Single-cell ecophysiology of key microbial taxa in wastewater treatment systems

A trip to unveil the microbial "dark matter"

Petriglieri, Francesca

Publication date:
2020

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

SINGLE-CELL ECOPHYSIOLOGY OF KEY MICROBIAL TAXA IN WASTEWATER TREATMENT SYSTEMS

A TRIP TO UNVEIL THE MICROBI AL “DARK MAT TER”

BY
FRANCESCA PETRIGLIERI

DISSERTATION SUBMITTED 2020

AALBORG UNIVERSITY
DENMARK

SINGLE-CELL ECOPHYSIOLOGY OF KEY MICROBIAL TAXA IN WASTEWATER TREATMENT SYSTEMS

A TRIP TO UNVEIL THE MICROBIAL "DARK MATTER"

By

Francesca Petriglieri

AALBORG UNIVERSITY
DENMARK

Dissertation submitted August 2020

.

Dissertation submitted: August 2020

PhD supervisor: Prof. Per Halkjær Nielsen,
Aalborg University, Aalborg

PhD committee: Associate Professor Niels Iversen (chairman)
Aalborg University

Professor Andreas Schramm
Aarhus University

Professor Malte Hermansson
University of Gothenburg

PhD Series: Faculty of Engineering and Science, Aalborg University

Department: Department of Chemistry and Bioscience

ISSN (online): 2446-1636
ISBN (online): 978-87-7210-693-9

Published by:
Aalborg University Press
Kroghstræde 3
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk

Printed in Denmark by Rosendahls, 2020

ENGLISH SUMMARY

Microorganisms play a key-role in wastewater treatment, a vital process to ensure not only the environment safety, but also essential resource recovery and production of bioenergy. However, the microbial community of these engineered systems is in large part a black box and the majority of the microorganisms belonging to it is still undescribed. It is an “old”, but still new, challenge for microbial ecologists to unravel this black box and explore the so-called microbial dark matter in these systems. Over the years, innovation in molecular techniques, have allowed to gain a first insight into the identity of the microorganisms abundant in these systems, but only recently, a major breakthrough was reached with the development of a method that would allow the retrieval of millions of full-length 16S rRNA genes from single experiments, enabling the construction of ecosystem-specific databases that would facilitate design of specific primers and probes.

The aim of this project was to gain a better understanding of the ecophysiology and functions of key microbial groups in activated sludge wastewater treatment plants, to aid with future process optimization and troubleshooting. The identity of key microorganisms was investigated by using the MIDAS database for amplicon sequencing profiling and Fluorescence in situ hybridization (FISH) probes design. Their metabolism and ecophysiology were also explored by using Raman microspectroscopy for *in situ* studies and through retrieval and annotation of metagenome-assembled genomes (MAGs).

Among the different functional groups, the polyphosphate-accumulating organisms (PAO) and filamentous bacteria belonging to the phylum Chloroflexi were selected for further characterization. The PAO community of Danish wastewater treatment plants was explored in detail, with the identification of several novel PAO through *in situ* analysis and MAGs annotation. Species belonging to the genus *Dechloromonas*, abundant in Danish WWTPs, possessed in *situ* a phenotype similar to the model PAO *Ca.* Accumulibacter, with dynamics of poly-P, PHA, and glycogen during feast/famine cycling. These results were supported by the annotation of several high-quality MAGs, showing also their possible involvement in nitrogen removal. The names *Ca.* Decloromonas phosphatis and *Ca.* Dechloromonas phopshovora were proposed for the two most abundant species. The individual contribution to P removal in full-scale WWTPs of all the known PAOs, *Ca.* Accumulibacter, *Tetrasphaera* and

Dechloromonas, and the unconventional PAO *Ca*. Microthrix was then assessed using a combination of different independent methods, to obtain for the first time a comprehensive mass balance of all organic and inorganic P-compounds, normally present in activated sludge. This showed that the majority of the abundant PAO in Danish wastewater treatment systems is now known, even though a small amount of potential PAOs are still uncharacterized. To discover them, screening of more than 1000 MAGs was performed, indicating new genera with potential for poly-P accumulation. *Ca*. Methylophosphatis was then selected for further *in situ* characterization, which revealed the presence of poly-P at similar levels to other known PAOs. The MAGs represent an invaluable resource to ink identity to function and discover more potential PAO or bacteria belonging to other important functional groups.

Filamentous Chloroflexi are often abundant in Danish or global activated sludge systems, sometimes involved in operational issues. However, only few genera have been characterized so far. A new FISH probe set was designed to target the most abundant members of the Chloroflexi community of Danish and global activated sludge, showing their morphology, spatial arrangement, metabolic potential and possible functions in these engineered systems. The different genera appeared to have diverse morphologies, from long filaments often creating inter-floc bridges to rod-shaped cells found deep into the activated sludge flocs. Interestingly, some of them were found consistently abundant, reaching up to 30% of the biovolume in some WWTPs, suggesting their potential involvement in bulking problems. FISH combined with microautoradiography (MAR) of some of the novel genera showed their potential to ferment sugars and suggested their role in the degradation of organic matter in activated sludge. The utilization of the MiDAS database for global activated sludge allowed also the comparison of Chloroflexi genera abundant in different plant designs and climate zones, enabling for the first time a comprehensive overview of the distribution of this group of microorganisms worldwide. This study provides a starting point for in-depth understanding of the ecophysiology of these microorganisms, also in relation to operational parameters and potential control measures.

DANSK RESUME

Mikroorganismer har en altafgørende rolle i forbindelse med rensning af spildevand, en essentiel proces, der ikke kun er med til at beskytte miljøet, men også vigtig i forbindelse med genbrug af ressourcer og produktion af bioenergi. Til trods for dette, så er den mikrobielle sammensætning i biologiske renseanlæg i høj grad et mysterium, hvor langt de fleste mikroorganismer endnu ikke er blevet beskrevet. Det er en "gammelkendt", men stadig yderst relevant udfordring at udforske disse ukendte mikoorganismer, ofte kaldet "microbial dark matter". Inden for de seneste år har nye molekylære teknikker gjort det muligt at få et indblik i disse mikroorganismers identitet og levevis. Det er dog først for nyligt, at nye teknologiske gennembrud har gjort det muligt at analysere tusindvis af "fingeraftryksgener" og etablere en økosystem-specifik database, som giver mulighed for at undersøge disse økosystemer detaljeret, bl.a. ved at designe specifikke primer og prober for ukendte mikroorganismer.

Formålet for dette projekt var at opnå en mere dybdegående forståelse af identitet og økofysiologi for nogle af de vigtigste mikroorganismer i aktiv slam anlæg, for at kunne identificere eventuelle problemer samt optimere anlæggenes drift. For at identificere de vigtigste mikroorganismer blev MIDAS databasen anvendt til profilering ved hjælp af af amplicon sekventering og fluorescerende *in situ* hybridisering (FISH). Udvalgte grupper af mikroorganismer blev udvalgt til nærmere undersøgelse af metabolisme og økofysiologi, bl.a. ved hjælp af Raman mikrospektroskopi til *in situ* studier kombineret med annotering af metagenome-assembled genomer (MAGs).

Polyfosfat-akkumulerende bakterier (PAO) og trådformede bakterier tilhørende phylum Chloroflexi blev udvalgt til detaljeret karakterisering. Kendte og nye PAO i danske renseanlæg blev undersøgt i detaljer, med henblik på at bestemme deres individuelle bidrag til P-fjernelse ved hjælp af in situ enkelt-celle studier. Yderligere blev flere/disse nye PAO identificeret og deres fysiologi analyseret med metabolisk rekonstruktion og verificeret eksperimentelt. Flere arter tilhørende slægten *Dechloromonas*, som er hyppig i de danske renseanlæg, viste sig ved hjælp af *in situ* studier at have en PAO fænotype meget lig den velkendte *Ca*. Accumulibacter, med sine dynamiske ændringr i intracellulært puljer af poly-P, PHA og glykogen. Dette blev støttet ved hjælp af annotering af flere høj-kvalitet MAGs, som ydermere viste

at disse også kan være involveret i fjernelse af nitrogen. De to hyppigste arter fik navnene *Ca.* Dechloromonas phosphatis og *Ca.* Dechloromonas phosphovora. De individuelle bidrag til P-fjernelse i renseanlæg for kendte PAOs, *Ca.* Accumulibacter, *Tetrasphaera* og *Dechloromonas*, samt den utraditionelle PAO *Ca.* Microthrix, blev estimeret ved at kombinere forskellige metoder for at opnå en meget fyldestgørende massebalance for alt organisk og uorganisk fosfor, der er til stede i det aktive slam. Dette studie viste bl.a. at der stadig måtte være ukendte PAOs på trods af at langt de fleste PAOs i de danske renseanlæg nu er kendte. En analyse af mere end 1000 MAGs fra de danske renseanlæg blev derfor foretaget. En ny potentiel poly-P akkumulerende slægt blev fundet og i*n situ* karakterisering bekræftede, at den kunne oplagre poly-P i tilsvarende mængder som andre kendte PAOs. Slægten har fået navnet *Ca.* Methylophosphatis. Anvendelsen af MAG er et uundværligt værktøj i forhold til at kæde identitet sammen med funktion, hvilket giver mulighed for at undersøge flere potentielle PAO eller andre bakteriers rolle i renseprocessen.

Trådformede mikroorganismer fra rækken (phylum) Chloroflexi forekommer ofte i danske og globale aktiv slam anlæg, hvor de til tider er involveret i driftsmæssige problemer. Diversiteten er dog dårligt kendt og kun få slægter er hidtil er blevet karakteriseret. Nye FISH-prober blev designet med henblik på at identificere de hyppigste mikroorganismer fra Chloroflexi i danske anlæg og fra globale prøver, hvored visualisering af morfologi, rummeligt placering, potentielle metabolisme og mulige funktioner blev kortlagt. De forskellige slægter synes at have forskellige morfologier, mange danner lange tråde, som ofte danner bro mellem slamflokke til stavformet celler som findes dybt inde i flokkene. Det er bemærkelsesværdigt, at nogle af disse regelmæssigt forekommer i store mængder, helt op til 30% af biomassen nogle renseanlæg, og dermed kan de skabe store problemer med dårlige slamegenskaber og slamflugt. FISH-MAR analyse af nogle af de nye slægter demonstrerede aspekter af deres levevis. De kan bl.a. fermentere sukre, er involveret i nedbrydning af komplekst organisk stof og er involveret i denitrifikation. Anvendelsen af den globale MiDAS database med data for over 650 renseanlæg over hele verden har gjort det muligt at sammenholde diversiteten af Chloroflexi slægter i forskellige typer af renseanlæg og klimatiskezoner. Det er første gang et så omfattende studie af denne gruppe af mikroorganismer er foretaget. Dette studie er med til at give en mere dybdegående forståelse for økofysiologien af disse bakterier også i relation til de driftsmæssige parametre og potentielle kontrolforanstaltninger.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Per H. Nielsen, for being a guidance during this scientific journey, for being understanding and patient, and, most important, for believing in the Italian girl that few years ago knocked on his door and wanted to become a scientist. I have enjoyed our scientific discussions, both when we agreed and when we didn't, and I feel that our collaboration made me grow as a scientist and as a person.

I would also like to express my gratitude to all my colleagues at the Center for Microbial Communities, for the practical help during the whole project and for the great memories of conferences and parties. In particular, I want to thank Marta for sharing her knowledge and being my "lighthouse in darkness", as I like to call her, and to my other office mates, Giulia and Dorka, for and being supportive all the time, for the laughter and also for listening to my complains (from time to time). Thanks to Caitlin, which has been the most patient "teacher" I ever had and is always so enthusiast about starting a new project together. Also a special thanks to Zivile, who has been, first, the best master student to supervise and, after, the best right hand I could ever wish for.

Lastly, I have to thank my amazing family, my boyfriend Morten and my friends, that supported me patiently and were always so good at reminding me that science and work are not everything in life… Finally, I need to give a special thank to my beloved father, because it´s only thanks to him that I am who I am. I hope that wherever you are now, you are proud of me today.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PAPERS

Paper 1: F. Petriglieri, C.M. Singleton, M. Peces, J. F. Petersen, M. Nierychlo, P. H. Nielsen. "*Candidatus* Dechloromonas phosphatis" and "*Candidatus* Dechloromonas phosphovorax", two novel polyphosphate accumulating organisms abundant in wastewater treatment systems. ***BioRxiv, (for submission to ISME Journal)***

Paper 2: F. Petriglieri, J.F. Petersen, M. Peces, M. Nierychlo, K. Hansen, U.G. Nielsen, K. Reitzel, P.H. Nielsen. Quantification of biologically and chemically bound phosphorus in activated sludge from full-scale plants. ***Manuscript (for submission to Water Research)***

Paper 3: C.M. Singleton, **F. Petriglieri**, J.M. Kristensen, R.H. Kirkegaard, T.Y. Michaelsen, M.H Andersen, Z. Kondrotaite, S.M. Karst, M.S. Dueholm, P.H. Nielsen, M. Albertsen. Connecting structure to function with the recovery of over 1000 high-quality activated sludge metagenome-assembled genomes encoding full-length rRNA genes using long-read sequencing. ***BioRxiv, doi: https://doi.org/10.1101/2020.05.12.088096***

Paper 4: M. Nierychlo, A. Miłobędzka, **F. Petriglieri**, B. McIlroy, P.H. Nielsen, S.J. McIlroy. The morphology and metabolic potential of the Chloroflexi in full-scale activated sludge wastewater treatment plants. ***FEMS, doi: https://doi-org.zorac.aub.aau.dk/10.1093/femsec/fiy228***

Paper 5: F. Petriglieri, Z. Kondrotaite, M. Nierychlo, P.H. Nielsen. A comprehensive overview of the Chloroflexi community in Danish and global wastewater treatment plants. ***Manuscript.***

Papers not included in this thesis:

Paper 6: F. Petriglieri, M. Nierychlo, P.H. Nielsen, S.J. McIlroy. *In situ* visualization of the abundant Chloroflexi populations in full-scale anaerobic digesters and the fate of immigrating species. ***PlosOne. doi: https://doi.org/10.1371/journal.pone.0206255***

Paper 7: E.Y. Fernando, S.J. McIlroy, M. Nierychlo, F-A. Herbst, **F. Petriglieri**, M.C. Schmid, M. Wagner, J.L. Nielsen, P.H Nielsen. Resolving the individual contribution of key microbial populations to enhanced biological phosphorus removal

with Raman–FISH. ***The ISME Journal, doi:*** ***https://doi.org/10.1038/s41396-019-0399-7***

Paper 8: M.S. Dueholm, K.S. Andersen, **F. Petriglieri,** S.J. McIlroy, M. Nierychlo, J.F. Petersen, J.M. Kristensen, E. Yashiro, S.M. Karst, M. Albertsen, and P.H. Nielsen. Comprehensive ecosystem-specific 16S rRNA gene databases with automated taxonomy assignment (AutoTax) provide species-level resolution in microbial ecology. ***BioRxiv, doi:*** ***https://doi.org/10.1101/672873***

Paper 9: P.H. Nielsen, M. Nierychlo, **F. Petriglieri**, K. Reitzel. Bakterier fjerner phosphate fra spildevand. ***Aktuel Naturvidenskab,*** ***https://aktuelnaturvidenskab.dk/fileadmin/Aktuel_Naturvidenskab/nr-5/AN5-2019-Bakterier-fjerner-P-spildevand.pdf***

Paper 10: JM. Kristensen, C. Singleton, **F. Petriglieri,** L. Clegg, PH. Nielsen. Characterization of the diversity, distribution, and functional potential of undescribed Acidobacteriota in Danish water resource recovery facilities. ***BioRxiv.***

CHAPTER 1. INTRODUCTION

In the last centuries, humans have greatly affected the natural environments on our planet and now we are facing a multitude of challenges, directly or indirectly connected with the anthropogenic activity, such as pollution, climate change, environment decay and resource depletion. However, over the last few years, our awareness of such problematics and the need to act on them has increased dramatically.

Wastewater represents one of the most common and universally diffused pollutant of the aquatic resources. Every year, tons of domestic sewage and industrial wastewater are produced worldwide and may contain organic and inorganic nutrients and contaminants, toxic compounds, bacteria and viruses (potentially pathogens). To prevent pollution and diffusion of waterbone diseases and to safeguard water supplies, wastewater undergoes a specific treatment in dedicated facilities, before it can be discharged into surface waters (Seviour et al., 2003). In recent years, wastewater treatment has also attracted an increased interest for its role in the circular economy, thanks to its high reuse possibilities, e.g. for agricultural purposes or landscaping, and for recovery of limited nutrients, such as phosphorus (P) and nitrogen (N) (Egle et al., 2015; Nielsen, 2017). Considering its high potential, it is not surprising the increasing tendency of the new generation facilities to evolve and improve wastewater treatment´s efficiency to reach high level performances (Seviour and Nielsen, 2010; Zhao et al., 2017).

1.1. WASTEWATER TREATMENT AND ACTIVATED SLUDGE

Typically, wastewater treatment is a multistep operation, which consists of a series of physical, chemical and biological steps to remove solids, organic matter and nutrients from wastewater. In the first step, called primary treatment, the larger solid particles are mechanically separated from wastewater, while the effluent undergoes further treatment. During the second phase, called secondary or biological treatment, the remaining organic materials are degraded by microorganisms, resulting in the removal of nutrients and potential pollutants. Some wastewater treatment plants may apply an extra step of treatment, called tertiary treatment, to further reduce the level of inorganic nutrients, bacteria, and toxic compounds from the final effluent (Gray, 2004; Madigan et al., 2017; Seviour and Nielsen, 2010).

The biological treatment, which usually applies the activated sludge process, acquired increasing relevance and a key role in the wastewater management during the years, as it helps to prevent severe damage to the aquatic environment (e.g. eutrophication) and the nutrients that can be actively removed represent a valuable economical

resource (Seviour and Nielsen, 2010; Wiesmann et al., 2007). Conventionally, an activated sludge system (Figure 1) consists of an aeration tank, where microorganisms (mainly bacteria and protozoa) are enclosed in a polymeric matrix to form peculiar structures called flocs, in close proximity to the organic compounds present in wastewater, allowing the aerobic oxidation of the nutrients (Bitton and Bitton, 2011, 2002; Seviour and Nielsen, 2010). From the aeration tank, the sludge is then moved into a clarifier, where the flocs can settle, allowing separation from the effluent, and can then be re-used to treat the incoming wastewater (Bitton and Bitton, 2011, 2002; Seviour and Nielsen, 2010; Wilén et al., 2008).

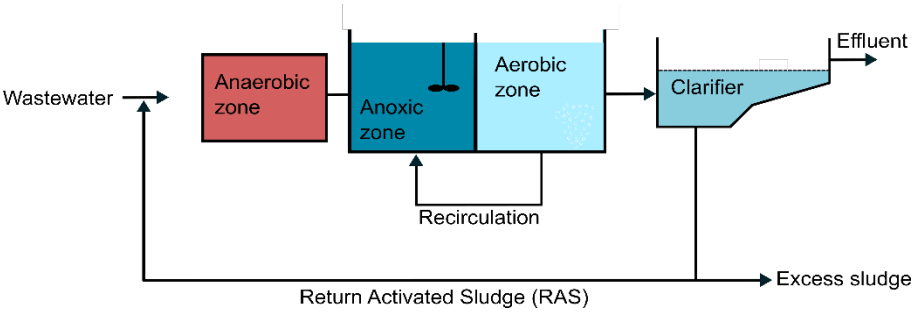

Figure 1. Schematic illustration of the activated sludge process with nutrient removal. Inspired by Henze et al. (2002).

Over the years, as discharge license requirements became more stringent, several modifications to this general process configuration have been designed to select for microorganisms able to reduce effluent N and P concentrations to environmentally acceptable levels. An example that has recently attracted more attention is the enhanced biological phosphorus removal (EBPR) process, a sustainable method to remove P without addition of chemicals and also for recovery of high concentration of soluble P without addition of chemicals, e.g. as struvite that can be directly used as fertilizer. The EBPR-process (Figure 1) is designed to enrich for specific microorganisms, called polyphosphate accumulating organisms (PAOs), which are able to store P as intracellular polyphosphate, allowing its removal with the surplus sludge (Nielsen et al., 2019; Seviour and Nielsen, 2010).

1.1.1. ACTIVATED SLUDGE FLOC

Microorganisms are the workhorses of wastewater treatment and all activated sludge technologies rely on a good flocculation and a microbial community stable over time. The flocs (Figure 2) are aggregates of microorganisms and extracellular polymeric substances (EPS), composed mainly by protein, humics, polysaccharides, lipids and nucleic acids. The matrix also contains extracellular enzymes, which hydrolyze the

organic matter before the cells can assimilate it. All these components are held together by intermolecular interactions, such as Van der Waals and electrostatic or hydrophobic interactions (Bitton and Bitton, 2002; Seviour and Nielsen, 2010; Wilén et al., 2008). Most bacteria are organized in microcolonies embedded into the floc structure, but single cells or filamentous bacteria are also frequently present (Seviour and Nielsen, 2010; Wilén et al., 2008). The latter, in particular, have an essential role in the floc formation, as they form a rigid backbone to which zoogloeal (floc-forming) microorganisms can attach (Bitton and Bitton, 2011; Madoni et al., 2000; Sezgin et al., 1978).

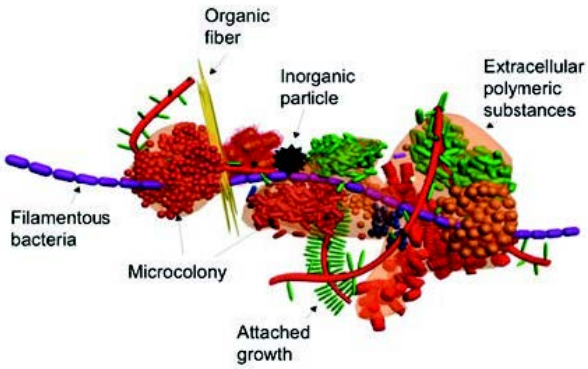

Figure 2. Activated sludge floc. Filamentous bacteria form the backbone to which zoogleal microorganisms, usually organized in microcolonies, can attach. EPS constitute the embedding matrix. Image adapted from Nielsen et al. (2012).

However, their excessive growth can be very problematic for the plant performance, as it can provoke poor settling and bulking sludge, causing its unwanted discharge together with the effluent into the environment (Deepnarain et al., 2015; Kragelund et al., 2008; Sezgin et al., 1978). Other operational problems, such as foaming in the aeration tanks are also often caused by filamentous bacteria characterized by a hydrophobic cell surface, such as *Candidatus* Microthrix parvicella (Blackall et al., 1996). Its presence in high abundance may cause the formation of a stable scum that can be released with the final effluent (Gray, 2004; Madoni et al., 2000).

1.2. MICROBIAL DARK MATTER IN WASTEWATER TREATMENT SYSTEMS

Despite their evident significance, a large amount of the microorganisms thriving in these engineered systems are still uncharacterized, sometimes belonging to poorly described phyla or candidate phyla without cultivated representatives, representing the so-called “microbial dark matter" (Lok, 2015; Marcy et al., 2007; Nobu et al.,

2015; Rinke et al., 2013; Solden et al., 2016). Activated sludge is a complex ecosystem that contains a wide microbial diversity, comprising a large amount of different ecological niches, and even with the wonderful progresses in microbiology and biotechnology of the past years, these microbial communities are still in large part a black-box, when it comes to the phylogeny and ecophysiology of their important members (Dueholm et al., 2019; Nielsen et al., 2019; M. Nierychlo et al., 2020a). The lack of this important information is partly to be attributed to the difficulties encountered for the cultivation of these microorganisms. Only a small proportion of bacteria can grow in the laboratory and cultivation-dependent methods proved to be heavily biased, as the microorganisms successfully isolated often reflected more the methods utilized than the microbial population actually present, resulting in an insufficient characterization of the community biodiversity (Seviour and Nielsen, 2010; Wagner and Loy, 2002). The uncultured majority may inevitably exert important roles in these systems, contributing to nutrient cycling and influencing other components of the surrounding microflora. Therefore, during the past decades, a set of different molecular techniques, comprising high-throughput sequencing or advanced microscopy methods, have been developed to study bacterial diversity in wastewater treatment systems in a cultivation-independent manner (Dueholm et al., 2019; Hao et al., 2018; Karst et al., 2019, 2018; Kono and Arakawa, 2019; Lawson et al., 2019). Understanding the roles and interactions of the uncultivated microorganisms in these engineered systems is of primary interest to unravel the microbial black-box of activated sludge and wastewater treatment plants (WWTPs) and use this information to improve wastewater treatment´s efficiency, control strategies and reach high-level performances.

1.3. AIM OF THE STUDY

The overall aim of this project was to obtain a comprehensive understanding of the key-microorganisms in activated sludge, in order to provide the basis for an improved design and performance of nutrient removal from wastewater.

The specific objectives of the project were:

- To investigate the diversity, abundance and ecophysiology of key-microorganisms involved in P removal in Danish WWTPs.
- To establish a comprehensive P mass-balance of activated sludge from full-scale wastewater treatment plants, combining several independent techniques and determine the individual contribution of conventional and unconventional PAOs.
- To obtain a comprehensive overview on the diversity and abundance of filamentous bacteria, with focus on the phylum Chloroflexi, in Danish and global WWTPs and their role in floc characteristics and nutrient removal.

1.4. NOVEL METHODS FOR THE CHARACTERIZATION OF MICROBIAL COMMUNITIES

The development of new molecular techniques for the analysis and characterization of microbial communities has dramatically increase in the last decades and with the advent of Next-Generation sequencing (NGS) technologies, it is now possible to comprehensively evaluate the complexity of these communities in a faster and cost-effective manner (Sanz and Ko, 2019). In parallel, microscopy and bioimaging analysis have advanced drastically over the past decade, with the development of high-resolution techniques that allow to visualize cell morphology and even intracellular molecular events (Berry et al., 2015; Huang et al., 2007, 2004; Majed et al., 2009). These new approaches, especially when applied in combination, provide a better insight into the identity and physiology of key-microorganisms, shedding light also on their complex interactions with the environmental system.

1.4.1. HIGH-THROUGHPUT SEQUENCING FOR RETRIEVAL OF FULL-LENGTH 16S rRNA SEQUENCES

NGS enables the generation of massive high throughput and high-quality sequencing results. Some of these modern culture-independent methods rely on the use of 16S ribosomal RNA (rRNA) gene as a phylogenetic marker gene for the identification of bacteria. This gene encodes for an RNA-molecule that is a functional part of the small subunit of bacterial ribosomes, thus involved in protein synthesis, universally present in all prokaryotic cells. Moreover, the gene contains both conserved and variable regions (Figure 3), which enables the identification of specific bacteria and discrimination between different organisms.

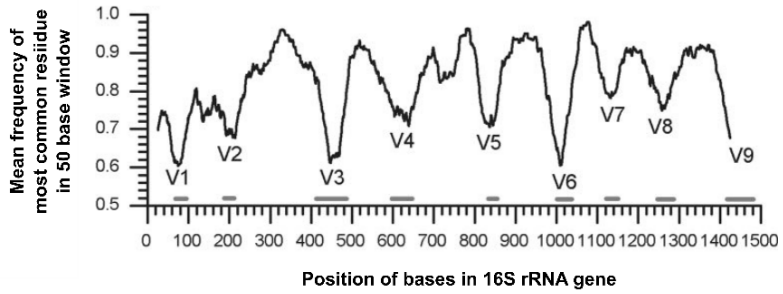

Figure 3. Distribution of conserved and variable regions in the 16S rRNA gene (illustration adapted from Ashelford et al. (2005). Variable regions are indicated from V1 to V9.

Nowadays, 16S rRNA amplicon sequencing is one of the preferred tools for profiling of microbial communities in environmental ecosystems, as it offers the chance to analyze simultaneously multiple samples, producing millions of sequences from each in a time- and cost-effective way (Caporaso et al., 2012). However, 16S rRNA amplicon sequencing still suffer from critical disadvantages, e.g. the effect of different DNA extraction methods or PCR primers applied in the study (Albertsen et al., 2015). Another critical shortcoming is the taxonomic classification, which identify the sequences and provide important biological information. However, the most commonly used reference databases, SILVA (Quast et al., 2013), Greengenes (Desantis et al., 2006) or RDP (Cole et al., 2014), often lack taxonomic information, as many of the sequences can only be classified at order of family level (McIlroy et al., 2017a; Werner et al., 2012). This issue has been partially addressed by the creation of the MiDAS database (McIlroy et al., 2015), a manually curated version of the SILVA database, which specifically aims to provide taxonomic and functional information about microorganisms significant in the wastewater treatment field. A major breakthrough was reached recently, when Karst et al., (2018) developed a method to retrieve millions of high-quality full-length 16S rRNA gene sequences from environmental samples, allowing an improved coverage of microorganisms that were previously underrepresented in the databases. The utilization of these full-length 16S rRNA gene sequences to build a comprehensive 16S rRNA gene sequences reference database, ecosystem-specific for Danish activated sludge, improved taxonomic assignments and enabled the design of specie-specific fluorescence *in situ* hybridization (FISH) probes, which will increase the resolution of single-cell physiology studies (Dueholm et al., 2019; M. Nierychlo et al., 2020a). Moreover, new innovations in the bioinformatics workflows allowed the use of amplicon sequencing variants (ASVs), which can differentiate sequence variants down to the level of single-nucleotide variances, yielding a better resolution of taxa compared to the commonly used operational taxonomic unit (OTU) (Callahan et al., 2017; M. Nierychlo et al., 2020a).

1.4.2. RETRIEVAL AND ANNOTATION OF HIGH-QUALITY METAGENOME-ASSEMBLED GENOMES

Despite its widespread use, sequencing of the 16S rRNA gene is not providing information about the metabolic potential and functions of the microorganisms belonging to a specific community. For this purpose, it is necessary to apply metagenomics, conventionally defined as the sequencing of the community of organisms living in the same environment. Since its advent in 1998 (Handelsman et al., 1998), the continuous innovation in sequencing technologies and its lower costs allowed the diffusion of a multitude of metagenomics studies from different environments, such as aquifer systems (Anantharaman et al., 2016), sediments (Hug et al., 2013), permafrost (Woodcroft et al., 2018) and also activated sludge (Singleton

et al., 2020). With this approach, near-complete genomes of dominant species can be retrieved directly from environmental samples, providing a "gene inventory" which allows to define the metabolic potential of selected organisms, including the presence/absence of important pathways (Figure 4).

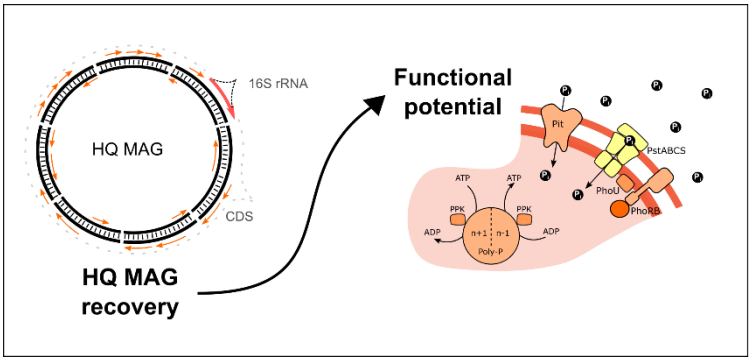

Figure 4. Schematic representation of the retriveal of biological information from genomic DNA sequences. Image adapted from **Paper 3.**

Recently, the development of new high-throughput sequencing platforms able to retrieve long-read sequences from complex communities, such as Oxford Nanopore Technologies (ONT) (Brown and Clarke, 2016), represented a major progress in sequencing technologies. Among the various advantages of the nanopore device, the most interesting is probably the extraordinary capability to sequence extremely long stretches of DNA, allowing also an improvement in recovery of genomes from metagenomes. However, the major drawback of nanopore sequencing is the relatively high error rate (5-20%) compared to more traditional short- read sequencing, such as Illumina sequencing. However, this throwback may be overcome with an initial quality assessment step and/or with the combined use of long-read and high-accuracy short reads for error correction (Kono and Arakawa, 2019; Singleton et al., 2020).

Thousands of metagenome-assembled genomes (MAGs) are currently present in the Genomes OnLine Database (GOLD) (Mukherjee et al., 2019), but there are increasing concerns regarding their quality. According to the Minimum Information about a Metagenome-Assembled Genome (MIMAG) standard, a high quality (HQ) genome draft should be >90% complete, with less than 5% contamination and it should include the 23S, 16S, and 5S rRNA genes, and at least 18 tRNAs (Bowers et al., 2017). The retrieval of the 16S rRNA gene sequences in metagenomics studies is particularly important, as it allows to connect the functional potential obtained by annotation of the genomes to the identity of key-microorganisms. The introduction of these standards in future metagenomics studies will hopefully ensure the publication of more and more high-quality MAGs from different environments. An example is the

study described in **Paper 3**, where we recovered for the first time 1083 high-quality MAGs from 23 Danish full-scale wastewater treatment plants, all meeting the MIMAG requirements and accounting for approx. 30% of the microbial community in these systems. These MAGs represent a fundamental resource for future studies aimed to the collection of physiological and functional information on the microorganisms with a key-role in activated sludge.

However, the sequencing itself is not enough to retrieve biological information from the genomes and a crucial step in this process is the interpretation of DNA sequences for the identification and annotation of genes and metabolic pathways. Typically, the annotation is performed using automatic bioinformatics pipelines, e.g. the MicroScope platform (Vallenet et al., 2009) or the KEGG project (Kanehisa et al., 2016), to predict the location of genes and to describe the cellular function of gene products by comparing them with annotation already present in commonly used databases, sometimes followed by manual curation (Digue and Moszerc, 2007). Therefore, the accuracy of these steps relies not only on the bioinformatics pipeline used for the automatic annotation, but also on the quality of the annotation databases and on the quality of the sequence itself. In addition, functional annotation without experimental verification remains still a complex task for novel microorganisms that may have unknown metabolic pathways (Digue and Moszerc, 2007). Even though metabolic reconstruction represents only an initial step toward understanding a microorganism´s metabolism, it is providing valuable information for an accurate design of successive experimental steps. The annotation of several high-quality MAGs allowed the identification of novel PAOs in **Paper 2** and in **Paper 3**, shedding light on their physiology, metabolic potential and potential role in activated sludge.

1.4.3. *IN SITU* STUDIES FOR RETRIEVAL OF IDENTITY, MORPHOLOGY AND ECOPHYSIOLOGY INFORMATION

Despite their great potential, sequencing techniques alone are not enough to investigate the actual community composition and the physiology and interactions of microorganisms in complex systems, but they must be supported by other molecular methods that allows to verify the identity and physiology of key-microorganisms *in situ.* One of the most widely applied *in situ* method is fluorescence *in situ* hybridization (FISH), which also rely on the 16S rRNA gene. In this method (Figure 5), short oligonucleotide probes, typically 5' end-labeled with a fluorescent dye, such as fluorescein or sulfoindocyanine, are designed to specifically target the 16S rRNAs of a narrow phylogenetic group (down to the species level) or any broader taxonomic group (Hugenholtz et al., 2002; Wagner et al., 2003).

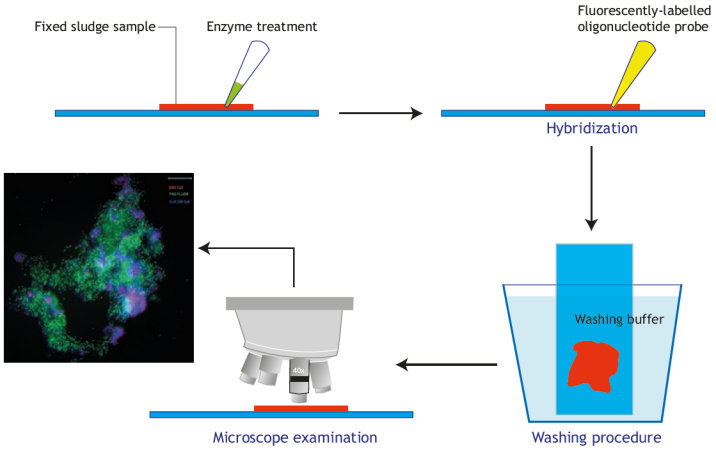

Figure 5. Schematic representation of the FISH method. From Van Loosdrecht et al. (2016).

FISH has been used for several years for *in situ* identification and characterization of key microbial taxa in various complex ecosystems, including activated sludge (Crocetti et al., 2000; Kong et al., 2005; Kragelund et al., 2007; Nielsen et al., 2002; Nierychlo et al., 2019). One of the direct advantages is that by using FISH it is possible to visualize the morphology and spatial arrangement and to quantify target microorganisms (Daims et al., 2005). However, FISH probe design is a complex task due to the enormous diversity among bacterial sequences and finding a target site shared by all of the microorganisms within the target group but absent in others may be problematic (Noguera et al., 2014). Maintaining a high specificity to the targeted organisms and, at the same time, high coverage of the targeted group is one of the main challenges in the design of oligonucleotides probes (Noguera et al., 2014; Yilmaz et al., 2011). This complication can be overcome with the utilization of ecosystem-specific databases with a comprehensive set of full-length high-quality 16s rRNA genes (Dueholm et al., 2019; Nierychlo et al., 2020a), which provides a more accurate assessment of probe specificity and coverage in the ecosystem of interest, allowing also the confident design and application of FISH probes with higher taxonomic resolution (e.g. approx. species-specific). The ecosystem-specific MiDAS databases for Danish (Nierychlo et al., 2020) and global (M.S. Dueholm et al., 2020) activated sludge have been used for evaluation of existing specie-specific FISH probes (**Paper 1**) and design of novel ones (**Paper 3** and **5**) to target microorganisms belonging to important functional groups in activated sludge.

In the last decades, several methods have been developed from the original FISH to improve its sensitivity or provide information about the physiology or activity. Common examples used in microbial ecology are: double labeling of oligonucleotide

probes (DOPE)-FISH, which uses double-labeling of the FISH probe to improve the signal intensity (Behnam et al., 2012); catalyzed reporter deposition (CARD-FISH), used for the identification and visualization of low-active microorganisms (Amann and Fuchs, 2008); microautoradiography (MAR)-FISH, which investigate substrate uptake and activity (Wagner et al., 2006); or more recently Raman-FISH microspectroscopy, which can be utilized for detection of intracellular compounds or analysis of substrate uptake in probe-defined organisms (Fernando et al., 2019; Huang et al., 2007).

1.4.3.1 Raman microspectroscopy

Raman spectroscopy is a vibrational spectroscopic technique, frequently used in chemistry and material science, for identification and characterization of different compounds. It relies on the so-called Raman effect, which is the inelastic scattering of excitation light (e.g. laser light) from the analyzed sample. Raman spectroscopy can be coupled with microscopy and the use of confocal pinholes allows high-resolution analysis of microscopic material (Baena and Lendl, 2004). In recent years, the use of Raman microspectroscopy has been exploited also for biological purposes, such as tumor diagnostics (Winterhalder and Zumbusch, 2015), rapid and accurate identification of microorganisms and pathogens (Buijtels et al., 2008; Huang et al., 2007, 2004; Kusi et al., 2014; Maquelin et al., 2002; Yang and Irudayaraj, 2003) and chemical characterization of important intracellular compounds (Majed et al., 2009; Winterhalder and Zumbusch, 2015). The main advantages of this technique lie in the minimal preparation required, allowing also analysis of biological samples *in vivo*, and its high sensitivity to specific intracellular molecules, which allow the acquisition of spectroscopic fingerprints that represents the molecular composition of a specific cell (Huang et al., 2007, 2004; Wagner, 2009). Raman microspectroscopy has also proved to be a useful tool for microbial ecologists and it was used for the identification of intracellular storage polymers (Figure 6) in randomly selected cells from EBPR systems (Majed et al., 2009). More recently, this tool has been combined with FISH to determine the level and dynamics of storage polymers present in specific microbial populations important for P removal in activated sludge (Fernando et al., 2019). In **Paper 1** and **3**, we used FISH Raman as a tool for identification of novel PAOs. The individual contribution of all known PAOs to P removal was then measured using the same approach in **Paper 2**, to establish a comprehensive P mass balance in full-scale wastewater treatment plants.

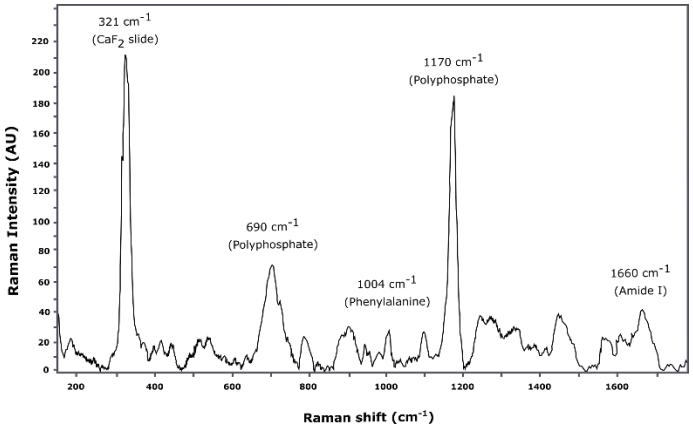

Figure 6. Example of a fingerprint spectrum from a PAO organism abundant in Danish activated sludge. Image adapted from **Paper 3.**

Another important advantage of Raman microspectroscopy, which still needs to be fully exploited, is its high sensitivity to biochemical changes, that can be readily observed as peak shifts after the incorporation of heavy isotopes, such as D_2O, ^{15}N, ^{13}C, or labelled ^{18}O (Atkins et al., 2017; Berry et al., 2015; Huang et al., 2007; Wagner, 2009). This effect has been used to analyze the uptake of labelled substrates into free-living bacteria and can represent a valuable tool for future studies. In addition, some advanced Raman spectrometers present a tweezers apparatus that can be used to separate single cells of interest of the remaining population (Huang et al., 2009). This feature has recently been upgraded, with the development of an automated microfluidic platform for Raman-activated microbial cell sorting (RACS), offering a potentially powerful tool for downstream analysis, such as single-cell genomics or cultivation (Jing et al., 2018; Nitta, 2020; Song et al., 2017).

1.4.3.2 Other methods

Several other methods are worth to be mentioned as useful tools for the cultivation-independent study of microbial ecophysiology, e.g. nanometer scale secondary ion mass spectroscopy (NanoSIMS) and biorthogonal non-canonical amino acid tagging method (BONCAT). NanoSIMS is a commonly applied technique in microbial ecology to study metabolic processes, as it provides a high-resolution chemical composition, but as for MAR-FISH, destroys the cells and is not suitable for downstream processes (Kopf et al., 2015; Wagner, 2009). BONCAT is one of the most recently developed methods for analysis of cells´ activity and is based on the incorporation of artificial amino acids fluorescently tagged in newly synthesized

proteins. When combined with fluorescence-activated cell sorting (FACS), it is then possible to sort bacteria of interest (Hatzenpichler et al., 2016, 2014; Singer et al., 2017). Also stable isotope probing (SIP) has proved to be a robust technique to identify metabolically active microorganisms that can incorporate a labeled substrate in their nucleic acids (Huang et al., 2009) and has previously applied for the study of key microorganisms in activated sludge (McIlroy et al., 2016b).

1.5. CHARACTERIZATION OF KEY-ROLE MICROORGANISMS IN ACTIVATED SLUDGE

Wastewater treatment plants (WWTPs) are designed to fully employ the metabolic abilities of microorganisms to remove nutrients potentially harmful for the environment, such as carbon, phosphorus and nitrogen. The activated sludge microbiome is, therefore, comprising multiple biological processes performed by various groups of bacteria and linked trophic interactions between the microorganisms, most of which (up to 60–90%) are still undescribed (Johnston et al., 2019). Some of the central processes carried out by the diverse functional groups belonging to the activated sludge microbiome are nitrification, nitrogen fixation, ammonification, denitrification and P cycling (Seviour and Nielsen, 2010). Moreover, bacteria are also often the cause of many technical problems in WWTPs, e.g. with the overgrowth of filamentous bacteria that can result in bulking and foaming. Understanding the physiology, functions and interaction´s mechanisms of the key-role microorganisms in these engineered systems is, therefore, of crucial importance for future application in process optimization and troubleshooting. The MiDAS (Microbial Database for Activated Sludge) project was established in 2015 (McIlroy et al., 2017b, 2015) to provide a reliable taxonomic classification for microorganisms abundant in wastewater treatment systems and it has now become a reference database based on millions of full-length 16S rRNA gene sequences specific for activated sludge (Nierychlo et al., 2020). An updated version of the MiDAS field guide (https://www.midasfieldguide.org/guide) is also now available as an open access knowledge repository for easy consulting. Analysis of the most abundant genera in the global MiDAS (Dueholm et al., 2020) dataset (**Table 1**) is highlighting the need for more studies to link identity and function, as a large fraction of the microorganisms present in activated sludge worldwide is unknown. Among the 50 most abundant genera (**Table 1**), some are involved in P removal, denitrification or are characterized by a filamentous morphology and are, therefore, essential elements of the functional groups in these systems.

Table 1. List of 50 most abundant speicies in global activated sludge. Data retrieved from global MiDAS (Dueholm et al., 2020).

Genus	FISH-probe (Ref.)	Role in activated sludge
Flavobacterium	Not available	Unknown
Tetrasphaera	(Dueholm et al., 2019; Kong et al., 2005)	PAO
Dechloromonas	(Kong et al., 2007; McIlroy et al., 2016b, **Paper 1**)	Denitrification and PAO
Zoogloea	(Oshiki et al., 2008; Rosselló-Mora et al., 1995)	Denitrification
Rhodoferax	(McIlroy et al., 2016b)	Denitrification
Thauera	(Lajoie et al., 2000)	Denitrification
Acidovorax	(Schulze et al., 1999)	Denitrification
Nitrospira	(Daims et al., 2001)	Nitrification
Ca. Competibacter	(McIlroy et al., 2014b)	GAO
Haliangium	(McIlroy et al., 2016b)	Denitrification
Rhodobacter	Not available	Unknown
Ferruginibacter	Not available	Unknown
Hydrogenophaga	Not available	Unknown
***Ca.* Accumulibater**	(Crocetti et al., 2000)	PAO
Terrimonas	Not available	Unknown
Novosphingobium	Not available	Unknown
OLB8	Not available	Unknown
Sulfuritalea	(McIlroy et al., 2016b)	Denitrification
Midas_g_81	Not available	Unknown
***Ca*. Epiflobacter**	(Xia et al., 2008)	Epiphytic bacteria
Sphaerotilus	(M. Wagner et al., 1994)	Filamentous
Nitrosomonas	(Lukumbuzya et al., 2020; Mobarry et al., 1996)	Nitrification
***Ca.* Microthix**	(Erhart et al., 1997)	Filament
Leptothrix	Not available	Filament
Arcobacter	(Snaidr et al., 1997)	Potential pathogen
***Ca.* Villigracilis**	**Paper 4**	Filament
Hyphomicrobium	Not available	Unknown
Midas_g_558	Not available	Unknown
Dokdonella	Not available	Unknown

Thermomonas	(Dolinšek et al., 2013)	Unknown
Thiothrix	(Kanagawa et al., 2000)	Filament
OLB12	Not available	Unknown
Trichoccoccus	(Liu and Seviour, 2001)	Filamentous
F_Comamonadaceae_ASV8	Not available	Unknown
Ottowia	Not available	Unknown
Defluviicoccus	(Burow et al., 2007; McIlroy and Seviour, 2009; McIlroy et al., 2010; Nittami et al., 2009)	GAO/Filaments
Comamonas	Not available	Unknown
F_Rhodocyclaceae_ASV5	Not available	Unknown
IMCC26207	Not available	Unknown
Pseudarcobacter	Not available	Unknown
Aquabacterium	Not available	Unknown
Midas_g_399	Not available	Unknown
Iamia	Not available	Unknown
Defluviimonas	Not available	Unknown
Methylotenera	Not available	Unknown
Midas_g_33	Not available	Unknown
Propionivibrio	(Albertsen et al., 2016)	GAO
JGI_0001001-H03	Not available	Unknown
Rhodoplanes	Not available	Unknown
Midas_g_70	Not available	Unknown

1.5.1. NOVEL POLYPHOSPHATE ACCUMULATING ORGANISMS (PAO) AND THEIR METABOLIC POTENTIAL

The removal of P from wastewater is one of the key process during the treatment, as excessive P released in aquatic ecosystems may induce an overgrowth of algae and oxygen depletion, a phenomenon called eutrophication. To reduce P entering water bodies and avoid this environmental damage, P discharge standards became more rigorous over the years, increasing the pressure on the WWTPs. The removal of P from wastewater can be carried out with utilization of chemicals, biological treatment or a combination of both (Nielsen et al., 2019). The enhanced biological phosphorus removal (EBPR) process is gaining more attention worldwide, as it doesn´t require the addition of chemicals and allows the recovery P (Nielsen et al., 2019). The PAOs

are the main players in the EBPR process and all the known genera belonging to this functional group or putative PAO can be found in **Table 2**.

Table 2. List of all known and putative PAOs.

Genus (Phylum)	Genome annotation	*In situ* verification	Pure culture
***Ca.* Accumulibacter (Proteobacteria)**	(Flowers et al., 2013; Skennerton et al., 2015; Weissbrodt et al., 2019)	(Crocetti et al., 2000; Fernando et al., 2019; Kong et al., 2004; **Paper 2**)	-
***Tetrasphaera* (Actinobacteria)**	(Kristiansen et al., 2013)	(Fernando et al., 2019; Kong et al., 2005, **Paper 2**)	(Fernando et al., 2019)
***Dechloromonas* (Proteobacteria)**	**Paper 1**	(Kong et al., 2007; McIlroy et al., 2016b**, Paper 1**)	(Terashima et al., 2016)
***Microlunatus* (Actinobacteria)**	(Kawakoshi et al., 2012)	-	(Nakamura et al., 1995)
***Ca.* Methylophosphatis (Proteobacteria)**	**Paper 3**	**Paper 3**	-
***Halomonas* (Proteobacteria)**	(Skennerton et al., 2015)	(Nguyen et al., 2012)	-
***Acinetobacter* (Proteobacteria)**	-	-	(Deinema et al., 1985)
***Ca.* Obscuribacter (Cyanobacteria)**	(Soo et al., 2014)	-	-
***Tessaracoccus* (Proteobacteria)**	-	-	(Maszenan et al., 1999a)

Gemmatimonas **(Gemmatimonadetes)**	-	-	(Zhang et al., 2003)
Pseudomonas **(Proteobacteria)**	-	-	(Gunther et al., 2009; Tobin et al., 2007)
Quadricoccus **(Proteobacteria)**	-	-	(Maszenan et al., 2002)
Malikia **(Proteobacteria)**	-	-	(Spring et al., 2005)
Friedmanniella **(Actinobacteria)**	-	-	(Maszenan et al., 1999b)

The metabolism of the canonical "model" organism *Ca.* Accumulibacter (Figure 7), genus within the Betaproteobacteria, has been deeply investigated using lab-scale enrichments with *in situ* and omics-based studies (Oyserman et al., 2016; Skennerton et al., 2015) and recently verified also in full-scale EBPR plants (Fernando et al., 2019). In the anaerobic stage, this organism is hydrolyzing polyphosphate (poly-P) and glycogen to assimilate volatile fatty acids (VFAs) and other organic substrates from the wastewater and convert them in storage compounds, such as polyhydroxyalkanoates (PHAs). In the following aerobic phase, stored PHAs are used to replenish glycogen and poly-P storages, resulting in net P removal with wastage of aerobic biomass (Oehmen et al., 2007a; Weissbrodt et al., 2019; Welles et al., 2015).

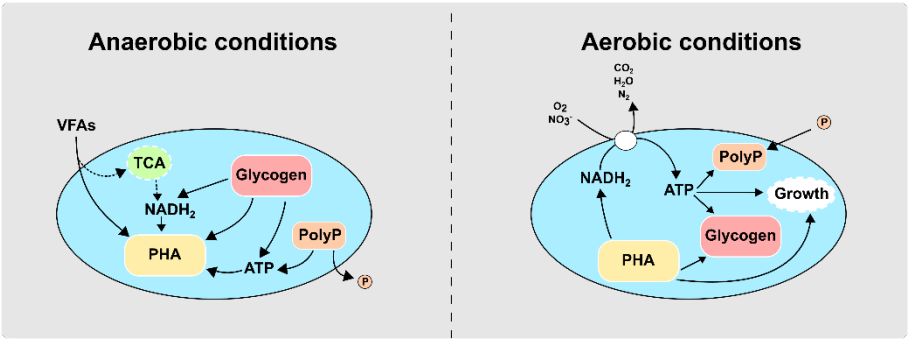

Figure 7. Schematic drawing of a canonical PAO metabolism, typical of *Ca.* Accumulibacter. Inspired from Yuan et al. (2012).

However, today is also known that other PAOs, such as *Tetrasphaera* spp., belonging to the phylum Actinobacteria, exhibit an alternative phenotype, as they cycle poly-P with dynamic feast–famine conditions without PHA or glycogen storage (Dueholm et al., 2019; Fernando et al., 2019). Some PAOs, comprising *Tetrasphaera*, can also have a fermentative metabolism and they are, therefore, called fermenting PAO (fPAO). Others can use nitrate or nitrite as electron acceptors and perform simultaneously denitrification and poly-P accumulation (Nielsen et al., 2019). Such variations in the PAOs metabolism emphasize the need for further investigation of their physiology. Moreover, our recent study that applied FISH in combination with Raman microspectroscopy (Fernando et al., 2019), showed that a large portion of P is potentially removed from wastewater by microorganisms that are still unknown. Several genera, such as *Dechloromonas* or *Tessaracoccus,* have been proposed to be putative PAOs, because they showed the presence of intracellular poly-P *in situ* or in pure culture using conventional staining methods (Kong et al., 2007; Nielsen et al., 2019). The genus *Dechloromonas,* in particular, has also been suggested to be the main responsible for P removal in lab-scale reactors (Goel et al., 2005) and has been found abundant in full-scale WWTPs in Denmark (Stokholm-Bjerregaard et al., 2017) and worldwide (Nielsen et al., 2019). Other studies suggested the possibility that some members of this genus may instead have a glycogen accumulating organisms (GAOs) phenotype. GAOs are considered as direct competitors of PAOs, as they do not accumulate poly-P, but they uptake VFAs and store them as PHA under anaerobic conditions, accumulating only glycogen under aerobic conditions (McIlroy et al., 2016b; Oehmen et al., 2006).

In **Paper 1** we supplied with the first insights into the physiology of novel *Dechloromonas* spp. (Figure 8), abundant in Danish WWTPs. FISH-Raman *in situ* analysis showed a phenotype similar to the model PAO *Ca.* Accumulibacter, with dynamics of all three known storage polymers (poly-P, PHA, and glycogen) during

feast/famine cycling in full-scale WWTPs. A similar analysis on biomass retrieved from a lab-scale reactor fed with different carbon sources (sugars and amino acids) gave a further insight into the ecophysiology of these organisms, showing their substrate preferences and potential ecological niche within the EBPR microbial community. The annotation of several high-quality MAGs retrieved from Danish activated sludge plants allowed the recovery of important information about the metabolic potential of these organisms, confirming the results from the *in situ* analysis and showing their possible involvement in nitrogen removal. The names *Ca.* Decloromonas phosphatis and *Ca.* Dechloromonas phopshovora were proposed for the two most abundant species. Although further experimental studies are needed, these findings have important implications for the study of activated sludge communities and the EBPR process, as these organisms appear to be often abundant and actively involved in nutrient removal.

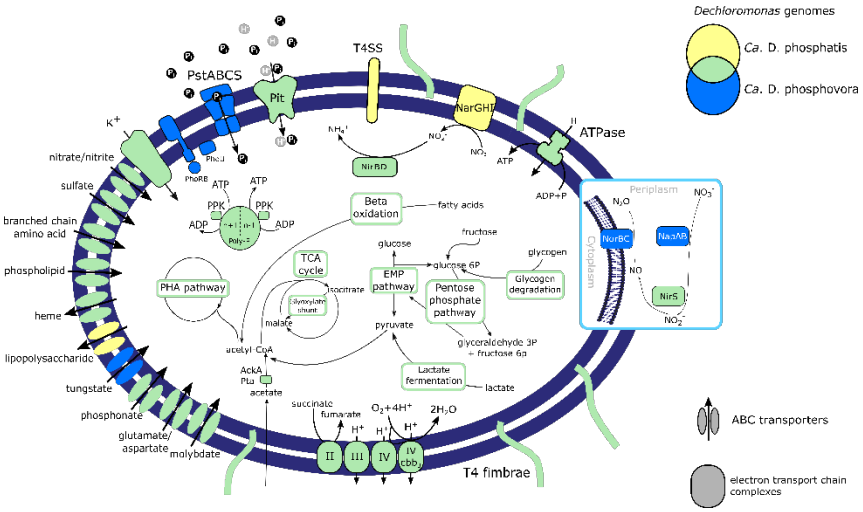

Figure 8. Metabolic model of the two novel PAO species *Ca.* Dechloromonas phosphatis and *Ca.* Dechloromonas phosphovora. More details about the physiology of these organisms can be found in **Paper 1.**

Another organism, which is not a conventional PAO but it is still able to uptake phosphate and store it intracellularly, is *Ca.* Microthrix. These bacteria are well-known for another reason, as they are filamentous, and often their overgrowth is causing foaming and bulking in WWTPs (M. Nierychlo et al., 2020b). However, they can store poly-P, but they do not cycle it under conventional feed/famine conditions (Wang et al., 2014).

The individual contribution to P removal in full-scale WWTPs of all the known PAOs, *Ca.* Accumulibacter, *Tetrasphaera* and *Dechloromonas*, and the unconventional PAO *Ca.* Microthrix was assessed in **Paper 2**. Moreover, a combination of different methods was applied to obtain for the first time a comprehensive P mass balance that would cover all P forms, both chemically and biologically-bound, normally present in activated sludge. The four largest full-scale WWTPs in Denmark were selected for the analysis. In all the plants, a substantial portion of P was present in the biomass, in the form of DNA, phospholipids or, when present, poly-P. The total amount of poly-P stored in unknown cells in activated sludge was measured by different, independent methods, showing their potential for future utilization. The specific contribution of the known PAOs and *Ca.* Microthrix to P removal was significant, but a significant fraction of poly-P (1-13%) could still not be assigned to any known PAO, indicating the potential presence of more unknown PAO and the need for further studies.

In the attempt to unveil undescribed microorganisms abundant in WWTPs and members of important functional groups, including PAOs, we retrieved 1083 high-quality MAGs from 23 Danish WWTPs, as described in **Paper 3**. Beside the MAGs for the well-known PAOs, *Ca.* Accumulibacter, *Tetrasphaera* and *Dechloromonas*, other genera showed potential for poly-P accumulation and a novel one, *Ca*. Methylophosphatis (previously midas_g_190), was selected for *in situ* verification of its metabolic potential. Newly designed FISH probes applied in combination with Raman microspectroscopy revealed the presence of poly-P at levels similar to the other known PAOs, but no other storage compounds, suggesting its potential role in P removal from wastewater. Even though the majority of the abundant PAOs is now known, new potential PAOs are still unknown and the study conducted in **Paper 3** can represent an invaluable resource to discover them through analysis of the MAG potential, followed by experimental verification.

1.5.2. FILAMENTOUS BACTERIA IN ACTIVATED SLUDGE AND THEIR ROLE IN ACTIVATED SLUDGE

As mentioned previously, filamentous bacteria are an essential part of the well-settling activated sludge floc, as they are providing the structural backbone to which other bacteria can attach (Seviour and Nielsen, 2010). However, overgrowth of some species is often associated to poor settling or foaming, if they possess hydrophobic cell surfaces, which may lead to a reduced treatment efficiency and release of untreated wastewater (Madoni et al., 2000; Martins et al., 2004; Sezgin et al., 1978; Vervaeren et al., 2005). Identification and characterization of filamentous bacteria abundant in these systems and may potentially cause these problematics is therefore crucial to determine proper control measures. Conventionally, microscopic observation and classification into different morphotypes has been the rule of thumb for the characterization of filamentous microorganisms in activated sludge

(Eikelboom, 1975; Eikelboom and Geurkink, 2001). However, with the advent of culture-independent molecular methods it is now possible to have a phylogenetically reliable identification and description of the diversity of filamentous bacteria present in the activated sludge (McIlroy et al., 2016a; Mielczarek et al., 2012; Nierychlo et al., 2019; Saunders et al., 2016; Speirs et al., 2017). A detailed list of the known genera with filamentous representative can be found in **Table 3**. Among the most abundant filamentous genera, the ones that are often linked to operational problems in WWTPs are the actinobacterial genera *Ca.* Microthrix (M. Nierychlo et al., 2020b; Seviour and Nielsen, 2010), Gordonia, and Skermania (Seviour and Nielsen, 2010), *Ca.* Nostocoida (Blackall et al., 2000), and several genera belonging to the phylum Chloroflexi (Björnsson et al., 2002; Kragelund et al., 2007; McIlroy et al., 2016a; Nierychlo et al., 2019; Seviour and Nielsen, 2010; Speirs et al., 2009, 2011).

Table 3. List of all known genera with filamentous representatives.

Genus	Pure culture	*In situ* studies	Genome available
***Anaerolinea* (Chloroflexi)**	(Sekiguchi et al., 2003; Yamada et al., 2006)	-	(Matsuura et al., 2015a)
***Leptolinea* (Chloroflexi)**	(Yamada et al., 2006)	(Petriglieri et al., 2018)	(Matsuura et al., 2015a)
***Bellilinea* (Chloroflexi)**	(Yamada et al., 2007)	-	(Matsuura et al., 2015a)
***Flexilinea* (Chloroflexi)**	(Sun et al., 2016)	-	(Matsuura et al., 2015b)
***Litorilinea* (Chloroflexi)**	(Kale et al., 2013)	-	-
***Levilinea* (Chloroflexi)**	(Yamada et al., 2006)	-	(Matsuura et al., 2015a)
***Ornatilinea* (Chloroflexi)**	(Podosokorskaya et al., 2013)	-	-
***Caldilinea* (Chloroflexi)**	(Grégoire et al., 2011)	-	-

***Ardenticatena* (Chloroflexi)**	(Kawaichi et al., 2013)	-	-
***Ca.* Amarolinea (Chloroflexi)**	**-**	**Paper 4**	(Andersen et al., 2019)
***Ca.* Promineofilum (Chloroflexi)**	-	(McIlroy et al., 2016a)	(McIlroy et al., 2016a)
***Ca.* Sarcinithrix (Chloroflexi)**	-	**Paper 4**	-
***Ca.* Villigracilis (Chloroflexi)**	-	**Paper 4**	-
***Kouleothrix* (Chloroflexi)**	(Kohno et al., 2002)	(Beer et al., 2002)	(Ward et al., 2018)
***Beggiatoa* (Proteobacteria)**	(Dubinina et al., 2017)	(Williams and Unz, 1985)	-
***Ca.* Nostocoida (Planctomycetes)**	-	(Liu and Seviour, 2001)	-
***Ca.* Microthrix (Actinobacteria)**	-	(Erhart et al., 1997)	(McIlroy et al., 2013)
***Ca.* Alysiosphaera (Proteobacteria)**	-	(Snaidr et al., 2001)	-
***Fodinicola* (Actinobacteria)**	(Carlsohon et al., 2008)	-	-
***Gordonia* (Actinobacteria)**	(de los Reyes et al., 1997, 1998)	(Lechevalier and Lechevalier, 1974)	-
***Haliscomenobacter* (Bacteroidetes)**	(van Veen et al., 1973)	(Schauer and Hahn, 2005)	(Daligault et al., 2011)
***Leucothrix* (Proteobacteria)**	(Brock, 1967)	-	-
***Neomegalonema* (Proteobacteria)**	(Thomsen et al., 2006)	(Kragelund et al., 2005)	(Mcilroy et al., 2015)

***Skermania* (Actinobacteria)**	(Chun et al., 1997)	-	-
***Sphaerotilus* (Proteobacteria)**	(Park et al., 2014)	(M. Wagner et al., 1994)	(Park et al., 2014)
***Thiothrix* (Proteobacteria)**	(Larkin, 1980)	(Williams and Unz, 1985)	-
***Turicibacter* (Firmicutes)**	(Bosshard et al., 2002)	-	(Auchtung et al., 2016; Klaassens et al., 2011)
***Tetrasphaera* (Actinobacteria)**	-	(Dueholm et al., 2019)	-
***Defluviicoccus* (Proteobacteria)**	-	(McIlroy et al., 2010)	-
***Acinetobacter* (Proteobacteria)**	-	(Seviour et al., 1997; Wagner et al., 1994)	(Hu et al., 2018; Qin et al., 2019)
***Dietzia* (Actinobacteria)**	(Duckworth et al., 1998; Mohammad and Gharibzahedi, 2014)	(Muller, 2006)	(Ganguly et al., 2016; Procopio et al., 2012)
***Leptothrix* (Proteobacteria)**	(Spring et al., 1996)	(Wagner et al., 1994)	*Leptothrix ochracea* L12 Taxonomy ID: 735332
***Mycobacterium* (Actinobacteria)**	(Guo et al., 2019)	(Davenport et al., 2000)	(Cole et al., 1998)
***Nocardioides* (Actinobacteria)**	(Choi et al., 2007; Dastager et al., 2008)	-	(Kimbrel et al., 2013; Nocardioides et al., 2011)
***Streptococcus* (Firmicutes)**	(Liu et al., 2000)	(Trebesius et al., 2000)	(Mcshan et al., 2002; Tettelin et al., 2001)

***Trichococcus* (Firmicutes)**	(Scheff et al., 1984)	(Liu and Seviour, 2001)	(Campanaro et al., 2020)
Midas_g_169 (Chloroflexi)	-	**Paper 5**	-
Midas_g_105 (Chloroflexi)	-	(Speirs et al., 2017)	-
Midas_g_361 (Chloroflexi)	-	**Paper 5**	-
Midas_g_550 (Chloroflexi)	-	**Paper 5**	-
Midas_g_344 (Chloroflexi)	-	(Speirs et al., 2017)	-
Midas_g_1668 (Chloroflexi)	-	(Speirs et al., 2017)	-
Midas_g_119 (Chloroflexi)	-	**Paper 5**	-
Midas_g_461 (Chloroflexi)	-	**Paper 5**	-
Midas_g_72 (Chloroflexi)	-	**Paper 5**	-
UTCFX1 (Chloroflexi)	-	**Paper 5**	-
Midas_g_9648 (Chloroflexi)	-	**Paper 5**	-

Members of the phylum Chloroflexi (Figure 8) are often abundant in Danish activated sludge or anaerobic digesters, suggesting their ecophysiological relevance in such habitats, and FISH probes have been designed for the characterization of many of them (Kragelund et al., 2007; McIlroy et al., 2016a; Nierychlo et al., 2019; Petriglieri et al., 2018; Speirs et al., 2009, 2017, 2011; Yamada and Sekiguchi, 2009). MAR-FISH *in situ* showed a preferential use of sugars as a source of energy (Kragelund et al., 2007; Nierychlo et al., 2019) and metabolic models for uncultured Chloroflexi

showed their ability to survive in both aerobic and anaerobic conditions, by respiration or fermentation of sugars, confirming the potential role of Chloroflexi filaments in the degradation of complex carbohydrates (Andersen et al., 2019; McIlroy et al., 2016a).

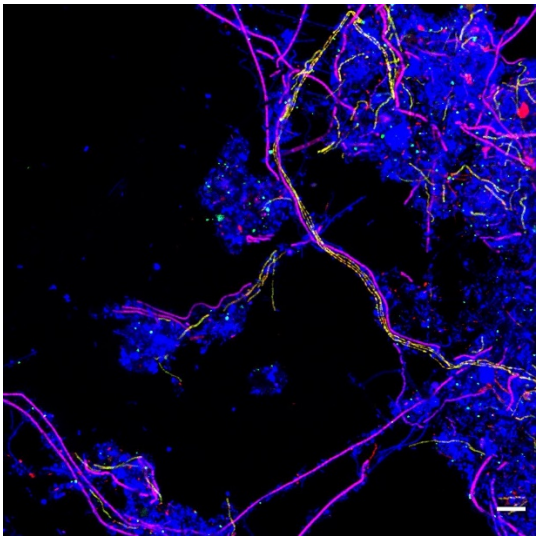

Figure 9. FISH micrograph of members of the phylum Chloroflexi (filaments in yellow and magenta) abundant in Danish activated sludge. The blue color indicates the rest of the bacterial biomass. Details about the FISH probes utilized for this image (EUBmix in blue, CFX in magenta and CFX758 in yellow) can be found in **Paper 5.**

A detailed morphological and physiological characterization of the most abundant uncultured genera belonging to the phylum Chloroflexi is reported in **Paper 4**. Novel FISH probes were designed and applied in situ for their phylogenetic identification. All the genera appeared to be filamentous and some of them were consistently abundant, reaching up to 30% of the biovolume in some WWTPs, suggesting their potential involvement in bulking problems. FISH-MAR showed that all the selected members of the phylum can ferment sugars, and most likely have an important role in the degradation of organic matter in activated sludge. Naming these novel genera and defining important features as their morphology, distribution and physiology, provides the start point for an in-depth understanding of their ecology and their relation to operational parameters and, eventually, control measures.

The Chloroflexi community of Danish and global activated sludge is further characterized in **Paper 5**, where the remaining genera abundant in Danish WWTPs were visualized *in situ* and partially characterized using FISH-Raman. The utilization of the MiDAS database for over 650 global activated sludge plants (Dueholm et al.,

2020) allowed also the comparison of Chloroflexi genera abundant in different countries, in different plant designs and in different climate zones. A higher diversity was observed in different climate areas, suggesting that this factor could influence the global Chloroflexi community. In **Paper 5**, we obtained for the first time a comprehensive characterization of this group of microorganisms important in activated sludge worldwide.

1.5.3. OTHER IMPORTANT GROUPS

Several groups of microorganisms are involved in the nitrogen cycle, to ensure the conversion of nitrogen compounds abundant in wastewater, such as ammonia (NH_3), nitrate (NO_3^-) and nitrite (NO_2^-), to dinitrogen gas and thus meet the environmental-safe requirements. For many years, nitrification has been considered as a two-step process happening in the aerobic tank, which involved two separate groups of organisms: first ammonia oxidizing bacteria (AOBs) or ammonia oxidizing archaea (AOA) that can convert ammonia to nitrite, and then nitrite oxidizing bacteria (NOBs) that convert nitrite to nitrate (Koch et al., 2019; Seviour and Nielsen, 2010). These groups of microorganisms and their metabolic traits have been extensively investigated in several studies both in situ and with the aid of "omics" methods (Juretschko et al., 2002; Lv et al., 2014; McIlroy et al., 2016b; Sorokin et al., 2012). However, recently some bacterial lineages that showed the capability to perform full ammonium oxidation were identified and information about these microorganisms, now called comammox (CMXs), is now available (Daims et al., 2015; Koch et al., 2019).

A second important process in wastewater treatment is the denitrification, used to remove nitrite and nitrate in the anoxic tank. Several microorganisms can use nitrate and/or nitrite as electron acceptors, converting them into dinitrogen gas via several intermediate steps. Not all denitrifiers are capable to perform full denitrification and the process can be incomplete, leading to the production of potentially toxic compounds as nitric oxide (NO) or nitrous oxide (N_2O) (Seviour and Nielsen, 2010). Several microbial genera have shown the potential for denitrification and are often abundant in activated sludge (McIlroy et al., 2016b). In **Paper 1**, all the *Dechloromonas* MAGs showed the potential for full or partial denitrification, but experimental evidence is needed to verify this metabolic trait. Another group of microorganisms recently identified are capable of anaerobic ammonia oxidation (anammox) and can convert ammonia directly into dinitrogen under anaerobic conditions, using nitrite as electron acceptor (Koch et al., 2019). Many PAO and also filamentous bacteria such as *Ca.* Microthrix (McIlroy et al., 2013) and some members of the phylum Chloroflexi (Andersen et al., 2019; McIlroy et al., 2016a) have been suggested to be involved in N removal. The MAGs retrieved in **Paper 3** can be a valuable starting point for further investigation of this group of microorganisms.

1.6. CONCLUSIONS

Microorganisms are the important key players in activated sludge and even though they perform all the fundamental biological processes to treat wastewater and recover resources, the microbial communities of these systems are still partly undescribed. To compensate to this lack of vital knowledge, the ecosystem-specific MiDAS database was used to design new FISH probes targeting novel microorganisms and investigate, in combination with Raman microspectroscopy, their ecophysiology (Chapter 2, 3, 4 and 5). Among the different functional groups of microorganisms in activated sludge, two were selected for in-depth characterization: the PAOs involved in P removal and filamentous bacteria belonging to the phylum Chloroflexi.

The PAO community in EBPR systems has been investigated for a long time using different approaches, and several genera with this peculiar metabolism, such as *Ca.* Accumulibacter and *Tetrasphaera*, are well-known and abundant worldwide. However, several putative PAO are still poorly described. Among these, the genus *Dechloromonas* was the most abundant and probably controversial, because the uncertainty regarding its effective role in activated sludge. In **Paper 1** we supplied the first insights into the ecophysiology of novel *Dechloromonas* spp., which showed *in situ* a phenotype similar to the model PAO *Ca.* Accumulibacter, with dynamics of all three known storage polymers (poly-P, PHA, and glycogen) during feast/famine cycling. FISH-Raman also gave a further insight into the substrate preferences of these organisms and their potential ecological niche within the EBPR microbial community. Annotation of several high-quality MAGs supported these results and indicated also their potential involvement in nitrogen removal. We proposed the names *Ca.* Decloromonas phosphatis and *Ca.* Dechloromonas phopshovora for the two most abundant species.

In **Paper 2**, the individual contribution of all the known PAO and, for the first time, of the unconventional PAO *Ca.* Microthrix, was measured using Raman microspectroscopy. The combination of several independent methods allowed to obtain for the first time a comprehensive P mass balance that would cover all P species, both chemically and biologically-bound. In all the plants, a large fraction of P was present in the biomass, as DNA, phospholipids or, when present, poly-P, but a significant fraction of the latter (15-25%) could still not be assigned to any known PAO, indicating the potential presence of more unknown PAO. The screening of more than 1000 MAGs retrieved in **Paper 3** indicated novel genera with potential for poly-P accumulation. The following design of novel FISH probes and the combination with Raman microspectroscopy allowed the identification of a novel putative PAO, named *Ca.* Methylophosphatis. The MAGs retrieved in **Paper 3** represent, therefore, an extraordinary resource of information for many future studies, potentially being a source of valuable knowledge to link identity to function and discover many more PAOs or other microorganisms of interest.

Filamentous bacteria have been studied for decades using microscopy techniques and often inspiring interest because of their potential involvement in operational problems in full-scale plants. The phylum Chloroflexi present several abundant genera in activated sludge, but it was still poorly described. **Paper 4** and **Paper 5** represent a first attempt into the characterization of the most abundant lineages belonging to this phylum. Both amplicon sequencing and FISH showed their high abundance in Danish (**Paper 4** and **Paper 5**) and global (**Paper 5**) WWTPs and a new FISH probe set was designed to target the most abundant genera. The application of the newly designed FISH probes showed very different morphologies, spanning from the classical long filaments often forming inter-floc bridges to rod-shaped cells found deep into the flocs and with unknown function. Important ecophysiology information was collected with the application of the FISH probes with MAR (**Paper 4**), showing their preference for sugars and capability to ferment, or Raman microspectroscopy (**Paper 5**), which confirmed the presence of glycogen storages, most likely used in period of substrate limitations. The utilization of the MiDAS database for global activated sludge in **Paper 5** allowed also to obtain for the first time a comparison of Chloroflexi genera abundant in different countries, plant designs and climate zones. The latter, in particular, showed a higher diversity depending on climate, indicating that this factor could have a large influence in shaping the global Chloroflexi community. **Paper 5** represent a first, comprehensive characterization of this group of microorganisms important in activated sludge.

1.7. PERSPECTIVES

The ecosystem specific MiDAS database proved to be a very useful tool for designing of FISH probes for novel microorganisms, and the combination of high-quality MAGs annotation and *in situ* studies allowed a comprehensive overview of the ecophysiology of selected organisms. This successful approach is particularly useful in the investigation of microbial dark matter in wastewater treatment systems, but it can be efficaciously extended to the study of any other environment and highlights the equal importance of both metagenome sequencing and *in situ* verification. The retrieval of thousands of MAGs, supported by experimental evidence, will with time shed light on the intricate phylogeny, functions and interactions of microbial communities in activated sludge and other environments. Many genera, also potential PAOs, abundant in both Danish and global activated sludge, still remain undescribed and can be discovered through screening and annotation of the MAGs, which represent an extraordinary resource for many future studies.

Raman microspectroscopy is a very powerful technique, especially for the study of PAO communities, for its extraordinary ability to detect intracellular storage compounds and, more in general, the total chemical environment in single-cells. If applied after stable isotopes incorporation, it can provide valuable information regarding substrate preference and activity level of any group of cells, offering important information for the deciphering of the complex interactions happening within microbial communities. With a more advanced setup, it is also possible to select or sort for specific cells, for example because of the presence of storage polymers or another interesting characteristic, without harming the biomass, which can then be used for example for cultivation or single-cell genomics. However, a major disadvantage of using Raman microspectroscopy is that it is a time-consuming technique, which requires an expert user to perform accurate analysis. Also the autofluorescence typical of biological samples is sometimes an obstacle difficult to overcome. It should be of primary importance to upgrade microscopy-based methods at a similar level of sequencing and omics techniques to allow a fastest and high-throughput analysis.

This PhD project has contributed with further deepening of the characterization of microbial communities in activated sludge, shedding some light into this “black box” and giving some indications of the significance and role in these engineering systems of important groups of microorganisms. The knowledge obtained is a valuable resource to reach a better understanding of the community ecology and connection with operational parameters and, with time, to translate this information to better wastewater treatment performances and, when needed, control measures.

LITERATURE LIST

Acevedo, B., Murgui, M., Borrás, L., Barat, R., 2017. New insights in the metabolic behaviour of PAO under negligible poly-P reserves. Chem. Eng. J. 311, 82–90. https://doi.org/10.1016/j.cej.2016.11.073

Acevedo, B., Oehmen, A., Carvalho, G., Seco, A., Borrás, L., Barat, R., 2012. Metabolic shift of polyphosphate-accumulating organisms with different levels of polyphosphate storage. Water Res. 6, 1889–1900. https://doi.org/10.1016/j.watres.2012.01.003

Achenbach, L.A., Michaelidou, U., Bruce, R.A., Fryman, J., Coates, J.D., 2001. *Dechloromonas agitata* gen. nov., sp. nov. and *Dechlorosoma suillum* gen. nov., sp. nov., two novel environmentally dominant (per)chlorate-reducing bacteria and their phylogenetic position. Int J Syst Evol Microbiol. 527–533.

Ahn, J., Schroeder, S., Beer, M., McIlroy, S., Bayly, R.C., May, J.W., Vasiliadis, G., Seviour, R.J., 2007. Ecology of the Microbial Community Removing Phosphate from Wastewater under Continuously Aerobic Conditions in a Sequencing Batch Reactor. Appl. Environ. Microbiol. 73, 2257–2270. https://doi.org/10.1128/AEM.02080-06

Albertsen, M., Hugenholtz, P., Skarshewski, A., Nielsen, K.L., Tyson, G.W., Nielsen, P.H., 2013. Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. Nat. Biotechnol. 31, 533–8. https://doi.org/10.1038/nbt.2579

Albertsen, M., Karst, S.M., Ziegler, A.S., Kirkegaard, R.H., Nielsen, P.H., 2015. Back to basics - The influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities. PLoS One 10, 1–15. https://doi.org/10.1371/journal.pone.0132783

Albertsen, M., Mcilroy, S.J., Stokholm-bjerregaard, M., Karst, S.M., Tyson, G.W., 2016. "*Candidatus* Propionivibrio aalborgensis": A Novel Glycogen Accumulating Organism Abundant in Full-Scale Enhanced Biological Phosphorus Removal Plants. Front Microbiol. 7, 1033. https://doi.org/10.3389/fmicb.2016.01033

Amann, R., Fuchs, B.M., 2008. Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. Nat. Rev. Microbiol. 6, 339–348. https://doi.org/10.1038/nrmicro1888

Anantharaman, K., Brown, C.T., Hug, L.A., Sharon, I., Castelle, C.J., Probst, A.J.,

Thomas, B.C., Singh, A., Wilkins, M.J., Karaoz, U., Brodie, E.L., Williams, K.H., Hubbard, S.S., Banfield, J.F., 2016. Thousands of microbial genomes shed light on interconnected biogeochemical processes in an aquifer system. Nat. Publ. Gr. 7, 1–11. https://doi.org/10.1038/ncomms13219

Andersen, M.H., Mcilroy, S.J., Nierychlo, M., Nielsen, P.H., Albertsen, M., 2019. Genomic insights into *Candidatus* Amarolinea aalborgensis gen. nov., sp. nov., associated with settleability problems in wastewater treatment plants. Syst. Appl. Microbiol. 42, 77–84. https://doi.org/10.1016/j.syapm.2018.08.001

Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., Weightman, A.J., 2005. At Least 1 in 20 16S rRNA Sequence Records Currently Held in Public Repositories Is Estimated To Contain Substantial Anomalies. Appl Environm Microbiol. 71, 7724–7736. https://doi.org/10.1128/AEM.71.12.7724

Atkins, C.G., Buckley, K., Blades, M.W., Turner, R.F.B., 2017. Raman Spectroscopy of Blood and Blood Components. Appl. Spectrosc. 71, 767–793. https://doi.org/10.1177/0003702816686593

Auchtung, T.A., Holder, M.E., Gesell, J.R., Ajami, N.J., Duarte, R.T.D., Itoh, K., Caspi, R.R., Petrosino, J.F., Horai, R., 2016. Complete Genome Sequence of *Turicibacter* sp . Strain H121 , Isolated from the Feces of a Contaminated Germ-Free Mouse. Genome Announc. 4, 2015–2016. https://doi.org/10.1128/genomeA.00114-16.Copyright

Baena, J.R., Lendl, B., 2004. Raman spectroscopy in chemical bioanalysis. Curr. Opin. Chem. Biol. 8, 534–539. https://doi.org/10.1016/j.cbpa.2004.08.014

Beer, M., Seviour, E.M., Kong, Y., Cunningham, M., Blackall, L.L., Y, R.J.S., 2002. Phylogeny of the filamentous bacterium Eikelboom Type 1851 , and design and application of a 16S rRNA targeted oligonucleotide probe for its fluorescence *in situ* identfication in activated sludge. FEMS Microbiol. Lett. 207, 179–183.

Behnam, F., Vilcinskas, A., Wagner, M., Stoecker, K., 2012. A Straightforward DOPE (Double Labeling of Oligonucleotide Probes) -FISH (Fluorescence *In Situ* Hybridization) Method for Simultaneous Multicolor Detection of Six Microbial Populations. AEM 78, 5138–5142. https://doi.org/10.1128/AEM.00977-12

Berry, D., Mader, E., Lee, T.K., Woebken, D., Wang, Y., Zhu, D., Palatinszky, M., Schintlmeister, A., Schmid, M.C., Hanson, B.T., Shterzer, N., Mizrahi, I., Rauch, I., Decker, T., Bocklitz, T., Popp, J., Gibson, C.M., Fowler, P.W., Huang, W.E., Wagner, M., 2015. Tracking heavy water (D_2O) incorporation for identifying and sorting active microbial cells. Proc. Natl. Acad. Sci. 112, E194–

E203. https://doi.org/10.1073/pnas.1420406112

Bitton, G., Bitton, G., 2011. Wastewater microbiology. Wiley-Blackwell, Hoboken.

Bitton, G., Bitton, G., 2002. Encyclopedia of environmental microbiology. Wiley, New York, N.Y.

Björnsson, L., Hugenholtz, P., Tyson, G.W., Blackall, L.L., 2002. Filamentous *Chloroflexi* (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removalc. Microbiology 148, 2309–2318. https://doi.org/10.1099/00221287-148-8-2309

Blackall, L.L., Seviour, E.M., Bradford, D., Rossetti, S., Tandoi, V., Seviour, R.J., 2000. '*Candidatus* Nostocoida limicola', a filamentous bacterium from activated sludge. Int. J. Syst. Evol. Microbiol. 703–709.

Blackall, L.L., Stratton, H., Bradford, D., Dot, T.D., Sjorup, C., Seviour, E.M., Seviour, R.J., 1996. "*Candidatus* Microthrix parvicella", a Filamentous Bacterium from Activated Sludge Sewage Treatment Plants. Int. J. Syst. Evol. Microbiol. 46, 344–346.

Bosshard, P.P., Zbinden, R., Altwegg, M., 2002. *Turicibacter sanguinis* gen. nov., sp. nov., a novel anaerobic, Gram-positive bacterium. Int. J. Syst. Evol. Microbiol. 12, 1263–1266. https://doi.org/10.1099/ijs.0.02056-0.A

Bowers, R.M., Kyrpides, N.C., Stepanauskas, R., Harmon-smith, M., Doud, D., Jarett, J., Rivers, A.R., Eloe-fadrosh, E.A., Tringe, S.G., Ivanova, N.N., Copeland, A., Clum, A., Becraft, E.D., Malmstrom, R.R., Birren, B., Podar, M., Bork, P., Weinstock, G.M., Garrity, G.M., Dodsworth, J.A., Yooseph, S., Sutton, G., Glöckner, F.O., Gilbert, J.A., Nelson, W.C., Hallam, S.J., 2017. Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. Nat. Biotechnol. 35. https://doi.org/10.1038/nbt.3893

Brock, T.D., 1967. Mode of Filamentous Growth of *Leucothrix mucor* in Pure Culture and in Nature , as Studied by Tritiated Thymidine Autoradiography. J. Bacteriol. 93, 985–990.

Brown, C.G., Clarke, J., 2016. Nanopore development at Oxford Nanopore Rapid , semi-automated protein terminal characterization using ISDetect. Nat. Publ. Gr. 34, 481–482. https://doi.org/10.1038/nbt.3622

Buijtels, P.C.A.M., Petit, P.L.C., Endtz, H.P., Puppels, G.J., Verbrugh, H.A., Belkum, A. Van, Soolingen, D. Van, Maquelin, K., 2008. Rapid Identification of

Mycobacteria by Raman Spectroscopy. J. Clin. Microbiol. 46, 961–965. https://doi.org/10.1128/JCM.01763-07

Burow, L.C., Kong, Y., Nielsen, J.L., Blackall, L.L., Nielsen, P.H., 2007. Abundance and ecophysiology of *Defluviicoccus* spp., glycogen-accumulating organisms in full-scale wastewater treatment processes. Microbiology 153, 178–185. https://doi.org/10.1099/mic.0.2006/001032-0

Callahan, B.J., McMurdie, P.J., Holmes, S.P., 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. ISME J. 11, 2639–2643. https://doi.org/10.1038/ismej.2017.119

Camejo, P.Y., Owen, B.R., Martirano, J., Ma, J., Kapoor, V., Santo, J., Mcmahon, K.D., Noguera, D.R., 2016. *Candidatus* Accumulibacter phosphatis clades enriched under cyclic anaerobic and microaerobic conditions simultaneously use different electron acceptors. Water Res. 102, 125–137. https://doi.org/10.1016/j.watres.2016.06.033

Camejo, P.Y., Oyserman, B.O., Mcmahon, K.D., Noguera, D.R., 2019. Integrated Omic Analyses Provide Evidence that a "*Candidatus* Accumulibacter phosphatis" Strain Performs Denitrification under Microaerobic Conditions. mSystems 4, 1–23.

Campanaro, S., Treu, L., R, L.M.R., Kovalovszki, A., Ziels, R.M., Maus, I., Zhu, X., Kougias, P.G., Basile, A., Luo, G., Schlüter, A., Konstantinidis, K.T., Angelidaki, I., 2020. New insights from the biogas microbiome by comprehensive genome - resolved metagenomics of nearly 1600 species originating from multiple anaerobic digesters. Biotechnol. Biofuels 13, 1–18. https://doi.org/10.1186/s13068-020-01679-y

Caporaso, J.G., Lauber, C.L., Walters, W. a, Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. a, Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 6, 1621–1624. https://doi.org/10.1038/ismej.2012.8

Carlsohon, M.R., Groth, I., Saluz, H., Schumann, P., Groth, I., 2008. *Fodinicola feengrottensis* gen. nov., sp. nov., an actinomycete isolated from a medieval mine. Int. J. Syst. Evol. Microbiol. 58, 1529–1536. https://doi.org/10.1099/ijs.0.65512-0

Chaumeil, P., Mussig, A.J., Parks, D.H., Hugenholtz, P., 2019. Genome analysis GTDB-Tk : a toolkit to classify genomes with the Genome Taxonomy Database. Bioinformatics 36, 1925–1927. https://doi.org/10.1093/bioinformatics/btz848

Choi, D.H., Kim, H.M., Noh, J., Cho, B.C., 2007. *Nocardioides marinus* sp. nov. Int. J. Syst. Evol. Microbiol. 57, 775–779. https://doi.org/10.1099/ijs.0.64649-0

Chun, J., Blackall, L., Kang, S.-O., Hah, Y.C., Googfellow, M., 1997. A Proposal To Reclassify Nocardia pinensis Blackall et al. as *Skermania pinifomis* gen. nov., comb. nov. Int. J. Syst. Bacteriol. 47, 127–131.

Coelho, C., Joao, M.R., 2015. Structural and mechanistic insights on nitrate reductases. Protein Sci. 24, 1901–1911. https://doi.org/10.1002/pro.2801

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2014. Ribosomal Database Project: Data and tools for high throughput rRNA analysis. Nucleic Acids Res. 42, 633–642. https://doi.org/10.1093/nar/gkt1244

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V, Eiglmeier, K., Gas, S., Barry, C.E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.-A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S., Barrell, B.G., 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393, 537–544. https://doi.org/10.1038/31159

Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A.J., Keller, J., Jenkins, D., Blackall, L.L., 2000. Identification of polyphosphate-accumulating organisms and design of 16SrRNA-directed probes for their detection and quantitation. Appl. Environ. Microbiol. 66, 1175–1182. https://doi.org/10.1128/AEM.66.3.1175-1182.2000.Updated

Daims, H., Elena, V., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R.H., Bergen, M. Von, Rattei, T., 2015. Complete nitrification by *Nitrospira* bacteria. Nature. https://doi.org/10.1038/nature16461

Daims, H., Lücker, S., Wagner, M., 2006. Daime, a Novel Image Analysis Program for Microbial Ecology and Biofilm Research. Environ. Microbiol. 8, 200–213. https://doi.org/10.1111/j.1462-2920.2005.00880.x

Daims, H., Nielsen, J.L., Nielsen, P.H., Schleifer, K.-H., Wagner, M., 2001. *In Situ* Characterization of *Nitrospira*-Like Nitrite-Oxidizing Bacteria Active in Wastewater Treatment Plants. Appl. Environ. Microbiol. 67, 5273 LP – 5284. https://doi.org/10.1128/AEM.67.11.5273-5284.2001

Daims, H., Stoecker, K., Wagner, M., 2005. Fluorescence *in situ* hybridization for the detection of prokaryotes, in: Osborn, A.M., Smith, C.J. (Eds.), Molecular Microbial Ecology. Taylor & Francis, New York, pp. 213–239.

Daligault, H., Lapidus, A., Zeytun, A., Nolan, M., Lucas, S., 2011. Complete genome sequence of *Haliscomenobacter hydrossis* type strain (OT). Stand. Genomic Sci. 4, 352–360. https://doi.org/10.4056/sigs.1964579

Dastager, S.G., Lee, J., Ju, Y., Park, D., Kim, C., Kim, C., 2008. *Nocardioides koreensis* sp. nov., *Nocardioides bigeumensis* sp. nov. and *Nocardioides agariphilus* sp. nov., isolated from soil from Bigeum Island , Korea. Int. J. Syst. Evol. Microbiol. 58, 2292–2296. https://doi.org/10.1099/ijs.0.65566-0

Davenport, R.J., Curtis, T.P., Goodfellow, M., Stainsby, F.M., Bingley, M., 2000. Quantitative Use of Fluorescent *In Situ* Hybridization To Examine Relationships between Mycolic Acid-Containing Actinomycetes and Foaming in Activated Sludge Plants. Appl. Environ. Microbiol. 66, 1158–1166.

de los Reyes, F.L., Ritter, W., Raskin, L., 1997. Group-specific small-subunit rRNA hybridization probes to characterize filamentous foaming in activated sludge systems. Appl. Environ. Microbiol. 63, 1107 LP – 1117.

de los Reyes, M.F., de los Reyes, F.L., Hernandez, M., Raskin, L., 1998. Quantification of *Gordona amarae* Strains in Foaming Activated Sludge and Anaerobic Digester Systems with Oligonucleotide Hybridization Probes. Appl. Environ. Microbiol. 64, 2503 LP – 2512. https://doi.org/10.1128/AEM.64.7.2503-2512.1998

Deepnarain, N., Kumari, S., Ramjith, J., Swalaha, F.M., Tandoi, V., Pillay, K., Bux, F., 2015. A logistic model for the remediation of filamentous bulking in a biological nutrient removal wastewater treatment plant. Water Sci. Technol. 72, 391. https://doi.org/10.2166/wst.2015.181

Deinema, M.H., Loosdrecht, M. Van, Scholten, A., 1985. Some physiological characteristics of *Acinetobacter* spp. accumulating large amounts of phosphate. Water Sci. Technol. 17, 119–125.

Desantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes , a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Appl Environ Microbiol. 72, 5069–5072. https://doi.org/10.1128/AEM.03006-05

Digue, C., Moszerc, I., 2007. Annotation, comparison and databases for hundreds of bacterial genomes. Res. Microbiol. 158, 724–736.

Dolinšek, J., Lagkouvardos, I., Wanek, W., Wagner, M., Daims, H., 2013. Interactions of Nitrifying Bacteria and Heterotrophs: Identification of a *Micavibrio*-Like Putative Predator of *Nitrospira* spp. Appl. Environ. Microbiol. 79, 2027 LP – 2037. https://doi.org/10.1128/AEM.03408-12

Dubinina, G., Savvichev, A., Orlova, M., Gavrish, E., Verbarg, S., Grabovich, M., 2017. *Beggiatoa leptomitoformis* sp. nov., the first freshwater member of the genus capable of chemolithoautotrophic growth. Int. J. Syst. Evol. Microbiol. 67, 197–204. https://doi.org/10.1099/ijsem.0.001584

Duckworth, A.W., Grant, S., Grant, W.D., Jones, B.E., Meijer, D., 1998. *Dietzia natronolimnaios* sp. nov., a new member of the genus Dietzia isolated from an East African soda lake. Extremophiles 2, 359–366.

Dueholm, M. S., Andersen, K.S., McIlroy, S.J., Kristensen, J.M., Yashiro, E., Karst, S.M., Albertsen, M., Nielsen, P.H., 2020. Generation of comprehensive ecosystems-specific reference databases with species-level resolution by high-throughput full-length 16S rRNA gene sequencing and automated taxonomy assignment (AutoTax). bioRxiv 672873. https://doi.org/10.1101/672873

Dueholm, M.S., Andersen, K.S., Petriglieri, F., McIlroy, S.J., Nierychlo, M., Petersen, J.F., Kristensen, J.M., Yashiro, E., Karst, S.M., Albertsen, M., Nielsen, P.H., 2019. Comprehensive ecosystem-specific 16S rRNA gene databases with automated taxonomy assignment (AutoTax) provide species-level resolution in microbial ecology. bioRxiv 672873. https://doi.org/10.1101/672873

Dueholm, M.S., Andersen, K.S., Rudkjøbing, V., Nierychlo, M., Knudsen, S., Albertsen, M., the Global Microbiome Consortia, Nielsen, P.H., 2020. The global microbiome of wastewater treatment plants uncovered at full-length 16S rRNA gene resolution. In preparation.

Egle, L., Rechberger, H., Zessner, M., 2015. Overview and description of technologies for recovering phosphorus from municipal wastewater. Resources, Conserv. Recycl. 105, 325–346. https://doi.org/10.1016/j.resconrec.2015.09.016

Eikelboom, D.H., 1975. Filamentous organisms observed in activated sludge. Water Res. 9, 365–388.

Eikelboom, D.H., Geurkink, B., 2001. Filamentous micro-organisms observed in industrial activated sludge plants. Water Sci. Technol. 46, 535–542.

Erdal, U.G., Erdal, Z.K., Daigger, G.T., Randall, C.W., 2008. Is it PAO-GAO competition or metabolic shift in EBPR system? Evidence from an

experimental study. Water Sci Technol. 58, 1329–1334. https://doi.org/10.2166/wst.2008.734

Erhart, R., Bradford, D., Seviour, R., Amann, R., 1997. Development and Use of Fluorescent *In Situ* Hybridization Probes for the Detection and Identification of "Microthrix parvicella" in Activated Sludge. Syst. Appl. Microbiol. 20, 310–318. https://doi.org/10.1016/S0723-2020(97)80078-1

Fernando, E.Y., McIlroy, S.J., Nierychlo, M., Herbst, F.-A., Petriglieri, F., Schmid, M.C., Wagner, M., Nielsen, J.L., Nielsen, P.H., 2019. Resolving the individual contribution of key microbial populations to enhanced biological phosphorus removal with Raman–FISH. ISME J. 2019. https://doi.org/10.1038/s41396-019-0399-7

Flowers, J.J., He, S., Malfatti, S., Glavina, T., Tringe, S.G., Hugenholtz, P., Mcmahon, K.D., 2013. Comparative genomics of two '*Candidatus* Accumulibacter' clades performing biological phosphorus removal. ISME J 7, 2301–2314. https://doi.org/10.1038/ismej.2013.117

Ganguly, S., Jimenez-galisteo, G., Pletzer, D., Winterhalter, M., Benz, R., 2016. Draft Genome Sequence of *Dietzia maris* DSM 43672 , a Gram-Positive Bacterium of the Mycolata Group. Genome Announc. 4, e00542-16. https://doi.org/10.1128/genomeA.00542-16.Copyright

Goel, R., Sanhueza, P., Noguera, D., 2005. Evidence of *Dechloromonas* sp. participating in enhanced biological phosphorous removal (EBPR) in a bench-scale aerated-anoxic reactor. Proc. Water Environ. Fed. 3864–3871.

Gray, N.F., 2004. Biology of wastewater treatment (Second Edition), Series on environmental science and management.

Grégoire, P., Bohli, M., Cayol, J.L., Joseph, M., Guasco, S., Dubourg, K., Cambar, J., Michotey, V., Bonin, P., Fardeau, M.L., Ollivier, B., 2011. *Caldilinea tarbellica* sp. nov., a filamentous, thermophilic, anaerobic bacterium isolated from a deep hot aquifer in the Aquitaine Basin. Int. J. Syst. Evol. Microbiol. 61, 1436–1441. https://doi.org/10.1099/ijs.0.025676-0

Gunther, S., Trutnau, M., Kleinsteuber, S., Hause, G., Bley, T., Roske, I., Harms, H., Muller, S., 2009. Dynamics of Polyphosphate-Accumulating Bacteria in Wastewater Treatment Plant Microbial Communities Detected via DAPI (4′, 6′-Diamidino-2-Phenylindole) and Tetracycline Labeling. Appl. Environ. Microbiol. 75, 2111–2121. https://doi.org/10.1128/AEM.01540-08

Guo, F., Zhang, T., Li, B., Wang, Z., Ju, F., Liang, Y., 2019. Mycobacterial species

and their contribution to cholesterol degradation in wastewater treatment plants. Sci. Rep. 1–10. https://doi.org/10.1038/s41598-018-37332-w

Handelsman, J., Rondon, M.R., Goodman, R.M., Brady, S.F., Clardy, J., 1998. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chem. Biol. 5, R245–R249.

Hao, L., McIlroy, S.J., Kirkegaard, R.H., Karst, S.M., Fernando, W.E.Y., Aslan, H., Meyer, R.L., Albertsen, M., Nielsen, P.H., Dueholm, M.S., 2018. Novel prosthecate bacteria from the candidate phylum Acetothermia. ISME J. 12, 2225–2237. https://doi.org/10.1038/s41396-018-0187-9

Hatzenpichler, R., Connon, S.A., Goudeau, D., Malmstrom, R.R., Woyke, T., 2016. Visualizing *in situ* translational activity for identifying and sorting slow-growing archaeal − bacterial consortia. PNAS 113, E4069–E4078. https://doi.org/10.1073/pnas.1603757113

Hatzenpichler, R., Scheller, S., Tavormina, P.L., Babin, B.M., Tirrell, D.A., Orphan, V.J., 2014. *In situ* visualization of newly synthesized proteins in environmental microbes using amino acid tagging and click chemistry. Environ. Microbiol. 16, 2568–2590. https://doi.org/10.1111/1462-2920.12436

Hendriks, J., Oubrie, A., Castresana, J., Urbani, A., Gemeinhardt, S., Saraste, M., 2000. Nitric oxide reductases in bacteria. Biochim. Biophys. Acta - Bioenerg. 1459, 266–273.

Henze, M., Harremoes, P., Jansen, J.L.C., Arvin, E., 2002. Wastewater Treatment: Biological and Chemical Processes (Environmental Science and Engineering).

Hesselmann, R.P.X., Von Rummel, R., Resnick, S.M., Hany, R., Zehnder, A.J.B., 2000. Anaerobic metabolism of bacteria performing enhanced biological phosphate removal. Water Res. 34, 3487–3494.

Hesselsoe, M., Fu, S., Schloter, M., Bodrossy, L., Iversen, N., Roslev, P., Nielsen, P.H., Wagner, M., Loy, A., 2009. Isotope array analysis of Rhodocyclales uncovers functional redundancy and versatility in an activated sludge. ISME J. 3, 1349–1364. https://doi.org/10.1038/ismej.2009.78

Horn, M.A., Ihssen, J., Matthies, C., Schramm, A., Acker, G., Drake, H.L., Drake, H.L., 2005. *Dechloromonas denitrificans* sp. nov., *Flavobacterium denitrificans* sp. nov., *Paenibacillus anaericanus* sp. nov. and *Paenibacillus terrae* strain MH72 , N_2O-producing bacteria isolated from the gut of the earthworm *Aporrectodea caliginosa*. Int. J. Syst. Evol. Microbiol. 55, 1255–1265. https://doi.org/10.1099/ijs.0.63484-0

Hu, Y., Feng, Y., Qin, J., Radolfova-krizova, L., Maixnerova, M., Zhang, X., 2018. *Acinetobacter wuhouensis* sp . nov ., isolated from hospital sewage. Int. J. Syst. Evol. Microbiol. 68, 3212–3216. https://doi.org/10.1099/ijsem.0.002963

Huang, W.E., Bailey, M.J., Thompson, I.P., Whiteley, A.S., Spiers, A.J., 2007. Single-cell Raman spectral profiles of *Pseudomonas fluorescens* SBW25 reflects in vitro and in planta metabolic history. Microb. Ecol. 53, 414–425. https://doi.org/10.1007/s00248-006-9138-5

Huang, W.E., Griffiths, R.I., Thompson, I.P., Bailey, M.J., Whiteley, A.S., 2004. Raman microscopic analysis of single microbial cells. Anal. Chem. 76, 4452–4458. https://doi.org/10.1021/ac049753k

Huang, W.E., Ward, A.D., Whiteley, A.S., 2009. Raman tweezers sorting of single microbial cells. Environ. Microbiol. Rep. 1, 44–49. https://doi.org/10.1111/j.1758-2229.2008.00002.x

Hug, L.A., Castelle, C.J., Wrighton, K.C., Thomas, B.C., Sharon, I., Frischkorn, K.R., Williams, K.H., Tringe, S.G., Banfield, J.F., 2013. Community genomic analyses constrain the distribution of metabolic traits across the Chloroflexi phylum and indicate roles in sediment carbon cycling. Microbiome 1, 22. https://doi.org/10.1186/2049-2618-1-22

Hugenholtz, P., Tyson, G.W., Blackall, L.L., 2002. Oligonucleotide probes for fluorescence *in situ* hybridization. Methods Mol. Biol. 179, 29–42. https://doi.org/10.1385/1-59259-238-4:029

Jing, X., Gou, H., Gong, Y., Su, X., Xu, L., Ji, Y., Song, Y., Thompson, I.P., Xu, J., Huang, W.E., 2018. Raman-activated cell sorting and metagenomic sequencing revealing carbon-fixing bacteria in the ocean. Environ. Microbiol. 20, 2241–2255. https://doi.org/10.1111/1462-2920.14268

Johnston, J., Lapara, T., Behrens, S., 2019. Composition and Dynamics of the Activated Sludge Microbiome during Seasonal Nitrification Failure. Sci. Reports Reports 9, 1–15. https://doi.org/10.1038/s41598-019-40872-4

Jørgensen, M.K., Nierychlo, M., Nielsen, A.H., Larsen, P., Christensen, M.L., Nielsen, P.H., 2017. Unified understanding of physico-chemical properties of activated sludge and fouling propensity. Water Res. 120, 117–132. https://doi.org/10.1016/j.watres.2017.04.056

Juretschko, S., Loy, A., Lehner, A., Wagner, M., 2002. The Microbial Community Composition of a Nitrifying-Denitrifying Activated Sludge from an Industrial Sewage Treatment Plant Analyzed by the Full-Cycle rRNA Approach. Syst

Appl Microbiol 25, 84–99.

Kale, V., Björnsdóttir, S.H., Fridjónsson, Ó.H., Pétursdóttir, S.K., Ómarsdóttir, S., Hreggvidsson, G.Ó., 2013. *Litorilinea aerophila* gen. nov., sp. nov., an aerobic member of the class Caldilineae, phylum Chloroflexi, isolated from an intertidal hot spring. Int. J. Syst. Evol. Microbiol. 63, 1149–1154. https://doi.org/10.1099/ijs.0.044115-0

Kanagawa, T., Kamagata, Y., Aruga, S., Kohno, T., Horn, M., Wagner, M., 2000. Phylogenetic Analysis of and Oligonucleotide Probe Development for Eikelboom Type 021N Filamentous Bacteria Isolated from Bulking Activated Sludge. Appl. Environ. Microbiol. 66, 5043 LP – 5052. https://doi.org/10.1128/AEM.66.11.5043-5052.2000

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., Tanabe, M., 2016. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res. 44, 457–462. https://doi.org/10.1093/nar/gkv1070

Karst, S.M., Dueholm, M.S., McIlroy, S.J., Kirkegaard, R.H., Nielsen, P.H., Albertsen, M., 2018. Retrieval of a million high-quality , full-length microbial 16S and 18S rRNA gene sequences without primer bias. Nat. Biotechnol. 36, 190–195. https://doi.org/10.1038/nbt.4045

Karst, S.M., Ziels, R.M., Kirkegaard, R.H., Albertsen, M., 2019. Enabling high-accuracy long-read amplicon sequences using unique molecular identifiers and Nanopore sequencing. bioRxiv 645903. https://doi.org/10.1101/645903

Kawaharasaki, M., Tanaka, H., Kanagawa, T., Nakamura, K., 1999. *In situ* identification of polyphosphate-accumulating bacteria in activated sludge by dual staining with rRNA-targeted oligonucleotide probes and 4',6-diaimidino-2-phenylindol (DAPI) at a polyphosphate-probing concentration. Water Res. 33, 257–265. https://doi.org/10.1016/S0043-1354(98)00183-3

Kawaichi, S., Ito, N., Kamikawa, R., Sugawara, T., Yoshida, T., Sako, Y., 2013. *Ardenticatena maritima* gen. nov., sp. nov., a ferric iron- and nitrate-reducing bacterium of the phylum "Chloroflexi" isolated from an iron-rich coastal hydrothermal field, and description of Ardenticatenia classis nov. Int. J. Syst. Evol. Microbiol. 63, 2992–3002. https://doi.org/10.1099/ijs.0.046532-0

Kawakoshi, A.K., Nakazawa, H.I., Fukada, J.U., Sasagawa, M.A., Katano, Y.O.K.O., Kamagata, Y.O., Nakamura, K.A., Yamazaki, S.H., Fujita, N.O., 2012. Deciphering the Genome of Polyphosphate Accumulating Actinobacterium *Microlunatus phosphovorus*. DNA Res. 19, 383–394.

Kimbrel, J.A., Chang, J., Arp, D.J., Sayavedra-soto, L.A., 2013. The Draft Genome Sequence of *Nocardioides* sp . Strain CF8 Reveals the Scope of Its Mmetabolic Capabilities. Genome Announc. 1, 1–2. https://doi.org/10.1128/genomeA.00439-13.Copyright

Klaassens, E.S., Durkin, A.S., Harkins, D.M., Foster, L., 2011. Draft Genome Sequence of *Turicibacter sanguinis* PC909 , Isolated from Human Feces. J. Bacteriol. 193, 1314–1315. https://doi.org/10.1128/JB.01328-10

Koch, H., Kessel, M.A.H.J. Van, Lücker, S., 2019. Complete nitrification : insights into the ecophysiology of comammox *Nitrospira*. Appl. Microbiol. Biotechnol. 103, 177–189.

Kohno, T., Sei, K., Mori, K., 2002. Characterization of type 1851 organism isolated from activated sludge samples. Water Sci. Technol. 46, 111–114.

Kong, Y., Nielsen, J.L., Nielsen, P.H., 2005. Identity and Ecophysiology of Uncultured Actinobacterial Polyphosphate-Accumulating Organisms in Full-Scale Enhanced Biological Phosphorus Removal Plants. Appl. Environ. Microbiol. 71, 4076–4085. https://doi.org/10.1128/AEM.71.7.4076-4085.2005

Kong, Y., Nielsen, J.L., Nielsen, P.H., 2004. Microautoradiographic Study of Rhodocyclus-Related Polyphosphate-Accumulating Bacteria in Full-Scale Enhanced Biological Phosphorus Removal Plants. Appl. Environ. Microbiol. 70, 5383–5390. https://doi.org/10.1128/AEM.70.9.5383

Kong, Y., Xia, Y., Nielsen, J.L., Nielsen, P.H., 2007. Structure and function of the microbial community in a full-scale enhanced biological phosphorus removal plant. Microbiology 153, 4061–4073. https://doi.org/10.1099/mic.0.2007/007245-0

Kono, N., Arakawa, K., 2019. Nanopore sequencing : Review of potential applications in functional genomics. Dev. Growth Differ. 316–326. https://doi.org/10.1111/dgd.12608

Kopf, S.H., Mcglynn, S.E., Green-saxena, A., Guan, Y., Newman, D.K., Orphan, V.J., 2015. Heavy water and ^{15}N labelling with NanoSIMS analysis reveals growth rate-dependent metabolic heterogeneity in chemostats. Environ. Microbiol. 17, 2542–2556. https://doi.org/10.1111/1462-2920.12752

Kragelund, C., Levantesi, C., Borger, A., Thelen, K., Eikelboom, D., Tandoi, V., Kong, Y., Krooneman, J., Larsen, P., Thomsen, T.R., Nielsen, P.H., 2008. Identity, abundance and ecophysiology of filamentous bacteria belonging to the Bacteroidetes present in activated sludge plants. Microbiology 154, 886–894.

https://doi.org/10.1099/mic.0.2007/011684-0

Kragelund, C., Levantesi, C., Borger, A., Thelen, K., Eikelboom, D., Tandoi, V., Kong, Y., Van Der Waarde, J., Krooneman, J., Rossetti, S., Thomsen, T.R., Nielsen, P.H., 2007. Identity, abundance and ecophysiology of filamentous Chloroflexi species present in activated sludge treatment plants. FEMS Microbiol. Ecol. 59, 671–682. https://doi.org/10.1111/j.1574-6941.2006.00251.x

Kragelund, C., Nielsen, J.L., Thomsen, T.R., Nielsen, P.H., 2005. Ecophysiology of the filamentous Alphaproteobacterium *Meganema perideroedes* in activated sludge. FEMS Microbiol. Ecol. 54, 111–122. https://doi.org/10.1016/j.femsec.2005.03.002

Kristiansen, R., Thi, H., Nguyen, T., Saunders, A.M., Nielsen, J.L., Wimmer, R., Le, V.Q., Mcilroy, S.J., Petrovski, S., 2013. A metabolic model for members of the genus *Tetrasphaera* involved in enhanced biological phosphorus removal. ISME J. 7, 543–554. https://doi.org/10.1038/ismej.2012.136

Kusi, D., Kampe, B., Ro, P., 2014. Identification of water pathogens by Raman microspectroscopy. Water Res. 48, 179–189. https://doi.org/10.1016/j.watres.2013.09.030

Lajoie, C.A., Layton, A.C., Gregory, I.R., Sayler, G.S., Taylor, D.E., Meyers, A.J., 2000. Zoogleal Clusters and Sludge Dewatering Potential in an Industrial Activated-Sludge Wastewater Treatment Plant. Water Environ. Res. 72, 56–64. https://doi.org/10.2175/106143000X137112

Larkin, J.M., 1980. Isolation of *Thiothrix* in pure culture and observation of a filamentous epiphyte on *Thiothrix*. Curr. Microbiol. 4, 155–158. https://doi.org/10.1007/BF02602820

Lawson, C.E., Harcombe, W.R., Hatzenpichler, R., Lindemann, S.R., Löffler, F.E., O'Malley, M.A., García Martín, H., Pfleger, B.F., Raskin, L., Venturelli, O.S., Weissbrodt, D.G., Noguera, D.R., McMahon, K.D., 2019. Common principles and best practices for engineering microbiomes. Nat. Rev. Microbiol. 17, 725–741. https://doi.org/10.1038/s41579-019-0255-9

Lechevalier, M.P., Lechevalier, H.A., 1974. *Nocardia amarae* sp. nov., an Actinomycete Common in Foaming Activated Sludge. Int. J. Syst. Bacteriol. 24, 278–288.

Liu, J.R., Burrell, P., Seviouri, E.M., Soddelli, J.A., Blackall, L.L., Seviour, R.J., 2000. The Filamentous Bacterial Morphotype Nostocoida limicola Contains at

least Two Previously Described Genera in the Low G+C Gram Positive Bacteria. Syst Appl Microbiol 23, 528–534. https://doi.org/10.1016/S0723-2020(00)80027-2

Liu, J.R., Seviour, R.J., 2001. Design and application of oligonucleotide probes for fluorescent *in situ* identification of the filamentous bacterial morphotype Nostocoida limicola in activated sludge. Environ. Microbiol. 3, 551–560. https://doi.org/10.1046/j.1462-2920.2001.00229.x

Lok, C., 2015. Mining the microbial dark matter. Nature 522, 270–273. https://doi.org/10.1038/522270a

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, a., Buchner, A., Lai, T., Steppi, S., Jacob, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüßbmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüßmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.H., 2004. ARB: A software environment for sequence data. Nucleic Acids Res. 32, 1363–1371. https://doi.org/10.1093/nar/gkh293

Lukumbuzya, M., Kristensen, J.M., Kitzinger, K., Pommerening-Röser, A., Nielsen, P.H., Wagner, M., Daims, H., Pjevac, P., 2020. A refined set of rRNA-targeted oligonucleotide probes for *in situ* detection and quantification of ammonia-oxidizing bacteria. bioRxiv 2020.05.27.119446. https://doi.org/10.1101/2020.05.27.119446

Lv, X., Shao, M., Li, C., Li, J., Gao, X., Sun, F., 2014. A Comparative Study of the Bacterial Community in Denitrifying and Traditional Enhanced Biological Phosphorus Removal Processes. Microbes Environ. 29, 261–268. https://doi.org/10.1264/jsme2.ME13132

Madigan, M., Bender, K.S., Buckley, D., Sattley, W.M., Stahl, D., 2017. Brock Biology of Microorganisms, 15th Edition, 15th Editi. ed. Pearson.

Madoni, P., Davoli, D., Gibin, G., 2000. Survey of filamentous microorganisms from bulking and foaming activated sludge plants in Italy. Water Res. 34, 1767–1772. https://doi.org/10.1016/S0043-1354(99)00352-8

Majed, N., Matthaus, C., Diem, M., Gu, A.Z., 2009. Evaluation of Intracellular Polyphosphate Dynamics in Enhanced Biological Phosphorus Removal Process

using Raman Microscopy. Environ. Sci. Technol. 43, 5436–5442. https://doi.org/10.1021/es900251n

Maquelin, K., Endtz, H.P., Bruining, H.A., Puppels, G.J., 2002. Rapid Identification of *Candida* Species by Confocal Raman Microspectroscopy. J. Clin. Microbiol. 40, 594–600. https://doi.org/10.1128/JCM.40.2.594

Marcy, Y., Ouverney, C., Bik, E.M., Lo, T., Ivanova, N., Garcia, H., Szeto, E., Platt, D., Hugenholtz, P., Relman, D.A., Quake, S.R., 2007. Dissecting biological 'dark matter' with single-cell genetic analysis of rare and uncultivated TM7 microbes from the human mouth. PNAS 104, 11889–11894.

Marques, R., Ribera-guardia, A., Santos, J., Carvalho, G., Reis, M.A.M., Pijuan, M., Oehmen, A., 2018. Denitrifying capabilities of *Tetrasphaera* and their contribution towards nitrous oxide production in enhanced biological phosphorus removal processes. Water Res. 137, 262–272. https://doi.org/10.1016/j.watres.2018.03.010

Marques, R., Santos, J., Nguyen, H., Carvalho, G., Noronha, J.P., Nielsen, P.H., Reis, M.A.M., Oehmen, A., 2017. Metabolism and ecological niche of *Tetrasphaera* and *Ca.* Accumulibacter in enhanced biological phosphorus removal. Water Res. 122, 159–171. https://doi.org/10.1016/j.watres.2017.04.072

Martins, A.M.P., Pagilla, K., Heijnen, J.J., Van Loosdrecht, M.C.M., 2004. Filamentous bulking sludge - A critical review. Water Res. 38, 793–817. https://doi.org/10.1016/j.watres.2003.11.005

Maszenan, A.M., Seviour, R.J., Jena, A., 1999a. *Tessaracoccus bendigoensis* gen. nov., sp. nov., a Gram-positive coccus occurring in regular packages or tetrads , isolated from activated sludge biomass. Int. J. Syst. Bacteriol. 49, 459–468.

Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Schumann, P., 2002. *Quadricoccus australiensis* gen. nov., sp. nov., a β-proteobacterium from activated sludge biomass. Int. J. Syst. Evol. Microbiol. 52, 223–228.

Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Schumann, P., Burghardt, J., Webb, R.I., Soddell, J.A., Rees, G.N., 1999b. *Friedmanniella spumicola* sp. nov. and *Friedmanniella capsulata* sp. nov. from activated sludge foam : Gram-positive cocci that grow in aggregates of repeating groups of cocci. Int. J. Syst. Bacteriol. 49, 1667–1680.

Matsuura, N., Tourlousse, D.M., Ohashi, A., Hugenholtz, P., Sekiguchi, Y., 2015a. Draft Genome Sequences of *Anaerolinea thermolimosa* IMO-1 , *Bellilinea caldifistulae* GOMI-1, *Leptolinea tardivitalis* YMTK-2, *Levilinea*

saccharolytica KIBI-1, *Longilinea arvoryzae* KOME-1, Previously Described as Members of the Class Anaerolineae (Chloroflexi). Genome Announc. 3, 3–4. https://doi.org/10.1128/genomeA.00975-15.Copyright

Matsuura, N., Tourlousse, D.M., Sun, L., Toyonaga, M., Kuroda, K., Ohashi, A., Cruz, R., 2015b. Draft Genome Sequence of Anaerolineae Strain TC1 , a Novel Isolate from a Methanogenic Wastewater Treatment System. Genome Announc. 3, 2012–2013. https://doi.org/10.1128/genomeA.01104-15.Copyright

McIlroy, S., Seviour, R.J., 2009. Elucidating further phylogenetic diversity among the *Defluviicoccus*-related glycogen-accumulating organisms in activated sludge. Environ. Microbiol. Rep. 1, 563–568. https://doi.org/10.1111/j.1758-2229.2009.00082.x

McIlroy, S.J., Albertsen, M., Andresen, E.K., Saunders, A.M., 2014a. '*Candidatus* Competibacter'-lineage genomes retrieved from metagenomes reveal functional metabolic diversity. ISME J. 8, 613–624. https://doi.org/10.1038/ismej.2013.162

McIlroy, S.J., Karst, S.M., Nierychlo, M., Dueholm, M.S., Albertsen, M., Kirkegaard, R.H., Seviour, R.J., Nielsen, P.H., 2016a. Genomic and *in situ* investigations of the novel uncultured Chloroflexi associated with 0092 morphotype filamentous bulking in activated sludge. ISME J. 10, 1–12. https://doi.org/10.1038/ismej.2016.14

McIlroy, S.J., Kirkegaard, R.H., McIlroy, B., Nierychlo, M., Kristensen, J.M., Karst, S.M., Albertsen, M., Nielsen, P.H., 2017. MiDAS 2.0: An ecosystem-specific taxonomy and online database for the organisms of wastewater treatment systems expanded for anaerobic digester groups. Database 2017, 1–9. https://doi.org/10.1093/database/bax016

McIlroy, S.J., Kristiansen, R., Albertsen, M., Karst, S.M., Rossetti, S., Nielsen, J.L., Tandoi, V., Seviour, R.J., 2013. Metabolic model for the filamentous '*Candidatus* Microthrix parvicella' based on genomic and metagenomic analyses. ISME J. 7, 1161–1172. https://doi.org/10.1038/ismej.2013.6

Mcilroy, S.J., Lapidus, A., Thomsen, T.R., Han, J., Haynes, M., Lobos, E., Huntemann, M., Pati, A., Ivanova, N.N., Markowitz, V., Verbarg, S., Woyke, T., Klenk, H., Kyrpides, N., Nielsen, P.H., 2015. High quality draft genome sequence of *Meganema perideroedes* str . Gr1 T and a proposal for its reclassification to the family Meganemaceae. Stand. Genomic Sci. 1–8. https://doi.org/10.1186/s40793-015-0013-1

McIlroy, S.J., Nittami, T., Kanai, E., Fukuda, J., Saunders, A.M., Nielsen, P.H.,

2014b. Re-appraisal of the phylogeny and fluorescence *in situ* hybridization probes for the analysis of the Competibacteraceae in wastewater treatment systems. Environ. Microbiol. Rep. 7, 166–174. https://doi.org/10.1111/1758-2229.12215

McIlroy, S.J., Nittami, T., Seviour, E.M., Seviour, R.J., 2010. Filamentous members of cluster III *Defluviicoccus* have the *in situ* phenotype expected of a glycogen-accumulating organism in activated sludge. FEMS Microbiol. Ecol. 74, 248–256. https://doi.org/10.1111/j.1574-6941.2010.00934.x

McIlroy, S.J., Saunders, A.M., Albertsen, M., Nierychlo, M., McIlroy, B., Hansen, A.A., Karst, S.M., Nielsen, J.L., Nielsen, P.H., 2015. MiDAS: The field guide to the microbes of activated sludge. Database 2015, 1–8. https://doi.org/10.1093/database/bav062

McIlroy, S.J., Starnawska, A., Starnawski, P., Saunders, A.M., Nierychlo, M., Nielsen, P.H., Nielsen, J.L., 2016b. Identification of active denitrifiers in full-scale nutrient removal wastewater treatment systems. Environ. Microbiol. 18, 50–64.

Mcshan, W.M., Mclaughlin, R.E., Savic, G., Chang, J., Carson, M.B., Primeaux, C., Tian, R., Kenton, S., Jia, H., Lin, S., Qian, Y., Li, S., Zhu, H., Najar, F., Lai, H., White, J., Roe, B.A., Ferretti, J.J., 2002. Genome sequence of *Streptococcus mutans* UA159 , a cariogenic dental pathogen. PNAS 99, 14434–14439.

Mielczarek, A.T., Kragelund, C., Eriksen, P.S., Nielsen, P.H., 2012. Population dynamics of filamentous bacteria in Danish wastewater treatment plants with nutrient removal. Water Res. 46, 3781–3795. https://doi.org/10.1016/j.watres.2012.04.009

Mobarry, B.K., Wagner, M., Urbain, V., Rittmann, B.E., Stahl, D.A., 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. 62, 2156 LP – 2162.

Mohammad, S., Gharibzahedi, T., 2014. Characterization of bacteria of the genus *Dietzia* : an updated review. Ann. Microbiol. 64, 1–11. https://doi.org/10.1007/s13213-013-0603-3

Mukherjee, S., Stamatis, D., Bertsch, J., Ovchinnikova, G., Katta, H.Y., Mojica, A., Chen, I.A., Kyrpides, N.C., Reddy, T.B.K., 2019. Genomes OnLine database (GOLD) v . 7 : updates and new features. Nucleic Acids Res. 47, 649–659. https://doi.org/10.1093/nar/gky977

Muller, E., 2006. Bacteria and extracellular polymeric substances in activated sludge

and scum formation. TU Munich, Germany.

Murray, R.G.E., Stackebrandt, E., 1995. Taxonomic Note : Implementation of the Provisional Status Candidatus for Incompletely Described Procaryotes. Int. J. Syst. Evol. Microbiolology 45, 186–187.

Nakamura, K., Hiraishi, A., Yoshimi, Y., Kawaharasaki, M., Masuda, K., Kamagata, Y., 1995. *Microlunatus phosphovoms* gen. nov., sp. nov., a New Gram-Positive Polyphosphate-Accumulating Bacterium Isolated from Activated Sludge. Int. J. Syst. Bacteriol. 45, 17–22.

Nawrocki, E.P., Kolbe, D.L., Eddy, S.R., 2009. Infernal 1.0 : inference of RNA alignments. Bioinformatics. 25, 1335–1337. https://doi.org/10.1093/bioinformatics/btp157

Nguyen, H.T.T., Nielsen, J.L., Nielsen, P.H., 2012. '*Candidatus* Halomonas phosphatis', a novel polyphosphate-accumulating organism in full-scale. Environ. Microbiol. 14, 2826–2837. https://doi.org/10.1111/j.1462-2920.2012.02826.x

Nguyen, L., Schmidt, H.A., Haeseler, A. Von, Minh, B.Q., 2014. IQ-TREE : A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. Mol Biol Evol. 32, 268–274. https://doi.org/10.1093/molbev/msu300

Nielsen, J.L., 2009. Protocol for fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotides, in: FISH Handbook for Biological Wastewater Treatment. p. Pages 73-84.

Nielsen, P.H., 2017. Microbial biotechnology and circular economy in wastewater treatment. Microb. Biotechnol. 10, 1102–1105. https://doi.org/10.1111/1751-7915.12821

Nielsen, P.H., Mcilroy, S.J., Albertsen, M., 2019. Re-evaluating the microbiology of the enhanced biological phosphorus removal process. Curr. Opin. Biotechnol. 57, 111–118. https://doi.org/10.1016/j.copbio.2019.03.008

Nielsen, P.H., Roslev, P., Dueholm, T.E., Nielsen, J.L., 2002. *Microthrix parvicella*, a specialized lipid consumer in anaerobic–aerobic activated sludge plants. Water Sci. Technol. 46, 73–80.

Nielsen, P.H., Saunders, A.M., Hansen, A.A., Larsen, P., Nielsen, J.L., 2012. Microbial communities involved in enhanced biological phosphorus removal from wastewater — a model system in environmental biotechnology. Curr.

Opin. Biotechnol. 23, 452–459. https://doi.org/10.1016/j.copbio.2011.11.027

Nierychlo, M., Andersen, K.S., Xu, Y., Green, N., Jiang, C., Albertsen, M., Dueholm, M.S., Nielsen, P.H., 2020a. MiDAS 3: An ecosystem-specific reference database, taxonomy and knowledge platform for activated sludge and anaerobic digesters reveals species-level microbiome composition of activated sludge. Water Res. 115955. https://doi.org/10.1016/j.watres.2020.115955

Nierychlo, M., McIlroy, S.J., Kucheryavskiy, S. V., Jiang, C., Ziegler, A.S., Kondrotaite, Z., Stokholm-Bjerregaard, M., Nielsen, P.H., 2020b. *Candidatus* Amarolinea and *Candidatus* Microthrix Are Mainly Responsible for Filamentous Bulking in Municipal Danish Wastewater Treatment Plants. Front. Microbiol. https://doi.org/https://doi.org/10.3389/fmicb.2020.01214

Nierychlo, M., Milobedzka, A., Petriglieri, F., Mcilroy, B., Nielsen, P.H., Mcilroy, S.J., 2019. The morphology and metabolic potential of the Chloroflexi in full-scale activated sludge wastewater treatment plants. FEMS Microbiol. Ecol. 95, 1–11. https://doi.org/10.1093/femsec/fiy228

Nitta, N., 2020. Raman image-activated cell sorting. Nat. Commun. 11, 1–16. https://doi.org/10.1038/s41467-020-17285-3

Nittami, T., McIlroy, S., Seviour, E.M., Schroeder, S., Seviour, R.J., 2009. Candidatus Monilibacter spp., common bulking filaments in activated sludge, are members of Cluster III *Defluviicoccus*. Syst. Appl. Microbiol. 32, 480–489. https://doi.org/https://doi.org/10.1016/j.syapm.2009.07.003

Nobu, M.K., Narihiro, T., Rinke, C., Kamagata, Y., Tringe, S.G., Woyke, T., Liu, W., 2015. Microbial dark matter ecogenomics reveals complex synergistic networks in a methanogenic bioreactor. ISME J. 9, 1710–1722. https://doi.org/10.1038/ismej.2014.256

Nocardioides, A., Coleman, N. V, Wilson, N.L., Barry, K., Brettin, T.S., Bruce, D.C., Copeland, A., Dalin, E., Detter, J.C., Glavina, T., Goodwin, L.A., Hammon, N.M., Han, S., Hauser, L.J., Israni, S., Kim, E., Kyrpides, N., Land, M.L., Lapidus, A., Larimer, F.W., Lucas, S., Pitluck, S., Richardson, P., Schmutz, J., Tapia, R., Thompson, S., Tice, H.N., Gossett, J.G., Mattes, T.E., 2011. Genome Sequence of the Ethene- and Vinyl Chloride-Oxidizing Actinomycete Nocardioides sp. Strain JS614. J. Bacteriol. 193, 3399–3400. https://doi.org/10.1128/JB.05109-11

Noguera, D.R., Wright, E.S., Camejo, P., Yilmaz, L.S., 2014. Mathematical tools to optimize the design of oligonucleotide probes and primers. Appl. Microbiol. Biotechnol. 98, 9595–9608. https://doi.org/10.1007/s00253-014-6165-x

Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Blackall, L.L., Reis, M.A.M., 2007. Advances in enhanced biological phosphorus removal: From micro to macro scale. Water Res. 41, 2271–2300. https://doi.org/10.1016/j.watres.2007.02.030

Oehmen, A., Saunders, A.M., Vives, M.T., Yuan, Z., Keller, J., 2006. Competition between polyphosphate and glycogen accumulating organisms in enhanced biological phosphorus removal systems with acetate and propionate as carbon sources. J. Biotechnol. 123, 22–32. https://doi.org/10.1016/j.jbiotec.2005.10.009

Olm, M.R., Brown, C.T., Brooks, B., Banfield, J.F., 2017. dRep : a tool for fast and accurate genomic comparisons that enables improved genome recovery from metagenomes through de-replication. ISME J. 11, 2864–2868. https://doi.org/10.1038/ismej.2017.126

Oshiki, M., Satoh, H., Mino, T., Onuki, M., 2008. PHA-accumulating microorganisms in full-scale wastewater treatment plants. Water Sci. Technol. 58, 13–20. https://doi.org/10.2166/wst.2008.652

Oyserman, B.O., Noguera, D.R., Del Rio, T.G., Tringe, S.G., McMahon, K.D., 2016. Metatranscriptomic insights on gene expression and regulatory controls in *Candidatus* Accumulibacter phosphatis. ISME J. 10, 810–822. https://doi.org/10.1038/ismej.2015.155

Park, S., Kim, D., Lee, J., Hur, H., 2014. *Sphaerotilus natans* encrusted with nanoball-shaped Fe(III) oxide minerals formed by nitrate-reducing mixotrophic Fe(II) oxidation. FEMS Microbiol. Ecol. 90, 68–77. https://doi.org/10.1111/1574-6941.12372

Parks, D.H., Rinke, C., Chuvochina, M., Chaumeil, P., Woodcroft, B.J., Evans, P.N., Hugenholtz, P., Tyson, G.W., 2017. Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life. Nat. Microbiol. 2. https://doi.org/10.1038/s41564-017-0012-7

Petriglieri, F., Nierychlo, M., Nielsen, P.H., Jon, S., Id, M., 2018. *In situ* visualisation of the abundant Chloroflexi populations in full-scale anaerobic digesters and the fate of immigrating species. PLoS One 13, e0206255. https://doi.org/https://doi.org/10.1371/journal.pone.0206255

Petriglieri, F., Petersen, J.F., Peces, M., Nierychlo, M., Hansen, K., Baastrand, C.E., Nielsen, U.G., Reitzel, K., Nielsen, P.H., 2020. Quantification of biologically and chemically bound phosphorus in activated sludge from EBPR plants. submitted.

Podosokorskaya, O.A., Bonch-Osmolovskaya, E.A., Novikov, A.A., Kolganova, T. V., Kublanov, I. V., 2013. *Ornatilinea apprima* gen. nov., sp. nov., a cellulolytic representative of the class Anaerolineae. Int. J. Syst. Evol. Microbiol. 63, 86–92. https://doi.org/10.1099/ijs.0.041012-0

Procopio, L., Alvarez, V.M., Jurelevicus, D.A., Hansen, L., Sørensen, S.J., Cardoso, J.S., Padula, M., Leitao, A.C., Seldin, L., van Elsas, J.D., 2012. Insight from the draft genome of *Dietzia cinnamea* P4 reveals mechanisms of survival in complex tropical soil habitats and biotechnology potential. Antonie Van Leeuwenhoek 101, 289–302. https://doi.org/10.1007/s10482-011-9633-7

Qin, J., Maixnerová, M., Nemec, M., Feng, Y., Zhang, X., Nemec, A., Zong, Z., 2019. *Acinetobacter cumulans* sp. nov., isolated from hospital sewage and capable of acquisition of multiple antibiotic resistance genes. Syst. Appl. Microbiol. 42, 319–325. https://doi.org/https://doi.org/10.1016/j.syapm.2019.02.001

Qiu, G., Zuniga-montanez, R., Law, Y., Swa, S., 2019. Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources. Water Res. 149, 469–510. https://doi.org/10.1016/j.watres.2018.11.011

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 41, 590–596. https://doi.org/10.1093/nar/gks1219

Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N.N., Anderson, I.J., Cheng, J.-F., Darling, A., Malfatti, S., Swan, B.K., Gies, E.A., Dodsworth, J.A., Hedlund, B.P., Tsiamis, G., Sievert, S.M., Liu, W.-T., Eisen, J.A., Hallam, S.J., Kyrpides, N.C., Stepanauskas, R., Rubin, E.M., Hugenholtz, P., Woyke, T., 2013. Insights into the phylogeny and coding potential of microbial dark matter. Nature 499, 431–437. https://doi.org/10.1038/nature12352

Rosselló-Mora, R.A., Wagner, M., Amann, R., Schleifer, K.H., 1995. The abundance of *Zoogloea ramigera* in sewage treatment plants. Appl. Environ. Microbiol. 61, 702 LP – 707.

RStudio Team, 2015. RStudio: Integrated Development Environment for R.

Salinero, K.K., Keller, K., Feil, W.S., Feil, H., Trong, S., Bartolo, G. Di, Lapidus, A., 2009. Metabolic analysis of the soil microbe *Dechloromonas aromatica* str . RCB anaerobic pathways for aromatic degradation. BMC Genomics 23, 1–23. https://doi.org/10.1186/1471-2164-10-351

Sanz, J.L., Ko, T., 2019. Next-generation sequencing and waste/wastewater treatment : a comprehensive overview. Ren Env. Sci Biotechnol 18, 635–680. https://doi.org/10.1007/s11157-019-09513-0

Saunders, A.M., Albertsen, M., Vollertsen, J., Nielsen, P.H., 2016. The activated sludge ecosystem contains a core community of abundant organisms. ISME J. 10, 11–20. https://doi.org/10.1038/ismej.2015.117

Saunders, A.M., Mabbett, A.N., Mcewan, A.G., Blackall, L.L., 2007. Proton motive force generation from stored polymers for the uptake of acetate under anaerobic conditions. FEMS Microbiol Lett. 274, 245-251. https://doi.org/10.1111/j.1574-6968.2007.00839.x

Schauer, M., Hahn, M.W., 2005. Diversity and Phylogenetic Affiliations of Morphologically Conspicuous Large Filamentous Bacteria Occurring in the Pelagic Zones of a Broad Spectrum of Freshwater Habitats. Appl. Environ. Microbiol. 71, 1931 LP – 1940. https://doi.org/10.1128/AEM.71.4.1931-1940.2005

Scheff, G., Salcher, O., Lingens, F., 1984. *Trichococcus flocculiformis* gen. nov. sp. nov. A new gram-positive filamentous bacterium isolated from bulking sludge. Appl. Microbiol. Biotechnol. 19, 114–119.

Schulze, R., Spring, S., Amann, R., Huber, I., Ludwigl, W., Schleiferl, K., Kampfer, P., 1999. Genotypic Diversity of *Acidovorax* Strains Isolated from Activated Sludge and Description of *Acidovorax defluvii* sp. nov. Syst. Appl. Microbiol. 205–214. https://doi.org/10.1016/S0723-2020(99)80067-8

Seemann, T., 2014. Prokka : rapid prokaryotic genome annotation. Bioinformatics 30, 2068–2069. https://doi.org/10.1093/bioinformatics/btu153

Sekiguchi, Y., Yamada, T., Hanada, S., Ohashi, A., Harada, H., Kamagata, Y., 2003. *Anaerolinea thermophila* gen. nov., sp. nov. and *Caldilinea aerophila* gen. nov., sp. nov., novel filamentous thermophiles that represent a previously uncultured lineage of the domain bacteria at the subphylum level. Int. J. Syst. Evol. Microbiol. 53, 1843–1851. https://doi.org/10.1099/ijs.0.02699-0

Seviour, E.M., Blackall, L.L., Christensson, C., Hugenholtz, P., Cunningham, M.A., 1997. The filamentous morphotype Eikelboom Type 1863 is not a single genetic entity. J. Appli 82, 411–421.

Seviour, R., Nielsen, P.H., 2010. Microbial Ecology of Activated Sludge. IWA Publishing.

Seviour, R.J., Mino, T., Onuki, M., 2003. The microbiology of biological phosphorus removal in activated sludge systems. FEMS Microbiol. Rev. 27, 99–127. https://doi.org/10.1016/S0168-6445(03)00021-4

Sezgin, M., Jenkins, D., Parker, D.S., 1978. A unified theory of filamentous activated sludge bulking. J. Water Pollut. Control Fed. 50, 362–381.

Singer, E., Wagner, M., Woyke, T., 2017. Capturing the genetic makeup of the active microbiome *in situ*. ISME J. 11, 1949–1963. https://doi.org/10.1038/ismej.2017.59

Singleton, C.M., Petriglieri, F., Kristensen, J.M., Kirkegaard, R.H., Michaelsen, T.Y., Andersen, M.H., Kondrotaite, Z., Karst, S.M., Dueholm, M.S., Nielsen, P.H., Albertsen, M., 2020. Connecting structure to function with the recovery of over 1000 high-quality activated sludge metagenome-assembled genomes encoding full-length rRNA genes using long-read sequencing. bioRxiv 2020.05.12.088096. https://doi.org/10.1101/2020.05.12.088096

Skennerton, C.T., Barr, J.J., Slater, F.R., Bond, P.L., Tyson, G.W., 2015. Expanding our view of genomic diversity in *Candidatus* Accumulibacter clades. Environ. Microbiol. n/a-n/a. https://doi.org/10.1111/1462-2920.12582

Smolders, G.J.F., Meij, J. Van Der, Loosdrecht, M.C.M. Van, Heijnen, J.J., 1994. Model of the Anaerobic Metabolism of the Biological Phosphorus Removal Process: Stoichiometry and pH Influence. Biotechnol. Bioeng. 43, 461–470.

Snaidr, J., Amann, R., Huber, I., Ludwig, W., Schleifer, K.H., 1997. Phylogenetic analysis and *in situ* identification of bacteria in activated sludge. Appl. Environ. Microbiol. 63, 2884 LP – 2896.

Snaidr, J., Beimfohr, C., Levantesi, C., Rossetti, S., Waarde, J. Van Der, Geurkink, B., Eikelboom, D., Lemaitre, M., Tandoi, V., Eurogentec, S.A., Sart-tilman, P.S., 2001. Phylogenetic analysis and in situ identification of "Nostocoida limicola" -like filamentous bacteria in activated sludge from industrial wastewater treatment plants. Water Sci. Technol. 46, 99–104.

Solden, L., Lloyd, K., Wrighton, K., 2016. The bright side of microbial dark matter: Lessons learned from the uncultivated majority. Curr. Opin. Microbiol. 31, 217–226. https://doi.org/10.1016/j.mib.2016.04.020

Song, Yizhi, Kaster, A., Vollmers, J., Song, Yanqing, Davison, P.A., Preston, G.M., Thompson, I.P., Murrell, J.C., Yin, H., Hunter, C.N., Huang, W.E., 2017. Single-cell genomics based on Raman sorting reveals novel carotenoid-containing bacteria in the Red Sea. Microb. Biotechnol. 10, 125–137.

https://doi.org/10.1111/1751-7915.12420

Soo, R.M., Skennerton, C.T., Sekiguchi, Y., Imelfort, M., Paech, S.J., Dennis, P.G., Steen, J.A., Parks, D.H., Tyson, G.W., Hugenholtz, P., 2014. An Expanded Genomic Representation of the Phylum Cyanobacteria. Genome Biol. Evol. 6, 1031–1045. https://doi.org/10.1093/gbe/evu073

Sorokin, D.Y., Lücker, S., Vejmelkova, D., Kostrikina, N.A., Kleerebezem, R., Rijpstra, W.I.C., Damsté, J.S.S., Le Paslier, D., Muyzer, G., Wagner, M., van Loosdrecht, M.C.M., Daims, H., 2012. Nitrification expanded: discovery, physiology and genomics of a nitrite-oxidizing bacterium from the phylum Chloroflexi. ISME J. 6, 2245–56. https://doi.org/10.1038/ismej.2012.70

Speirs, L., Nittami, T., McIlroy, S., Schroeder, S., Seviour, R.J., 2009. Filamentous bacterium Eikelboom Type 0092 in activated sludge plants in Australia is a member of the phylum chloroflexi. Appl. Environ. Microbiol. 75, 2446–2452. https://doi.org/10.1128/AEM.02310-08

Speirs, L.B.M., Dyson, Z.A., Tucci, J., Seviour, R.J., 2017. Eikelboom filamentous morphotypes 0675 and 0041 embrace members of the Chloroflexi : resolving their phylogeny , and design of fluorescence in situ hybridisation probes for their identification. FEMS Microbiol. Ecol. 93, 1–13. https://doi.org/10.1093/femsec/fix115

Speirs, L.B.M., McIlroy, S.J., Petrovski, S., Seviour, R.J., Seviour, R.J., 2011. The activated sludge bulking filament Eikelboom morphotype 0914 is a member of the Chloroflexi. Environ. Microbiol. Rep. 3, 159–65. https://doi.org/10.1111/j.1758-2229.2010.00201.x

Spring, S., Kampfer, P., Ludwig, W., Schleifer, K., Mikrobiologe, L., Universitiit, T., 1996. Polyphasic Characterization of the Genus *Leptothrix*: New Descriptions of *Leptothrix mobilis* sp. nov. and Emended Description of *Leptothrix cholodnii* emend. Syst. Appl. Microbiol. 19, 634–643. https://doi.org/10.1016/S0723-2020(96)80036-1

Spring, S., Wagner, M., Schumann, P., Ka, P., 2005. *Malikia granosa* gen. nov., sp. nov., a novel accumulating bacterium isolated from activated sludge, and reclassification of *Pseudomonas spinosa* as *Malikia spinosa* comb. nov. Int. J. Syst. Evol. Microbiol. 55, 621–629. https://doi.org/10.1099/ijs.0.63356-0

Stokholm-Bjerregaard, M., McIlroy, S.J., Nierychlo, M., Karst, S.M., Albertsen, M., Nielsen, P.H., 2017. A critical assessment of the microorganisms proposed to be important to enhanced biological phosphorus removal in full-scale wastewater treatment systems. Front. Microbiol. 8, 718.

https://doi.org/10.3389/FMICB.2017.00718

Sun, L., Toyonaga, M., Ohashi, A., Matsuura, N., Tourlousse, D.M., Meng, X.Y., Tamaki, H., Hanada, S., Cruz, R., Yamaguchi, T., Sekiguchi, Y., 2016. Isolation and characterization of *Flexilinea flocculi* gen. Nov., sp. Nov., a filamentous, anaerobic bacterium belonging to the class anaerolineae in the phylum chloroflexi. Int. J. Syst. Evol. Microbiol. 66, 988–996. https://doi.org/10.1099/ijsem.0.000822

Terashima, M., Yama, A., Sato, M., Yumoto, I., Kamagata, Y., Kato, S., 2016. Culture-Dependent and -Independent Identification of Polyphosphate-Accumulating *Dechloromonas* spp. Predominating in a Full-Scale Oxidation Ditch Wastewater Treatment Plant. Microbes Environ. Environ. 31, 449–455. https://doi.org/10.1264/jsme2.me16097

Tettelin, H., Nelson, K.E., Paulsen, I.T., Eisen, J.A., Read, T.D., Peterson, S., Heidelberg, J., DeBoy, R.T., Haft, D.H., Dodson, R.J., Durkin, A.S., Gwinn, M., Kolonay, J.F., Nelson, W.C., Peterson, J.D., Umayam, L.A., White, O., Salzberg, S.L., Lewis, M.R., Radune, D., Holtzapple, E., Khouri, H., Wolf, A.M., Utterback, T.R., Hansen, C.L., McDonald, L.A., Feldblyum, T. V, Angiuoli, S., Dickinson, T., Hickey, E.K., Holt, I.E., Loftus, B.J., Yang, F., Smith, H.O., Venter, J.C., Dougherty, B.A., Morrison, D.A., Hollingshead, S.K., Fraser, C.M., 2001. Complete Genome Sequence of a Virulent Isolate of *Streptococcus pneumoniae*. Science. 80, 293,498 LP – 506. https://doi.org/10.1126/science.1061217

Thomsen, T.R., Blackall, L.L., Muro, M.A. De, Nielsen, J.L., Nielsen, P.H., 2006. *Meganema perideroedes* gen. nov., sp. nov., a filamentous alphaproteobacterium from activated sludge. Int. J. Syst. Evol. Microbiol. 56, 1865–1868. https://doi.org/10.1099/ijs.0.02916-0

Tobin, K.M., McGrath, J.W., Mullan, A., Quinn, J.P., O'Connor, K.E., 2007. Polyphosphate Accumulation by *Pseudomonas putida* CA-3 and Other Medium-Chain-Length Polyhydroxyalkanoate-Accumulating Bacteria under Aerobic Growth Conditions. Appl. Environ. Microbiol. 73, 1383–1387. https://doi.org/10.1128/AEM.02007-06

Trebesius, K., Leitritz, L., Adler, K., Schubert, S., Autenrieth, I.B., Heesemann, J., 2000. Culture independent and rapid identification of bacterial pathogens in necrotising fasciitis and streptococcal toxic shock syndrome by fluorescence *in situ* hybridisation. Med. Microbiol. Immunol. 188, 169–175. https://doi.org/10.1007/s004300000035

Tu, Y., Schuler, A.J., 2013. Low acetate concentrations favor polyphosphate-

accumulating organisms over glycogen-accumulating organisms in enhanced biological phosphorus removal from wastewater. Environ. Sci. Technol. 47, 3816–3824. https://doi.org/10.1021/es304846s

Vallenet, D., Engelen, S., Mornico, D., Cruveiller, S., Fleury, L., Lajus, A., Rouy, Z., Roche, D., 2009. MicroScope : a platform for microbial genome annotation and comparative genomics. Data 2009, 1–12. https://doi.org/10.1093/database/bap021

van Loosdrecht, M.C., Nielsen, P.H., Lopez-Vazquez, C.M., Brdjanovic, D., 2016. Experimental Methods in Wastewater Treatment | IWA Publishing, Water Intelligence Online.

van Veen, W.L., van der Kooij, D., Geuze, E.C.W.A., van der Vlies, A.W., 1973. Investigations on the sheathed bacterium *Haliscomenobacter hydrossis* gen.n., sp.n., isolated from activated sludge. Antonie Van Leeuwenhoek 39, 207–216. https://doi.org/10.1007/BF02578853

Vervaeren, H., Wilde, K. De, Matthys, J., 2005. Quantification of an Eikelboom type 021N bulking event with fluorescence *in situ* hybridization and real-time PCR. Appl. Microbiol. Biotechnol. 68, 695–704. https://doi.org/10.1007/s00253-005-1963-9

Wagner, M., 2009. Single-Cell Ecophysiology of Microbes as Revealed by Raman Microspectroscopy or Secondary Ion Mass Spectrometry Imaging. Annu. Rev. Microbiol. 63, 411–429. https://doi.org/10.1146/annurev.micro.091208.073233

Wagner, M., Amann, R., Kampfer, P., Assmus, B., Hartmann, A., Hutzler, P., Springer, N., Schleifer, K.H., 1994. Identification and *in situ* detection of Gram-negative filamentous bacteria in activated sludge. Syst. Appl. Microbiol. 17, 405–417.

Wagner, Michael, Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D., Schleifer, K., Munich, D., Abfallwirtschaft, W., 1994. Development of an rRNA-Targeted Oligonucleotide Probe Specific for the Genus Acinetobacter and Its Application for *In Situ* Monitoring in Activated Sludge. Appl. Environ. Microbiol. 60, 792–800.

Wagner, M., Horn, M., Daims, H., 2003. Fluorescence *in situ* hybridisation for the identification and characterisation of prokaryotes. Curr. Opin. Microbiol. 6, 302–309. https://doi.org/10.1016/s1369-5274(03)00054-7

Wagner, M., Loy, A., 2002. Bacterial community composition and function in sewage treatment systems. Curr. Opin. Biotechnol. 13, 218–227.

https://doi.org/10.1016/S0958-1669(02)00315-4

Wagner, M., Nielsen, P.H., Loy, A., Nielsen, J.L., Daims, H., 2006. Linking microbial community structure with function: Fluorescence *in situ* hybridization-microautoradiography and isotope arrays. Curr. Opin. Biotechnol. 17, 83–91. https://doi.org/10.1016/j.copbio.2005.12.006

Wang, B., Jiao, E., Guo, Y., Zhang, L., Meng, Q., Zeng, W., Peng, Y., 2020. Investigation of the polyphosphate-accumulating organism population in the full-scale simultaneous chemical phosphorus removal system. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-020-09912-9

Wang, J., Qi, R., Liu, M., Li, Q., Bao, H., Li, Y., Wang, S., Tandoi, V., Yang, M., 2014. The potential role of '*Candidatus* Microthrix parvicella' in phosphorus removal during sludge bulking in two full-scale enhanced biological phosphorus removal plants. Water Sci. Technol. 70, 367–375. https://doi.org/10.2166/wst.2014.216

Ward, L.M., Hemp, J., Shih, P.M., Mcglynn, S.E., Fischer, W.W., 2018. Evolution of Phototrophy in the Chloroflexi Phylum Driven by Horizontal Gene Transfer. Front. Microbiol. 9, 1–16. https://doi.org/10.3389/fmicb.2018.00260

Weissbrodt, D.G., Lopez-vazquez, C.M., Welles, L., 2019. "*Candidatus* Accumulibacter delftensis": A clade IC novel polyphosphate-accumulating organism without denitrifying activity on nitrate. Water Res. 161, 136–151. https://doi.org/10.1016/j.watres.2019.03.053

Welles, L., Tian, W.D., Saad, S., Abbas, B., Lopez-Vazquez, C.M., Hooijmans, C.M., van Loosdrecht, M.C.M., Brdjanovic, D., 2015. Accumulibacter clades Type I and II performing kinetically different glycogen-accumulating organisms metabolisms for anaerobic substrate uptake. Water Res. 83, 354–366. https://doi.org/10.1016/j.watres.2015.06.045

Werner, J.J., Koren, O., Hugenholtz, P., Desantis, T.Z., Walters, W.A., Caporaso, J.G., Angenent, L.T., Knight, R., Ley, R.E., 2012. Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys. ISME J. 94–103. https://doi.org/10.1038/ismej.2011.82

Wickham, H., 2009. ggplot2 - Elegant Graphics for Data Analysis, Springer. Springer Science & Business Media. https://doi.org/10.1007/978-0-387-98141-3

Wiesmann, U., Choi, I., Dombrowski, E., 2007. Wastewater Characterization and Regulations, in: Fundamentals of Biological Wastewater Treatment. pp. 25–42.

Wilén, B.M., Onuki, M., Hermansson, M., Lumley, D., Mino, T., 2008. Microbial community structure in activated sludge floc analysed by fluorescence *in situ* hybridization and its relation to floc stability. Water Res. 42, 2300–2308. https://doi.org/10.1016/j.watres.2007.12.013

Williams, T.M., Unz, R.F., 1985. Filamentous Sulfur Bacteria of Activated Sludge : Characterization of *Thiothrix*, *Beggiatoa*, and Eikelboom Type 021N Strains. Appl. Environ. Microbiol. 49, 887–898.

Winterhalder, M.J., Zumbusch, A., 2015. Beyond the borders — Biomedical applications of non-linear Raman microscopy. Adv. Drug Deliv. Rev. 89, 135–144. https://doi.org/10.1016/j.addr.2015.04.024

Wolterink, A., Kim, S., Muusse, M., Kim, I.S., Roholl, P.J.M., Ginkel, C.G. Van, Stams, A.J.M., Kengen, W.M., 2005. *Dechloromonas hortensis* sp. nov. and strain ASK-1, two novel (per)chlorate-reducing bacteria, and taxonomic description of strain GR-1 1, 2063–2068. https://doi.org/10.1099/ijs.0.63404-0

Woodcroft, B.J., Singleton, C.M., Boyd, J.A., Evans, P.N., Emerson, J.B., Zayed, A.A.F., Hoelzle, R.D., Lamberton, T.O., Mccalley, C.K., Hodgkins, S.B., Wilson, R.M., Crill, P.M., Saleska, S.R., Purvine, S.O., Nicora, C.D., Li, C., Frolking, S., Jeffrey, P., Rich, V.I., Gene, W., 2018. Genome-centric view of carbon processing in thawing permafrost. Nature 560, 49–54. https://doi.org/10.1038/s41586-018-0338-1

Xia, Y., Kong, Y., Thomsen, T.R., Nielsen, P.H., 2008. Identification and Ecophysiological Characterization of Epiphytic Protein-Hydrolyzing Saprospiraceae ("*Candidatus* Epiflobacter" spp .) in Activated Sludge. Appl. Environ. Microbiol. 74, 2229–2238. https://doi.org/10.1128/AEM.02502-07

Yamada, T., Imachi, H., Ohashi, A., Harada, H., Hanada, S., Kamagata, Y., Sekiguchi, Y., 2007. *Bellilinea caldifistulae* gen. nov., sp. nov. and *Longilinea arvoryzae* gen. nov., sp. nov., strictly anaerobic, filamentous bacteria of the phylum Chloroflexi isolated from methanogenic propionate-degrading consortia. Int. J. Syst. Evol. Microbiol. 57, 2299–2306. https://doi.org/10.1099/ijs.0.65098-0

Yamada, T., Sekiguchi, Y., 2009. Cultivation of Uncultured Chloroflexi Subphyla: Significance and Ecophysiology of Formerly Uncultured Chloroflexi "Subphylum I" with Natural and Biotechnological Relevance. Microbes Environ. 24, 205–216. https://doi.org/10.1264/jsme2.ME09151S

Yamada, T., Sekiguchi, Y., Hanada, S., Imachi, H., Ohashi, A., Harada, H., Kamagata, Y., 2006. *Anaerolinea thermolimosa* sp. nov., *Levilinea saccharolytica* gen. nov., sp. nov. and *Leptolinea tardivitalis* gen. nov., sp. nov., novel filamentous

anaerobes, and description of the new classes Anaerolineae classis nov. and Caldilineae classis nov. in the . Int. J. Syst. Evol. Microbiol. 56, 1331–1340. https://doi.org/10.1099/ijs.0.64169-0

Yang, H., Irudayaraj, J., 2003. Rapid detection of foodborne microorganisms on food surface using Fourier transform Raman spectroscopy. J. Mol. Struct. 646, 35–43. https://doi.org/10.1016/S0022-2860(02)00575-6

Yilmaz, L.S., Parnerkar, S., Noguera, D.R., 2011. MathFISH, a web tool that uses thermodynamics-based mathematical models for *in silico* evaluation of oligonucleotide probes for fluorescence *in situ* hybridization. Appl. Environ. Microbiol. 77, 1118–1122. https://doi.org/10.1128/AEM.01733-10

Yuan, Y., Liu, J., Ma, B., Liu, Y., Wang, B., Peng, Y., 2016. Improving municipal wastewater nitrogen and phosphorous removal by feeding sludge fermentation products to sequencing batch reactor (SBR). Bioresour. Technol. 222, 326–334. https://doi.org/10.1016/j.biortech.2016.09.103

Yuan, Z., Pratt, S., Batstone, D.J., 2012. Phosphorus recovery from wastewater through microbial processes. Curr. Opin. Biotechnol. 23, 878–83.

Zhang, H., Sekiguchi, Y., Hanada, S., Hugenholtz, P., Kim, H., Kamagata, Y., Nakamura, K., 2003. *Gemmatimonas aurantiaca* gen. nov., sp. nov., a Gram-negative, aerobic, polyphosphate-accumulating micro-organism , the first cultured representative of the new bacterial phylum Gemmatimonadetes phyl. nov. Int. J. Syst. Evol. Microbiol. 53, 1155–1163. https://doi.org/10.1099/ijs.0.02520-0

Zhao, Y., Liu, R., Zhao, J., Xu, L., Sibille, C., 2017. A fancy eco-compatible wastewater treatment system: Green Bio-sorption Reactor. Bioresour. Technol. 234, 224–232. https://doi.org/10.1016/j.biortech.2017.03.037

Zhou, Y., Pijuan, M., Zeng, R.J., Lu, H., Ã, Z.Y., 2008. Could polyphosphate-accumulating organisms (PAOs) be glycogen-accumulating organisms (GAOs)? Water Res. 42, 2361–2368. https://doi.org/10.1016/j.watres.2008.01.003

CHAPTER 2. PAPER 1

"*Candidatus* Dechloromonas phosphatis" and "*Candidatus* Dechloromonas phosphovorax", two novel polyphosphate accumulating organisms abundant in wastewater treatment systems

F. Petriglieri, C. M. Singleton, M. Peces, J. F. Petersen, M. Nierychlo, P. H. Nielsen.

Preprint on BioRxiv (for submission to ISME Journal)

CHAPTER 3. PAPER 2

Quantification of biologically and chemically bound phosphorus in activated sludge from full-scale plants with biological P-removal

Francesca Petriglieri, J. F. Petersen, M. Peces, M. Nierychlo, K. Hansen, U. G. Nielsen, K. Reitzel, P. H. Nielsen

Manuscript (for submission to Water Reasearch)

CHAPTER 4. PAPER 3

Connecting structure to function with the recovery of over 1000 high-quality activated sludge metagenome-assembled genomes encoding full-length rRNA genes using long-read sequencing

C.M. Singleton, **F. Petriglieri**, J.M. Kristensen, R.H. Kirkegaard, T.Y. Michaelsen, M.H Andersen, Z. Kondrotaite, S.M. Karst, M.S. Dueholm, P.H. Nielsen, M. Albertsen.

Preprint on BioRxiv

CHAPTER 5. PAPER 4

The morphology and metabolic potential of the Chloroflexi in full-scale activated sludge wastewater treatment plants

M. Nierychlo, A. Miłobędzka, **F. Petriglieri**, B. McIlroy, P.H. Nielsen, S.J. McIlroy.

Published in FEMS

CHAPTER 6. PAPER 5

A comprehensive overview of the Chloroflexi community in Danish and global wastewater treatment plant

F. Petriglieri, Z. Kondrotaite, M. Nierychlo, M. S. Dueholm, P. H. Nielsen.

Manuscript

ISSN (online): 2446-1636
ISBN (online): 978-87-7210-693-9

AALBORG UNIVERSITY PRESS