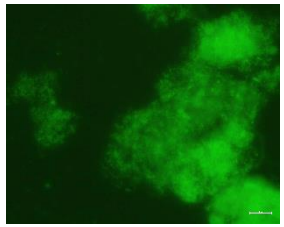
2-(2-((2,6-dichlorophenyl)amino)-5,6-dioxocyclohexa-1,3-dien-1-yl)acetic acid		$C_{14}H_9Cl_2NO_4$ 324.9909	8.306	326 (100%) 294 (30%) 263 (40%) 219 (40%) 179 (70%)	TP 19
2-(2-((2,6-dichloro-3-hydroxy-4-oxocyclohexa-1,5-dien-1-yl)amino)-4,5,6-trioxocyclohex-1-en-1-yl)acetic acid		$C_{14}H_{11}Cl_2NO_7$ 372.9756	1.273	372 (50%) 362 (100%) 228 (20%) 199 (20%)	TP 20
2-(2-((2,6-dichloro-3,4,5-trihydroxyphenyl)amino)-3,4-dihydroxy-5,6-dioxocyclohexa-1,3-dien-1-yl)acetic acid		DCF 17 $C_{14}H_9Cl_2NO_9$ 404.9654	12.773	403 (100%) 393 (40%) 339 (30%) 263 (40%)	TP 21
2-(2,3,4-trihydroxy-6-((3,4,5-trihydroxyphenyl)amino)phenyl)acetic acid		$C_{14}H_{13}NO_8$ 323.0641	5.462	321 (100%) 291 (50%) 277 (20%)	TP 22
2-(3,4-dihydroxy-2-((5-hydroxy-3,4-dioxocyclohexa-1,5-dien-1-yl)amino)-5,6-dioxocyclohexa-1,3-dien-1-yl)acetic acid		$C_{14}H_9NO_9$ 335.0277	5.793	355 (50%) 311 (100%) 296 (40%) 161 (40%)	TP 23

APPENDIX B

Table B2 Qualitative FISH images of *Accumulibacter* Types I and II, *Competibacter*, *Propionivibrio* and *Defluvvicocus* in the four operation periods. All bacteria are in green, while specific populations are in yellow (bar is 10 μm).

Period	**PAOmix** (*Accumulibacter*)	**Acc-I-444** (PAO Type I)	**Acc-II-444** (PAO Type II)	**CPB_654** (*Competibacter*)	**Prop207** (*Propionivibrio*)	**SuperDFmix** (*Defluviicoccus - rhodospirillaceae*)
1						
2						
3						
4						

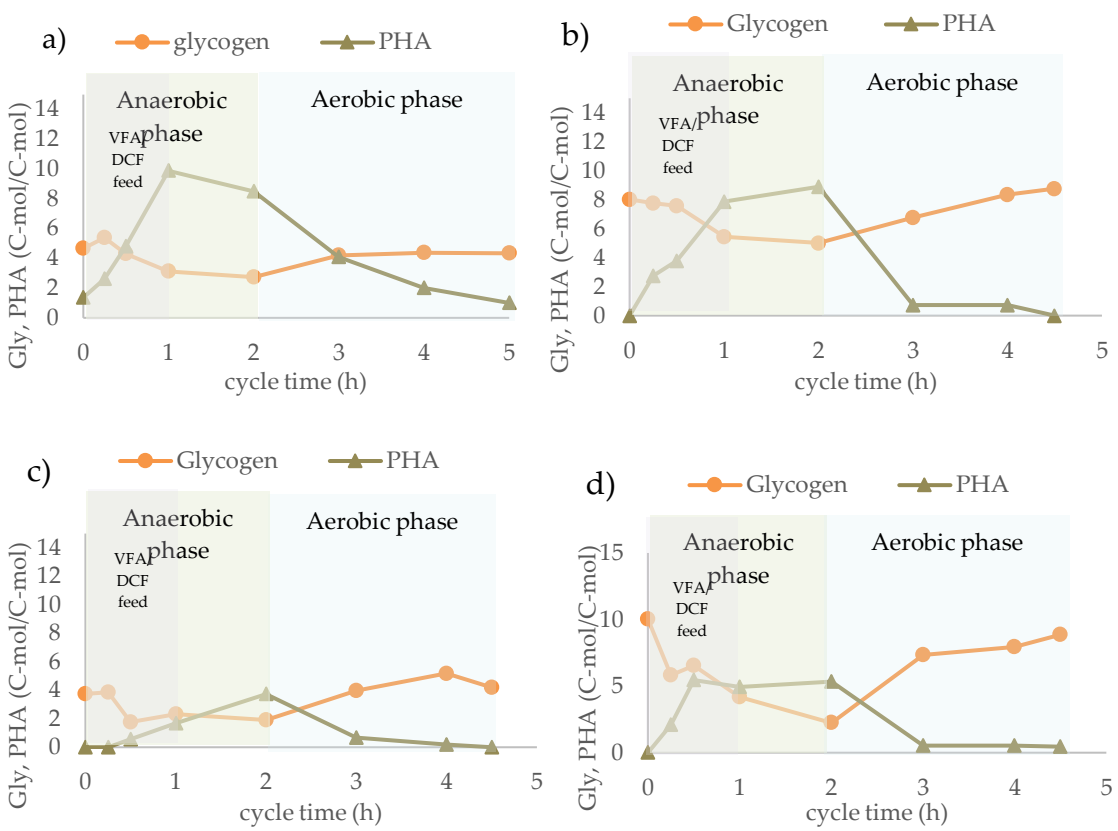


Figure B1 Profile of glycogen and PHA in the reactor during a) steady state period 1; b) steady state period; 2 c) steady state period 3 and d) steady state period 4.

Text B1 Mass balance for diclofenac adsorption to biomass

DCF adsorbed to biomass ($DCF_{biomass}$) was determined by measuring the concentration of DCF obtained after biomass washing (DCF_{wash}), as described in section 4.2.3, and divided by the dilution factor (D) applied during the washing step and the total number of cycles during one SRT (N) of bioreactor operation, as described below:

$$DCF_{biomass}\left(\frac{\mu g}{L.cycle}\right) = \frac{DCF_{wash}}{D * N}$$

$$N\ (cycles) = \left(\frac{cycles}{day}\right) * SRT(day)$$

The percentage of DCF removed by adsorption ($DCF_{adsorbed}$) was determined from the ratio of the DCF concentration adsorbed to the biomass divided by the average total (i.e., biotransformation + adsorption) DCF (DCF_{tot}) transformed during a cycle, as described below:

$$DCF_{adsorbed}(\%) = \frac{DCF_{biomass}}{DCF_{tot}} * 100$$

Finally, the percentage of DCF removed by biotransformation ($DCF_{biotransformation}$) to other transformation products was determined by subtracting the percentage of DCF removed by adsorption from 100 %:

$$DCF_{biotransformed}(\%) = 100 - DCF_{adsorbed}$$

APPENDIX C

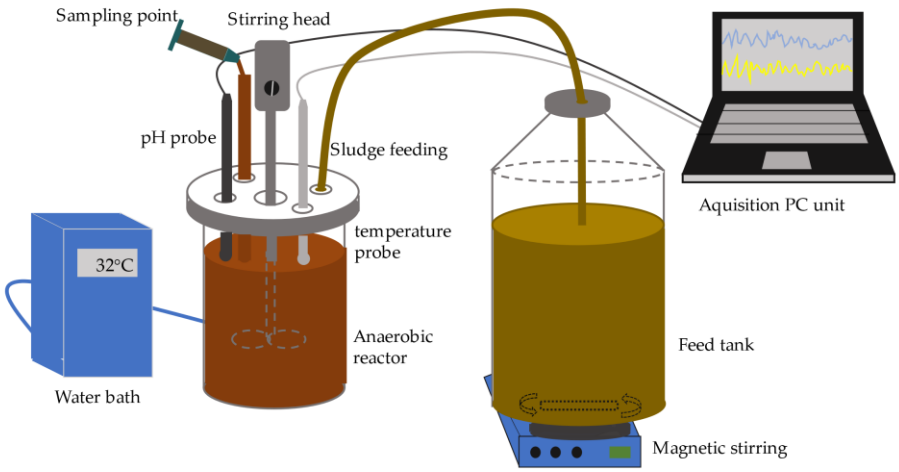


Figure C1 Scheme for setup of the experiments.

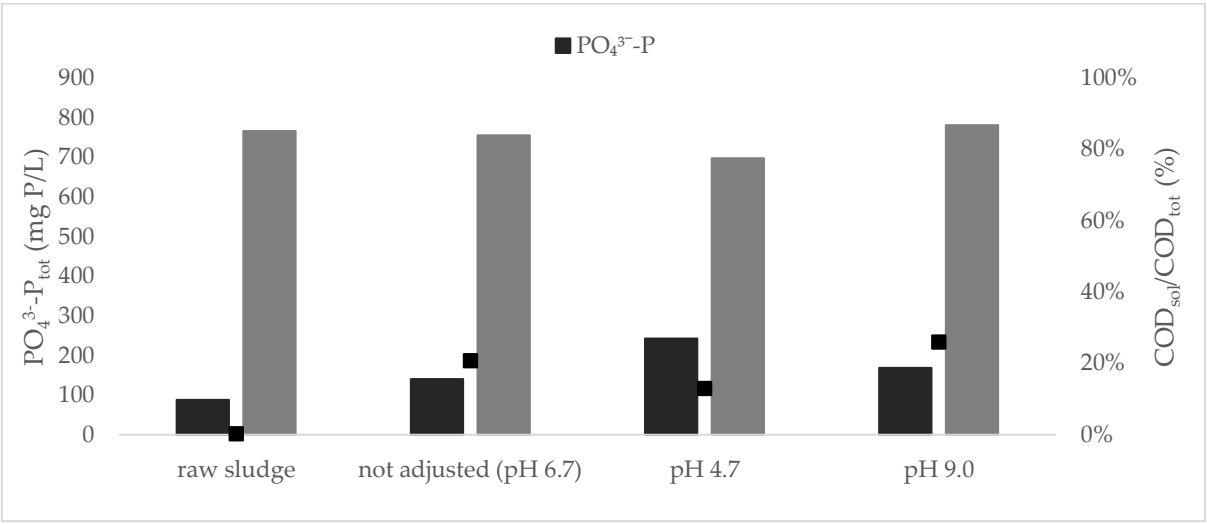


Figure C2 PO_4^{3-}-P and P_{tot} concentrations and COD_{sol}/COD_{tot} solubilisation ratios in thermal hydrolysis sludge pre-treatment at 120 °C, 1h and different pH (not adjusted, 4.7 and 9) using non-EBPR WAS.

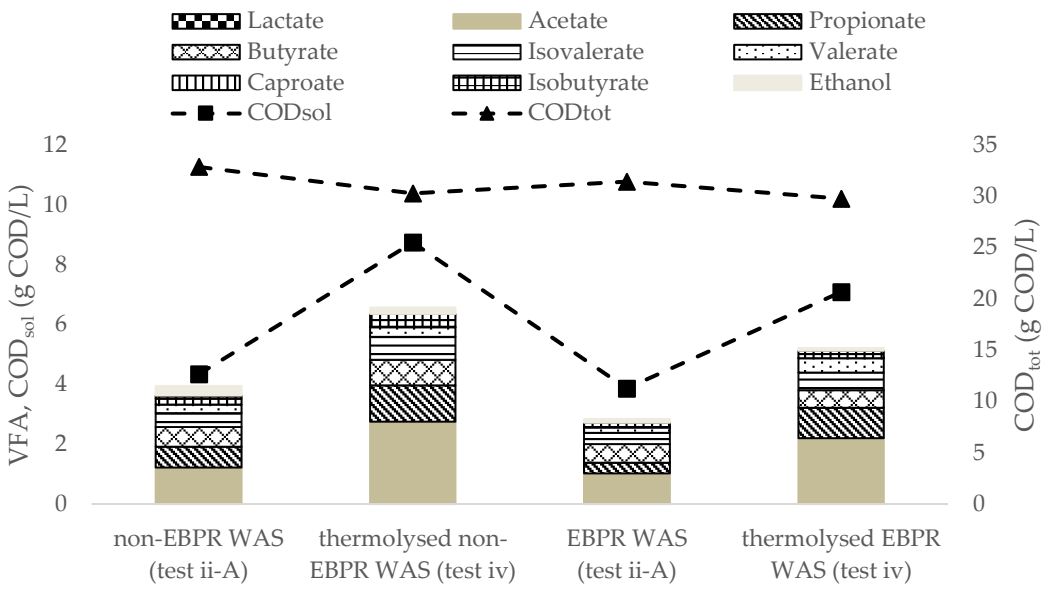


Figure C3 Compared VFA composition, COD_{sol} and COD_{tot} in the sole bioacidification (test ii-A) and combined thermal alkaline hydrolysis pre-treatment with bioacidification process (test iv).

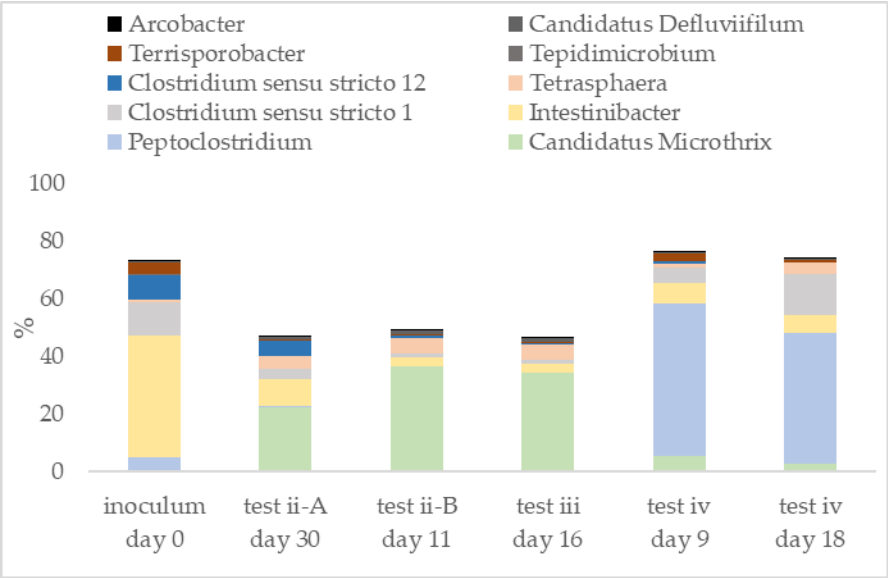


Figure C4 Compared microbial community DNA sequencing results with most abundant microorganisms on genus level.

Table C1 Average effluent ratios obtained in the pilot bioacidification reactor.

Phase	PO_4^{3-}-P /P_{tot} (%)	NHx-N/ N_{tot} (%)	COD_{sol}/COD_{tot} (%)	COD_{VFA}/VS (mg COD_{VFA}/g VS)	Fe_{sol}/Fe_{tot} (%)	Ca_{sol}/Ca_{tot} (%)	Mg_{sol}/Mg_{tot} (%)
I	68 ± 16	20 ± 3	11 ± 2	134 ± 64	-	-	-
II	74 ± 11	19 ± 9	13 ± 2	163 ± 51	-	-	-
III	67 ± 11	35 ± 12	19 ± 1	276 ± 44	26 ± 11	58 ± 9	77 ± 5
IV	78 ± 10	45 ± 6	28 ± 3	423 ± 55	41 ± 9	68 ± 10	80 ± 4

LOMBADA

INVESTIGATION OF KEY OPERATIONAL FACTORS IMPACTING PHOSPHORUS REMOVAL AND RECOVERY FROM WASTEWATER TREATMENT PLANTS

SRĐANA KOLAKOVIĆ OLIVEIRA BARREIROS

2021

Овај Образац чини саставни део докторске дисертације, односно докторског уметничког пројекта који се брани на Универзитету у Новом Саду. Попуњен Образац укоричити иза текста докторске дисертације, односно докторског уметничког пројекта.

План третмана података

Назив пројекта/истраживања
Ispitivanje ključnih procesnih parametara separacije i rekuperacije fosfora na postrojenju za tretman otpadne vode
Назив институције/институција у оквиру којих се спроводи истраживање
а) Fakultet Nauke i Tehnologije, Univerzitet Nova u Lisabonu, Portugalija (FCT, UNL) б) Fakultet Tehničkih Nauka, Univerzitet u Novom Sadu, Srbija (FTN, UNS)
Назив програма у оквиру ког се реализује истраживање
-
1. Опис података

1.1 Врста студије

Укратко описати тип студије у оквиру које се подаци прикупљају

U ovoj studiji nisu prikupljani podaci .

1.2 Врсте података

а) квантитативни

б) квалитативни

1.3. Начин прикупљања података

а) анкете, упитници, тестови

б) клиничке процене, медицински записи, електронски здравствени записи

в) генотипови: навести врсту _____

г) административни подаци: навести врсту _____

д) узорци ткива: навести врсту_____

ђ) снимци, фотографије: навести врсту_____

е) текст, навести врсту _____

ж) мапа, навести врсту _____

з) остало: описати _____

1.3 Формат података, употребљене скале, количина података

1.3.1 Употребљени софтвер и формат датотеке:

a) Excel фајл, датотека _____

b) SPSS фајл, датотека _____

c) PDF фајл, датотека _____

d) Текст фајл, датотека _____

e) JPG фајл, датотека _____

f) Остало, датотека _____

1.3.2. Број записа (код квантитативних података)

а) број варијабли _____

б) број мерења (испитаника, процена, снимака и сл.) _____

1.3.3. Поновљена мерења

а) да

б) не

Уколико је одговор да, одговорити на следећа питања:

а) временски размак између поновљених мера је _____

б) варијабле које се више пута мере односе се на _____

в) нове верзије фајлова који садрже поновљена мерења су именоване као _____

Напомене: _____

Да ли формати и софтвер омогућавају дељење и дугорочну валидност података?

а) Да

б) Не

Ако је одговор не, образложити _____

_____

2. Прикупљање података

2.1 Методологија за прикупљање/генерисање података

2.1.1. У оквиру ког истраживачког нацрта су подаци прикупљени?

а) експеримент, навести тип _____

б) корелационо истраживање, навести тип _____

ц) анализа текста, навести тип _____

д) остало, навести шта _____

2.1.2 Навести врсте мерних инструмената или стандарде података специфичних за одређену научну дисциплину (ако постоје).

_____

_____

2.2 Квалитет података и стандарди

2.2.1. Третман недостајућих података

а) Да ли матрица садржи недостајуће податке? Да Не

Ако је одговор да, одговорити на следећа питања:

а) Колики је број недостајућих података? _____

б) Да ли се кориснику матрице препоручује замена недостајућих података? Да Не

в) Ако је одговор да, навести сугестије за третман замене недостајућих података

_____

2.2.2. На који начин је контролисан квалитет података? Описати

_____

_____

2.2.3. На који начин је извршена контрола уноса података у матрицу?

_____

_____

3. Третман података и пратећа документација

3.1. Третман и чување података

3.1.1. Подаци ће бити депоновани у _____ репозиторијум.

3.1.2. URL адреса _____

3.1.3. DOI _____

3.1.4. Да ли ће подаци бити у отвореном приступу?

а) Да

б) Да, али после ембарга који ће трајати до _____

в) Не

Ако је одговор не, навести разлог _____

3.1.5. Подаци неће бити депоновани у репозиторијум, али ће бити чувани.

Образложење

_____

_____

3.2 Метаподаци и документација података

3.2.1. Који стандард за метаподатке ће бити примењен? _____

3.2.1. Навести метаподатке на основу којих су подаци депоновани у репозиторијум.

_____

_____

Ако је потребно, навести методе које се користе за преузимање података, аналитичке и процедуралне информације, њихово кодирање, детаљне описе варијабли, записа итд.

_____

_____

_____

_____

3.3 Стратегија и стандарди за чување података

3.3.1. До ког периода ће подаци бити чувани у репозиторијуму? _____

3.3.2. Да ли ће подаци бити депоновани под шифром? Да Не

3.3.3. Да ли ће шифра бити доступна одређеном кругу истраживача? Да Не

3.3.4. Да ли се подаци морају уклонити из отвореног приступа после извесног времена?

Да Не

Образложити

_____

_____

4. Безбедност података и заштита поверљивих информација

Овај одељак МОРА бити попуњен ако ваши подаци укључују личне податке који се односе на учеснике у истраживању. За друга истраживања треба такође размотрити заштиту и сигурност података.

4.1 Формални стандарди за сигурност информација/података

Истраживачи који спроводе испитивања с људима морају да се придржавају Закона о заштити података о личности *(https://www.paragraf.rs/propisi/zakon_o_zastiti_podataka_o_licnosti.html)* и одговарајућег институционалног кодекса о академском интегритету.

4.1.2. Да ли је истраживање одобрено од стране етичке комисије? Да Не

Ако је одговор Да, навести датум и назив етичке комисије која је одобрила истраживање

_____

4.1.2. Да ли подаци укључују личне податке учесника у истраживању? Да Не

Ако је одговор да, наведите на који начин сте осигурали поверљивост и сигурност информација везаних за испитанике:

а) Подаци нису у отвореном приступу

б) Подаци су анонимизирани

ц) Остало, навести шта

_____

_____

5. Доступност података

5.1. Подаци ће бити

а) јавно доступни

б) доступни само уском кругу истраживача у одређеној научној области

ц) затворени

Ако су подаци доступни само уском кругу истраживача, навести под којим условима могу да их користе:

_____

_____

Ако су подаци доступни само уском кругу истраживача, навести на који начин могу приступити подацима:

_____

_____

5.4. Навести лиценцу под којом ће прикупљени подаци бити архивирани.

_____

6. Улоге и одговорност

6.1. Навести име и презиме и мејл адресу власника (аутора) података

_____

6.2. Навести име и презиме и мејл адресу особе која одржава матрицу с подацима

_____

6.3. Навести име и презиме и мејл адресу особе која омогућује приступ подацима другим истраживачима

_____