An Investigation of Polyphosphate Accumulating Organisms in Lab and Full-Scale Wastewater Treatment Processes

By

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List of Abbreviations

A/O	Anaerobic-Aerobic
A2O	Anaerobic-Anoxic-Aerobic
ANI	Average Nucleotide Identity
AOB	Ammonia-Oxidizing Bacteria
ASVs	Amplicon Sequence Variants
BNR	Biological Nutrient Removal
DAPI	4′,6-Diamidino-2-Phenylindole
DDH	DNA-DNA Hybridization
EBPR	Enhanced Biological Phosphorus Removal
ffCOD	Flocculated and Filtered COD
GAO	Glycogen-Accumulating Organism
GBDP	Genome BLAST Distance Phylogeny
HAc	Acetate
HPLC	High Performance Liquid Chromatography
HPr	Propionate
HRT	Hydraulic Retention Time
ITS	Internal Transcribed Spacer
MIS	Microbial Identification System
MLE	Modified Ludzak-Ettinger
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
MSV Medium	Mineral Salts-Vitamin Medium
N	Nitrogen
NOB	Nitrite-Oxidizing Bacteria
NSTI	Nearest-Sequenced Taxon Index
OD	Optical Density
P	Phosphorus
PAO	Polyphosphate-Accumulating Organism
PCA	Principle Component Analysis
PCoA	Principle Coordinate Analysis
pCOD	Particulate COD
PHA	Polyhydroxyalkanoates
Pi	Orthophosphate
poly-P	Polyphosphate

PSD	Particle Size Distribution
qFISH	Quantitative Fluorescence In Situ Hybridization
qPCR	Quantitative Real-Time Polymerase Chain Reaction
RAS	Return Activated Sludge
SF	San Francisco
SRT	Sludge Retention Time
SVI	Sludge Volume Index
TCA	Tricarboxylic Acid
TIN	Total Inorganic Nitrogen
VFA	Volatile Fatty Acid
WWTP	Wastewater Treatment Plant

Abstract

Enhanced biological phosphorus removal (EBPR) process is an economic and sustainable approach for phosphorus (P) removal from municipal wastewater. A typical EBPR process is composed of an alternating anaerobic-aerobic zone which selectively enriches a group of phosphorus removing bacteria named polyphosphate-accumulating organisms (PAOs). While *Candidatus* Accumulibacter has been widely recognized as a model PAO in the past, recent studies have identified a diverse group of putative PAOs in activated sludge systems that may also contribute to P removal from wastewater. Among these putative PAOs, *Tetrasphaera* is the most promising group, and has been identified with high abundance in wastewater treatment plants (WWTPs) worldwide. However, current knowledge about these novel PAOs is limited. To utilize the full benefits of the EBPR process, a thorough understanding of the microbiology and characteristics of these putative PAOs is needed.

This dissertation investigated the diversity and kinetics of putative PAOs in both lab-scale and full-scale wastewater treatment processes with a focus on *Tetrasphaera*. In Chapter 2, a detailed literature review was conducted to systematically evaluate the identification history of *Tetrasphaera* genus and summarize our current knowledge about this important PAO. Chapter 3 investigated important biokinetic parameters of *Tetrasphaera elongata* in lab-scale pure cultures. The specific growth rate determined in this study (1.37-1.42 d^{-1}) appeared to be slightly higher than typical values for other PAOs: 0.12-1.0 d^{-1}. The specific anaerobic carbon uptake rates with glucose, acetate and gluconate were 0.0421, 0.0181 and 0.0159 C-mol·C-mol biomass^{-1}·h^{-1}, respectively. This data suggests that the carbon uptake capacity of *Tetrasphaera* is dependent on

substrate type. In Chapter 4, a field study was conducted to investigate the nutrient removal rates and microbial community structure (e.g., PAOs and the core community) in seven full-scale WWTPs with unintended P removal in the San Francisco Bay Area. The nutrient removal rates in the surveyed facilities appeared to be lower than values seen elsewhere that operate in EBPR mode. A wide variety of PAOs were identified in all seven WWTPs surveyed, even in those facilities with little or no observed P-removal activity. Those facilities surveyed that operate with a pure oxygen aeration configuration was found to significantly impact community diversity, functional gene profile, and the core community structure of activated sludge systems.

Overall, this research systematically evaluated the activity of putative PAOs in both lab-scale and full-scale conditions and the results observed may contribute to the design, modeling, and optimization of EBPR processes.

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Chapter 1. Introduction

Phosphorus (P) is a key macro-nutrient for life. However, there is a balance in P levels within the environment, whereby excessive P loading to the environment has caused eutrophication in surface waters worldwide (Conley et al. 2009; Schindler et al. 2016; Smith and Schindler 2009). For perspective, coastal waters around the world representing approximately 1.15 million km^2 have eutrophic potential (Maúre et al. 2021). Furthermore, it is estimated that 48 percent of lakes and reservoirs in North America are eutrophic (Cai, Park, and Li 2013).

Such elevated P levels in surface waters can be caused by both point source and non-point source pollution. Wastewater treatment plants (WWTPs) are thought to be the major contributor of point source loads (A Drolc and Zagorc Koncan 2002). In an effort to reduce P loading in surface waters, the Enhanced Biological Phosphorus Removal (EBPR) process was invented as an economic and sustainable approach for P removal (Barnard 1975). The traditional EBPR process comprises an anaerobic zone, followed by an aerobic zone. This configuration selects for microorganisms with high P removal capacity (Oehmen et al. 2007; Seviour, Mino, and Onuki 2003). The microorganisms that perform this function are referred to as polyphosphate-accumulating organisms (PAOs) as they can take up more orthophosphate (Pi) than their metabolic demand and store it as intracellular polyphosphate (poly-P) granules (He and McMahon 2011; Mino, van Loosdrecht, and Heijnen 1998).

Although the EBPR process has been studied for almost 50 years (Barnard 1975), previous research has mainly focused on *'Candidatus* Accumulibacter phosphatis' (referred to as *Accumulibacter* hereafter) as the only PAO in the EBPR process (He and McMahon 2011;

Oehmen et al. 2007). Through decades of investigation, the metabolisms of *Accumulibacter* are well established and have been widely accepted (Martín et al. 2006; Smolders et al. 1994; Smolders, van Loosdrecht, and Heijnen 1995). As a result, current EBPR processes at WWTPs are mainly designed and modeled based on the metabolisms and kinetics of *Accumulibacter* (Oehmen et al. 2005; Whang, Filipe, and Park 2007).

Recent studies have shown that in addition to *Accumulibacter*, there are a diverse group of PAOs in the EBPR processes that may also contribute to P removal (Fernando et al. 2019; Nielsen et al. 2019; Wang and He 2020). Preliminary research has shown that these putative PAOs are significantly different from *Accumulibacter* in both carbon substrate and metabolism (Kristiansen et al. 2013; Marques et al. 2017; Wang and He 2020). The presence of this diverse group of PAOs is beneficial to the overall EBPR performance as the design and modeling of wastewater treatment is no longer limited by the activity of *Accumulibacter.* Before the professional community can harness the benefits of these putative PAOs, expanding our fundamental knowledge of their kinetics and metabolism in activated sludge systems is essential.

This dissertation aims to understand the kinetics and functional roles of novel PAOs in both lab-scale and full-scale WWTPs. Specifically, this dissertation focuses on *Tetrasphaera* as it has been identified at high abundances (e.g., >10% biovolume) in many EBPR systems worldwide and is considered to be an important PAO (Liu et al. 2019; Marques et al. 2017; Nielsen et al. 2019; Singleton et al. 2022; Zhang and Kinyua 2020). To date, *Tetrasphaera* and *Accumulibacter* are the only known groups that have been consistently found in high abundances in EBPR systems (Nielsen et al. 2019). However, knowledge about the kinetics, metabolism, and ecological roles of *Tetrasphaera* is still limited, which limits the application of this important PAO in the EBPR

process. This dissertation aims to address these knowledge gaps by resolving three key research objectives:

- Objective 1 (Chapter 2): Provide a historical review of the taxonomy of *Tetrasphaera* and discuss opportunities and challenges in the application of the *Tetrasphaera* genus in the EBPR process.
- Objective 2 (Chapter 3): Measure the kinetic parameters (e.g., specific growth rate and carbon uptake rate) of *Tetrasphaera* in lab-scale pure cultures.
- Objective 3 (Chapter 4): Investigate the activity of PAO communities in facilities with unintended P removal processes in the San Francisco (SF) Bay area.

This research systematically evaluated the growth kinetics and carbon uptake rate of *Tetrasphaera and* evaluated the PAO communities in WWTPs in the San Francisco (SF) Bay Area. This research contributes to the body of literature on a promising PAO, and the results observed may contribute to the design, modeling, and optimization of EBPR processes.

Chapter 2. Literature Review of *Tetrasphaera* in the Enhanced Biological Phosphorus Removal Process[1]

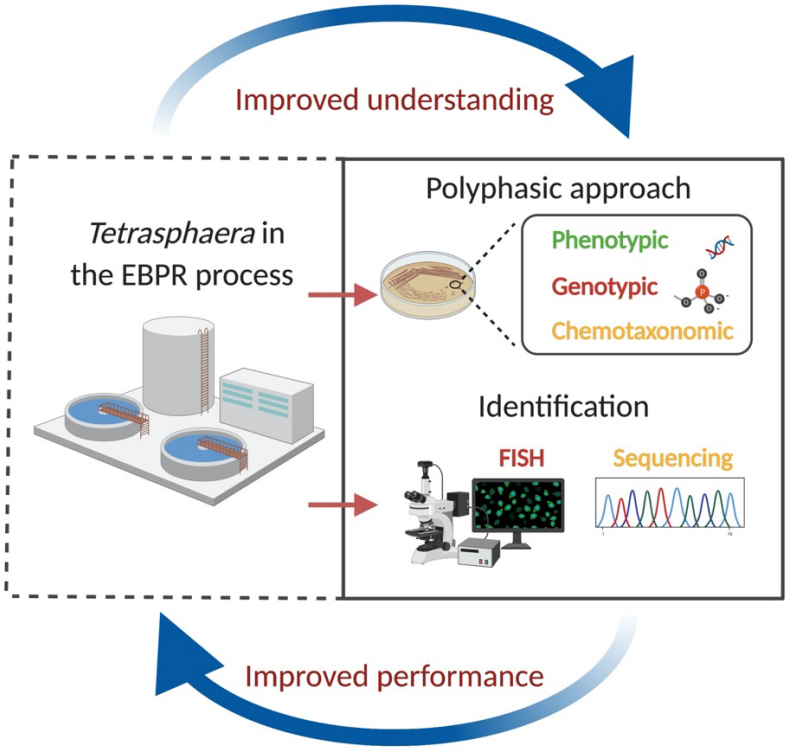
Abstract

Tetrasphaera are putative group of polyphosphate accumulating organisms (PAOs) that may play an important role in the Enhanced Biological Phosphorus Removal (EBPR) process. Unlike conventional PAOs that mostly rely on volatile fatty acids (VFAs), *Tetrasphaera* can assimilate a wide range of carbon substrates including glucose and various amino acids (e.g., glycine, glutamate, and aspartate). In addition, they have the ability to produce VFAs. This functional versatility confers significant performance and economic benefits for its application in EBPR processes. Before the wastewater industry can leverage such benefits, fundamental knowledge of their taxonomy, classification, and identification in EBPR and activated sludge systems must be obtained.

This review provides a summary of the polyphasic approach: use of phenotypic, genotypic, and chemotaxonomic methods for bacterial classification and its application in the classification of *Tetrasphaera*. A review of molecular tools currently used to identify *Tetrasphaera* in activated sludge systems is also provided. In addition, this review discusses how challenges with molecular tools limit our understanding and application of *Tetrasphaera* in EBPR processes.

[1] Adapted from Zhang, Yihan, and Maureen N. Kinyua. "Identification and classification of the Tetrasphaera genus in enhanced biological phosphorus removal process: a review." *Reviews in Environmental Science and Bio/Technology* 19.4 (2020): 699-715.

Graphic Abstract



2.1. Introduction

The enhanced biological phosphorus removal (EBPR) process has been widely implemented in wastewater treatment plants (WWTPs) as an economic and sustainable approach for phosphorus removal (Barnard 1975; Nielsen et al. 2019). The EBPR process relies on the group of microorganisms termed as polyphosphate accumulating organisms (PAOs), which have the ability to take up excessive orthophosphate from wastewater and store it as intracellular polyphosphate (poly-P) granules (Mino et al. 1998). Besides the most well-known PAO *Candidatus* Accumulibacter, there are a variety of promising putative PAOs that have been

identified in full-scale EBPR processes (e.g., *Candidatus* Halomonas phosphatis and *Tessaracoccus* (Nguyen, Nielsen, and Nielsen 2012).

This review specifically focuses on *Tetrasphaera* because: (1) they are consistently found in high abundance (1.3-11.9%) in EBPR processes (Nielsen et al. 2019), (2) their utilization of diverse carbon sources and ability to produce volatile fatty acids (VFAs) has the potential to reduce chemical costs for treatment plants (Barnard, Dunlap, and Steichen 2017; Marques et al. 2017), and (3) they have the potential for simultaneous denitrification and phosphorus removal (Kristiansen et al. 2013; Marques et al. 2018).

Tetrasphaera is a bacterial genus that belongs to the *Intrasporangiaceae* family within the Actinobacteria class and contains eight proposed species (Nguyen et al. 2011). Specifically, *Tetrasphaera japonica*, *Tetrasphaera australiensis*, *Tetrasphaera elongata*, *Tetrasphaera jenkinsii*, *Tetrasphaera vanveenii*, *Tetrasphaera veronensis*, *Tetrasphaera duodecadis* and *Tetrasphaera remsis* (Hanada et al. 2002; Ishikawa and Yokota 2006; Maszenan et al. 2000; McKenzie et al. 2006; Onda and Takii 2002; Osman et al. 2007). *Tetrasphaera* have the ability to store glycogen or intracellular amino acids from glucose and amino acids (Kong, Nielsen, and Nielsen 2005; Kristiansen et al. 2013; Nguyen et al. 2011, 2015) and have been regarded as a promising putative PAO group for the EBPR process (Barnard et al. 2017; Nielsen et al. 2019; Onnis-Hayden et al. 2020; Stokholm-Bjerregaard et al. 2017). However, to the best of our knowledge, only *T. elongata* has demonstrated archetypical PAO characteristics. Anaerobic phosphorus release and aerobic/anoxic uptake patterns for the other species as well as their ability to accumulate intracellular poly-P granules remain inconclusive (Kristiansen et al. 2013; Nguyen et al. 2015; Onda and Takii 2002).

The aim of this review is to provide a detailed summary of the molecular tools that have been used to improve our current understanding of *Tetrasphaera* in EBPR processes. Since the metabolic characteristics that confer *Tetrasphaera*'s application in EBPR processes are provided elsewhere (Liu et al. 2019); this review describes the polyphasic approach to bacterium taxonomic classification, including a discussion of the advantages and disadvantages of specific genotypic, chemotaxonomic and phenotypic methods. Next, a historical review of the application of the polyphasic approach in the determination of *Tetrasphaera* taxonomic position is provided along with a discussion of the application of molecular methods for the identification of *Tetrasphaera* and their metabolites in EBPR processes. Lastly, a discussion of how challenges with molecular tools limit our understanding and application of *Tetrasphaera* in EBPR processes is provided.

2.2. Polyphasic approach to bacterial classification

The polyphasic approach refers to the use of phenotypic, genotypic, and chemotaxonomic methods to characterize a bacterium and determine its taxonomic status (Colwell 1970; Schleifer 2009; Vandamme et al. 1996). This approach is attractive because it incorporates different types of information of a bacterium and determines its taxonomy based on a consensus of data (Chun and Rainey 2014; Varghese et al. 2015). In this section, we discuss the characteristics, advantages and disadvantages of genotypic, chemotaxonomic and phenotypic methods used to determine the morphological, biochemical and molecular characteristics of bacteria.

2.2.1. Genotypic methods

Genotypic methods use information derived from genetic material and genomes (i.e., genotypic characteristics) of a bacterium for classification (Kämpfer and Glaeser 2012). These

methods include DNA-DNA hybridization (DDH), 16S rRNA gene analysis, and DNA G+C content analysis (Tindall et al. 2010; Xu and Côté 2003). Whole-genome sequence analysis is also discussed because it has begun to play a significant role in bacterial genotypic characterization (Henz et al. 2005; Nouioui et al. 2018; Varghese et al. 2015). Although 16S-23S rDNA internal transcribed spacer (ITS) gene analysis and rpoC1 gene analysis are genotypic methods that supplement 16S rRNA gene analysis (Rocap et al. 2002; Toledo and Palenik 1997), they will not be discussed here.

2.2.1.1. DNA-DNA hybridization

DNA-DNA hybridization (DDH) method, also known as DNA-DNA re-association method was developed in the 1960s and measures the overall genetic relatedness between microorganisms based on their phylogenetic relationships (Goris et al. 2007; Rosselló-Mora 2006). In DDH analysis, denatured single-strand DNA molecules from two microorganisms are mixed and hybridized to yield their degree of relatedness (Rosselló-Mora 2001). If bacterial strains have a DDH value ≥ 70%, they are classified as belonging to the same species (Kim et al. 2014; Tindall et al. 2010). This artificial threshold was recommended by Wayne et al. (1987) based on studies that compared DDH values between well-defined species. Before the advent of 16S rRNA gene analysis, the DDH method was regarded as the "gold standard" for bacterial species delineation (Kim et al. 2014; Rosselló-Mora 2006; Tindall et al. 2010). However, this technique is tedious, error-prone, and cannot be used to build a comparative database that allows for easy comparisons between microorganisms (Auch et al. 2010), thus limiting its application. The DDH method was gradually replaced by the 16S rRNA gene analysis, in which a 70% DDH threshold corresponds to 97% 16S rRNA gene similarity (Rosselló-Mora 2001, 2006; Tindall et al. 2010). However, the 16S rRNA gene analysis alone is not always sufficient to guarantee the accuracy of species-level

identification because two species with a high degree of 16S rRNA gene similarity (>99%) may have distinct DDH values (Fox, Wisotzkey, and Jurtshuk 1992). Therefore, the 97% 16S rRNA gene similarity serves as the first indicator of a single species, and the 70% DDH value serves as the ultimate threshold to determine species boundary (Klenk and Göker 2010; Tindall et al. 2010).

2.2.1.2. DNA G+C content

The DNA G+C content, expressed as the percent ratio of guanine (G) and cytosine (C) to the overall number of DNA nucleotides in a genome, is an important parameter for taxonomic determination of species and genera (Meier-Kolthoff, Klenk, and Göker 2014; Nouioui et al. 2018). The G+C content of bacteria varies between 24% and 76% (Vandamme et al. 1996). Conventional techniques such as high performance liquid chromatography (HPLC) and quantitative real-time polymerase chain reaction (qPCR), indirectly estimate the genomic DNA G+C content of a bacterium based on its physical properties (Meier-Kolthoff et al. 2014; Mesbah, Premachandran, and Whitman 1989; Mesbah, Whitman, and Mesbah 2011). Whole-genome sequencing has also been utilized as a more accurate method to directly quantify the G+C content of microorganisms (Meier-Kolthoff et al. 2014). Variation of G+C content within a species is considered within 1 % using whole-genome sequencing (Meier-Kolthoff et al. 2014; Nouioui et al. 2018). While differences in G+C content serve as taxonomic markers for species, similarities in G+C content does not necessarily indicate a close relationship between microorganisms (Rosselló-Mora 2001). If the difference between the G+C contents of two microorganisms is > 1%, it is assumed they belong to different species. However, a variance of < 1% does not mean the microorganisms belong to the same species.

2.2.1.3. 16S rRNA gene analysis

The 16S rRNA gene is the standard taxonomic marker for bacterial classification because it is ubiquitous among bacteria and different regions of the gene evolve at different rates, which allows for the identification of both ancient lineages (e.g., domains) and modern lineages (e.g., genera) (Schleifer 2009; Woese 1987; Yarza et al. 2014). In taxonomic identifications, full-length 16S rRNA gene sequences from different microorganisms are used to calculate pairwise sequence similarities or for phylogenetic analyses (Kim and Chun 2014). While the 97% similarity was widely used as a species boundary, a higher threshold: 98.7-99.0% is now accepted as the new species boundary based on recent studies on large and comprehensive datasets (Kim et al. 2014; Stackebrandt and Ebers 2006). For higher taxa, a similarity threshold of 94.5% is proposed as the genus boundary, 86.5% for family, 82.0% for order, 78.5% for class, and 75.0% as the phylum boundary (Yarza et al. 2014). Since the new species threshold was only revised recently, studies related to the identification of *Tetrasphaera* used the 97% similarity as the species boundary. The 16S rRNA gene analysis is used alongside DDH and G+C content analyses for better classification (Rosselló-Mora 2001) because its reliance on a few thousand nucleotides means it often lacks enough resolution to distinguish between closely related species (Nouioui et al. 2018; Poretsky et al. 2014; Rosselló-Mora 2001).

2.2.1.4. Whole-genome sequence analysis

Unlike 16S rRNA gene analysis, the whole-genome sequence analysis has the ability to capture the genome-wide divergence between two microbial strains (Chan et al. 2012). This leads to two major applications in taxonomic identification: (1) calculation of the average nucleotide identity (ANI) between two genomes, and (2) construction of a genome BLAST distance phylogeny (GBDP) between microorganisms (Chun and Rainey 2014). ANI represents the

similarity between the homologous genomic regions shared by any two strains (Konstantinidis, Ramette, and Tiedje 2006). A 95-96% ANI value corresponds to a 70% DDH value making whole-genome sequencing an attractive replacement to the otherwise tedious DDH method for species delineation (Goris et al. 2007; Kim et al. 2014; Richter and Rosselló-Móra 2009). While the ANI analysis evaluates the genomic similarity between strains, the GBDP algorithm calculates the phylogenic distance between microorganisms using whole-genome sequences (Henz et al. 2005). Similar to 16S rRNA gene analysis, in the GBDP analysis, genome sequences from different microorganisms are aligned, a distance matrix is calculated, then a phylogenetic tree is constructed to represent the phylogenetic relationships between microorganisms (Chun and Rainey 2014; Henz et al. 2005). However, for species delineation, the whole-genome sequence analysis provides better resolving power than the 16S rRNA gene analysis, because it calculates the whole genomic distance instead of the divergence of a single gene (Chun and Rainey 2014; Klenk and Göker 2010; Varghese et al. 2015). Although the whole-genome sequence analysis is considered part of the polyphasic approach, some studies have used it as the sole criterion for taxonomic identifications (Breider et al. 2014; Nouioui et al. 2018). Their argument has been that the phenotypic and chemotaxonomic properties of a microorganism are based on its genomic information (Chan et al. 2012). However, other studies have argued that while the whole-genome sequence analysis should be regarded as the primary guide for species delineation, other phenotypic and chemotaxonomic information must also be considered (Ramasamy et al. 2014; Varghese et al. 2015).

2.2.2. Chemotaxonomic characteristics

Introduction of chemotaxonomy in the 1960s was a major milestone in bacterial classification (Schleifer 2009; Vandamme et al. 1996). Chemotaxonomic properties of a microorganism refer to the chemical composition of its cell structures: the cell wall (i.e.,

peptidoglycan), the cell membrane (e.g., fatty acids and lipids) and the cytoplasm (e.g., polyamines) (Tindall et al. 2010). In particular, the cell wall peptidoglycan structure, polar lipids composition, cellular fatty acids profile, and menaquinone composition are the major chemotaxonomic parameters used for bacterial classification.

2.2.2.1. Cell wall peptidoglycan

The cell wall of both Gram-positive and Gram-negative bacteria contains peptidoglycan also known as murein (Schleifer and Stackebrandt 1983). Peptidoglycan is composed of linear glycan strands cross-linked through short peptides and is responsible for the rigidity and shape of bacterial cells (Schleifer and Stackebrandt 1983; Schumann 2011). While there is a great deal of variability in the peptidoglycan structure of Gram-positive bacteria, the structure is remarkably uniform among Gram-negative bacteria, hence its application as a chemotaxonomic marker is restricted to Gram-positive bacteria (Schleifer and Kandler 1972). The peptidoglycan analysis compares the (1) identity of diamino acid in the cross-linking peptide, (2) type of peptidoglycan, (3) type of cross-linkage and (4) complete amino acid composition (Tindall et al. 2010). The amino acid composition in the peptide side chain and the identity of diamino acid are usually consistent among all species within a genus making this a useful criterion to determine whether a novel species can be classified into a recognized genus (Tindall et al. 2010). While closely related bacteria usually possess the same peptidoglycan structure, peptidoglycan analysis is not a rigid criterion to differentiate bacteria because some microorganisms that belong to a single genus or species can exhibit a high degree of variations in the mode of cross-linkage between the peptide side chains (Schleifer 1985; Tindall et al. 2010) .

2.2.2.2. Cellular fatty acid profile

Cellular fatty acid profile is a valuable tool in the species delineation and taxonomy identification (Imhoff and Bias-lmhoff 2006; Rosselló-Mora 2001). Fatty acids are usually linked to glycerol through an ester bond and are rarely present in free form (Imhoff and Bias-lmhoff 1995). In bacteria, fatty acids have varying chain lengths from C_2 to over C_{90} (Barka et al. 2016). However, only those chain lengths between C_{10} and C_{20} are of taxonomic significance because they are widely present across bacteria and provide the greatest taxonomic information (Shaw 1974). The Sherlock Microbial Identification System (MIS) has been widely used to identify cellular fatty acids contents of bacteria (Tindall et al. 2010). It provides both standard procedures and reference libraries that allows comparisons between unknown strains and reference strains (Sasser 1990). The use of the Sherlock MIS has aided the identification of *Tetrasphaera* species including *T. duodecadis* and *T. remsis* (Ishikawa and Yokota 2006; Osman et al. 2007). The members within a single taxonomic group usually have similar patterns of fatty acids and the occurrence of branched and unsaturated fatty acids, as well as the presence or absence of hydroxylated fatty acids are generally regarded as unique characteristics (Tindall et al. 2010). However, the resolution of this technique is dependent on the specific group of bacteria. For some genera, cellular fatty acid profile can be used to differentiate different species, while for others there is no variation in fatty acid compositions among species (Welch 1991). Therefore, the cellular fatty acid analysis is not a rigid criterion for bacteria classification.

2.2.2.3. Cell membrane composition

Polar lipids composition: Polar lipids are amphipathic molecules that are essential for the formation of cellular membranes in all prokaryotes (Schleifer and Stackebrandt 1983). Bacteria contain a variety of polar lipids, of which phospholipids (PL), glycolipids (GL) and

glycophospholipids (GP), aminolipids (AL), and sulfolipids (SL) are the most common classes (da Costa et al. 2011). The specific pattern of polar lipids is an important taxonomic marker to distinguish bacteria (Rosselló-Mora 2001; Schleifer and Stackebrandt 1983; Vandamme et al. 1996). For instance, bacteria belonging to the same genus within the phylum Actinobacteria possess the same type of phospholipids (Barka et al. 2016), which is a useful criterion when assigning new species to a specific genus of Actinobacteria.

Isoprenoid quinone: These are components of cytoplasmic membranes and play an important role in electron transport and oxidative phosphorylation (Schleifer and Stackebrandt 1983; Vandamme et al. 1996). Quinones are divided into several types, of which menaquinones (MK) are commonly found in actinomycete cell envelops and ubiquinones are present in all known purple nonsulfur bacteria (Barka et al. 2016; Imhoff and Bias-lmhoff 1995). The variations of quinones in the ring structure and in the side chain can be used as a taxonomic marker to differentiate bacteria at different levels (Collins and Jones 1981). Species of the same genus generally have identical quinone compositions (Imhoff and Bias-lmhoff 1995).

2.2.3. Phenotypic characteristics

The phenotypic properties of a microorganism refer to its morphological (e.g., cell size and shape, colony morphology, endospore, and flagella) and physiological features (e.g., pH, temperature, and nutrients requirement) (Schleifer 2009; Tindall et al. 2010; Vandamme et al. 1996). In the late 19th century, phenotypic properties were the only basis for bacterial classification (Ramasamy et al. 2014), however, numerical taxonomy was invented to compare and analyze phenotypic traits among large groups of microorganisms (Schlee et al. 1975). In numerical taxonomy, each phenotypic trait is weighted equally and the coefficients of phenotypic similarities between microorganisms are calculated (Schleifer 2009). Unfortunately, changes in

phenotypic properties based on growth conditions and difficulties in standardizing techniques decreases the reliability of phenotypic properties as the sole method to classify microorganisms (Chan et al. 2012; Schleifer and Stackebrandt 1983). Therefore, for the polyphasic approach, phenotypic properties are used in conjunction with chemotaxonomic and genotypic data for bacterial classification.

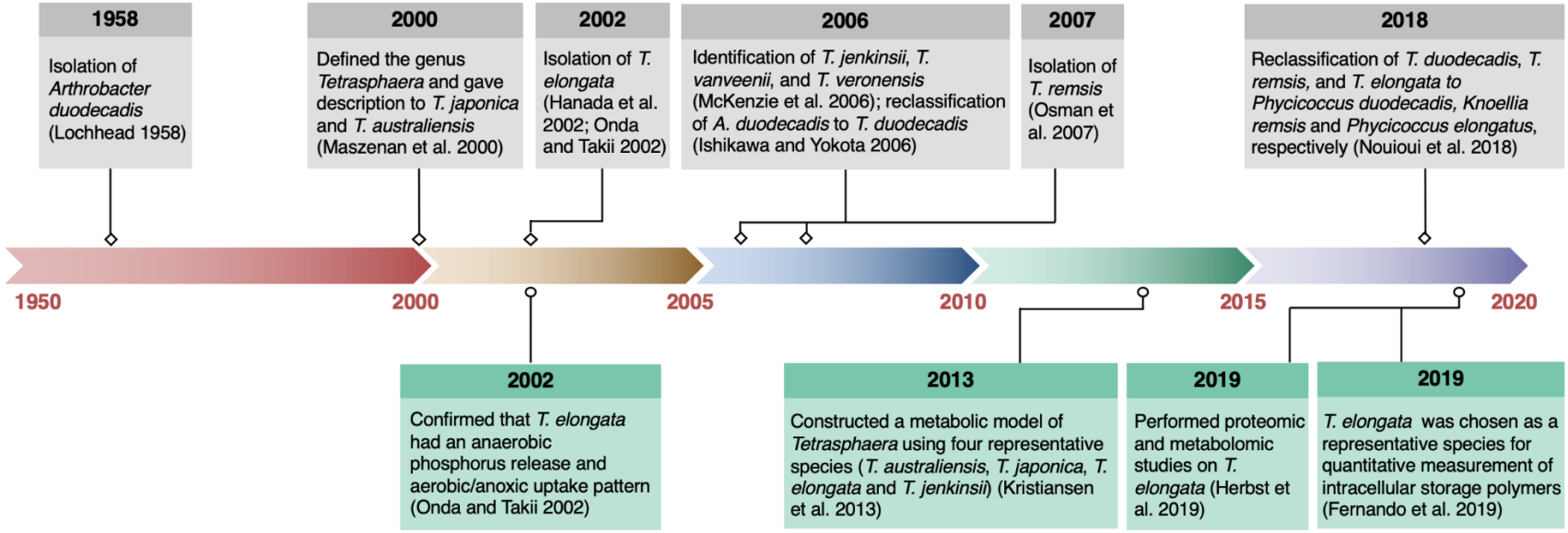


Figure 2.1. Timeline of identification history and research progress of Tetrasphaera species. Grey boxes represent the identification and reclassification history of Tetrasphaera species, and green boxes represent the current research progress of Tetrasphaera species (Fernando et al. 2019; Hanada et al. 2002; Herbst et al. 2019; Ishikawa and Yokota 2006; Kristiansen et al. 2013; Lochhead 1958; Maszenan et al. 2000; McKenzie et al. 2006; Onda and Takii 2002; Osman et al. 2007)

2.3. A historical review of the taxonomy of *Tetrasphaera* bacteria

This section provides a historical overview of the application of the polyphasic approach in the determination of *Tetrasphaera* as a novel genus and the classification and reclassification of its species. A timeline of *Tetrasphaera* identification and reclassification history as well as current research progress of its species is provided in Figure 2.1.

2.3.1. Determination of the *Tetrasphaera* genus

The genus *Tetrasphaera* was first described by Maszenan et al. (2000) after performing a detailed polyphasic taxonomic study on three bacterial strains (i.e., Ben 109, Ben 110, and strain T1-X7) that were isolated from activated sludge and had the capacity for poly-P accumulation. Specifically, 16S rRNA gene analysis showed that the three strains formed a well-supported cluster with a > 97% sequence similarity in the suborder *Micrococcineae* within the family *Intrasporangiaceae*. Their closest relatives were the genera *Janibacter*, *Intrasporangium*, *Terracoccus* and *Terrabacter*. DDH analysis showed that strain Ben 109 and Ben 110 were highly related (89% DDH value), but each had <50% DDH value with strain T1-X7, suggesting that the three strains belonged to a single genus and strain Ben 109 and Ben 110 represented a different species from strain T1-X7. Phenotypic and chemotaxonomic analyses showed that the strains shared two similar characteristics. First, all strains had type A1γ peptidoglycan containing meso-diaminopimelic acid (m-A_2pm) as the diagnostic diamino acid and MK-8(H_4) as the major menaquinone. Second, the strains were slow-growing Gram-positive cocci that accumulated poly-P but did not store PHA. However, cellular fatty acid profiles between the strains differed. While all three strains possessed 14-methylpentadecanoic (iso-$C_{16:0}$) and 14-methylhexadecanoic acid

(anteiso-$C_{17:0}$) as the dominant cellular fatty acids, strain Ben 109 and Ben 110 contained 12-methyltridecanoic acid (iso-$C_{14:0}$) and hexadecanoic acid ($C_{16:0}$), which were absent in strain T1-X7. Instead, strain T1-X7 contained iso-2OH-$C_{16:0}$, 2OH-$C_{17:0}$, iso-$C_{18:0}$, iso-$C_{18:1}$, and tuberculostearic acid. Based on these genotypic, phenotypic and chemotaxonomic similarities and differences, (Maszenan et al. 2000) classified strain Ben 109^T and Ben 110 as *Tetrasphaera australiensis* sp. nov., and strain T1-$X7^T$ as *Tetrasphaera japonica* sp. nov.

2.3.2. Identification of *Tetrasphaera* species

From the 2000s onward, several other species have been proposed to belong to the *Tetrasphaera* genus. In section 2.3.2 and 2.3.3, we will discuss the additional six species that have been cultured including three of which have been reclassified to different genera. A summary of the phenotypic and chemotaxonomic characteristics of these species is provided in Table 2.1.

Table 2.1. Chemotaxonomic and phenotypic characteristics of eight proposed species of *Tetrasphaera*

Polyphasic approach component	**Component characteristics**	**Indicator**		***T. australiensis***	***T. japonica***	***T. elongata (Phycicoccus elongatus)***	***T. vanveenii***	***T. veronensis***	***T. jenkinsii***	***T. remsis (Knoellia remsis)***	***T. duodecadis (Phycicoccus duodecadis)***
Chemotaxonomic	Cell wall peptidoglycan	Diamino acid (peptidoglycan type)	m-A_2pm (A1γ)	+	+	+	+	+	+	+ (ND)	3-OH m-A_2pm & m-A_2pm (A4γ)
	Cellular fatty acid	Major components		iso-$C_{16:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$	iso-$C_{16:0}$, anteiso-$C_{17:0}$	anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{14:0}$, $C_{16:0}$	iso-$C_{16:0}$, iso-$C_{15:0}$	iso-$_{16:0}$, iso-$C_{16:1}$, iso-$C_{15:0}$	anteiso-$C_{17:0}$, iso-$C_{16:0}$, iso-$C_{15:0}$, iso-$C_{16:1}$	$C_{18:1}$, iso-$C_{16:0}$, $C_{18:0}$, iso-$C_{15:0}$	10-methyl-$C_{17:0}$, iso-$C_{16:0}$, iso-$C_{15:0}$
	Cell membrane composition	Polar lipid	Composition	DPG, PG, PI, PL	DPG, PG, PI, PL, APL	DPG, PE, PG, PI, APL	DPG, PG, PI, APL	DPG, PG, PI, APL	PI, PG, APL	ND	ND
		Major menaquinone	MK-8(H_4)	+	+	+	–	ND	+	ND	+
Phenotypic	Cell structure	Morphology	Rod (diameter x length)	–	–	+ (0.7-1.0 µm x 1.0-1.8 µm)	–	–	–	–	+ (0.5-0.6 µm x 1.5-4.0 µm)
			Cocci (diameter)	+ (0.5-1.0 µm)	+ (0.6-1.4 µm)	–	+ (ND)	+ (ND)	+ (ND)	+ (0.5-1.0 µm)	+ (0.4-0.6 µm)
			Filament (length)	–	–	–	+ (ND)	+ (ND)	+ (ND)	–	–
		Gram stain	Gram positive	+	+	+	+	+	+	+	Gram-variable
	Environmental conditions	pH	6.0-9.0	+	+	+	ND	ND	ND	+	ND
		Temperature	15-37 °C	+	+	+	ND	+	+	+	+

	Carbon utilization	Carbohydrates	Glucose	+	+	+	+	+	+	+	+
		Proteins	Amino acids	+ (Lysine)	– (No lysine)	+ (Alanine, arginine, glycine)	ND	ND	ND	– (No alanine or glutamate)	– (No arginine)
		Lipids	VFA	+ (Acetate, propionate)	+ (Acetate, propionate)	+ (Acetate)	+ (Acetate, propionate)	+ (Acetate, propionate)	+ (Acetate, propionate)	+ (Acetate)	ND
		Other	LCFA [a]	+ (Palmitic and oleic acid ester)	– (No palmitic and oleic acid ester)	ND	+ (Oleic acid ester)	+ (Oleic acid ester)	+ (Oleic acid ester)	+ (Oleic acid ester)	ND
	Synthesis of intracellular storage granules	poly-P	Present	+	+	+	+	+	+	CD [b]	CD [c]
		PHA	Present	–	–	–	+	+	+	ND	ND
		Glycogen	Present	ND	ND	CD [d]	ND	ND	ND	ND	ND
		Other	Present	ND	ND	+ (Intracellular amino acids)	ND	ND	ND	ND	ND
Reference				(Maszenan et al. 2000)	(Maszenan et al. 2000)	(Hanada et al. 2002; Kristiansen et al. 2013; Nguyen et al. 2015; Seviour and Maszenan 2015)	(McKenzie et al. 2006; Seviour and Maszenan 2015)	(Blackall et al. 2000; McKenzie et al. 2006; Seviour and Maszenan 2015)	(Blackall et al. 2000; McKenzie et al. 2006; Seviour and Maszenan 2015)	(Osman et al. 2007)	(Ishikawa and Yokota 2006)

+, Positive results; –, negative results; ND, no data; CD, conflicting data; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PL, unknown phospholipid(s); APL, unknown amino phospholipid; LCFA, long chain fatty acid. If there is more than one strain in a species, then the data represents the type strain.

[a] Data is referred from substrate utilization ability of Tween 40 and Tween 80, of which the major component is palmitic acid ester and oleic acid ester, respectively.

[b] The original paper by (Osman et al. 2007) observed poly-P while (Stackebrandt et al. 2014) reported no poly-P storage.

[c] The original paper by (Ishikawa and Yokota 2006) did not examine poly-P while (Stackebrandt et al. 2014) and (Seviour and Maszenan 2015) reported no poly-P storage.

[d] (Kristiansen et al. 2013) identified glycogen according acid hydrolysis method. However, (Fernando et al. 2019) did not observe glycogen in T. elongata using FISH-Raman technique.

To begin, *T. elongata* was classified as a novel *Tetrasphaera* species after two strains: LP2 and ASP12, isolated from EBPR sludge demonstrated similar features to *T. japonica* and *T. australiensis* (Hanada et al. 2002; Onda and Takii 2002). Specifically, strain LP2 contained m-A_2pm in the cell wall peptidoglycan, MK-8(H_4) as the major menaquinone and a 96.9% and 96.7% sequence similarity to *T. australiensis*, and *T. japonica*, respectively (Hanada et al. 2002). While these features indicated that LP2 was a member of the genus *Tetrasphaera*, it was classified as a novel *Tetrasphaera* species for three reasons. First, LP2 had less than 97% 16S rRNA gene similarity with *T. australiensis* and *T. japonica*. Second, this strain mainly contained saturated fatty acids including anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{14:0}$ and $C_{16:0}$ while *T. australiensis* and *T. japonica* had large amounts of unsaturated fatty acids. Lastly, LP2 formed elongated linear or L-shaped clumps while *T. australiensis* and *T. japonica* occurred in tetrad and clusters. As a result, the name *Tetrasphaera elongata* sp. nov was proposed to reflect its elongated clump shape. For the second strain; ASP12, 16S rRNA gene analysis showed that its closest relatives were *Tetrasphaera elongata* (Strain LP2^T, 99.6% similarity) '*Candidatus* Nostocoida limicola' (97.1% similarity), *T. australiensis* (96.8% similarity), and *Janibacter limosus* (96.0% similarity) (Onda and Takii 2002). In addition, this strain shared similar G+C content and menaquinone type with *T. elongata* (strain LP2), thus Onda and Takii (2002) classified strain ASP12 as a member of T. elongata. It is worth mentioning here that '*Candidatus* Nostocoida limicola' was originally isolated from activated sludge by Blackall et al. (2000), and was later reclassified as novel *Tetrasphaera* species by McKenzie et al. (2006) as discussed below.

In 2000, Blackall et al. isolated six filamentous bacterial strains from activated sludge from Australia and Italy. A 16S rRNA gene based phylogenetic tree, showed that the six strains formed a tight cluster (97.4% similarity) within the phylum Actinobacteria. Four of the strains (Ben 17,

18, 67 and 68) formed a subgroup with 99.4% sequence similarity and the other two strains (Ver 1 and 2) formed a subgroup with 99.9% sequence similarity. Unfortunately, due to the strains' slow growth rate, Blackall et al. (2000) failed to obtain enough biomass to perform chemotaxonomic analyses and tentatively named the strains '*Candidatus* Nostocoida limicola' (Blackall et al. 2000). Later, McKenzie et al. (2006) continued this work by performing a detailed taxonomic study on the six strains obtained by Blackall et al. (2000) and added two more strains: Ben 70 and Ben 74 to the analysis. Following the polyphasic approach, all strains had type A1γ peptidoglycan containing m-A_2pm and formed a well-supported cluster with members of *Tetrasphaera* (>94% similarity) but DDH and isoprenoid quinone analysis indicated that these strains represented three different *Tetrasphaera* species. Five strains: Ben 17, 18, 67, 68 and 74 formed a sub-cluster with >99% sequence similarity and this cluster was separate from strain Ben 70 and Ver 1 and 2. To elucidate the taxonomic positions of these strains, strains Ben 17, Ben 74, Ben 70 and Ver 1 were selected as representatives for chemotaxonomic analyses. Strains Ben 17 and 74 had MK-8(H_4) as the major menaquinone, strain Ben 70 contained MK-8, MK-8(H_2) and MK-8(H_4) at a ratio of 39:29:6 and no major menaquinone was detected in strain Ver 1. Based on these analyses, strain Ben 17, Ben 18, Ben 67, Ben 68 and Ben 74^T were assigned to *Tetrasphaera jenkinsii* sp. nov., strain Ben 70^T was assigned to *Tetrasphaera vanveenii* sp. nov., and strain Ver 1^T and Ver 2 were classified as *Tetrasphaera veronensis* sp. nov.

In addition to isolating strains from activated sludge, Osman et al. (2007) isolated two strains: 3-M5-R-4 and 3-M5-R-7 from air samples collected from a Regenerative Enclosed Life Support Module Simulator system in Pasadena, CA. The two strains were highly similar in 16S rRNA gene sequences (>99.9%) and shared a high degree of DNA relatedness (>70% DDH value). In addition, 16S rRNA gene based phylogenetic analysis showed that the two strains formed a

well-supported cluster with members of *Tetrasphaera* and shared a high sequence similarity (>97%) with all known species of *Tetrasphaera*, with their closest relative being *T. japonica* (98%). The two strains also possessed m-A_2pm as the diagnostic amino acid in the peptidoglycan. The two strains were proposed to be a novel species in the genus *Tetrasphaera* because (1) they exhibited a low degree of DNA relatedness to their closest relative *T. japonica* (<16% DDH value), and (2) they contained higher amounts cellular fatty acids: octadecanoic acid ($C_{18:0}$) and cis-9-octadecenoic acid ($C_{18:1}$) compared to other *Tetrasphaera* species. This species was named *Tetrasphaera remsis* sp. nov. (Osman et al. 2007).

2.3.3. Reclassification of *Tetrasphaera* species

Ongoing technological advancement of molecular methods has allowed us to understand the fundamental microbial structures (genotypic, phenotypic and chemotaxonomic) that reveal relationships between microorganisms. In addition, these advancements have alleviated identification and classification limitations observed by prior researchers leading to reclassification of various bacteria. Three *Tetrasphaera* species have undergone this process of reclassification. For example, *T. duodecadis* has undergone several reclassification phases. *T. duodecadis* was initially classified as *Arthrobacter duodecadis*, by Lochhead (1958) based on its morphological features. Then in 2006, Ishikawa and Yokota (2006) performed a full chemotaxonomic and genotypic study on *A. duodecadis*, and compared its 16S rRNA gene sequence to members of *Tetrasphaera* and *Arthrobacter*. The results showed that *A. duodecadis* shared a 97.9% sequence similarity with *T. elongata*, 96.8% with *T. australiensis*, 97.4% with *T. japonica* and only 92.3% sequence similarity with the type species of the genus *Arthrobacter*, *Arthrobacter globiformis*. Together with other genotypic and chemotaxonomic information, Ishikawa and Yokota (2006) reclassified *A. duodecadis* as a new species within *Tetrasphaera* and

named it *T. duodecadis* because it had lower than 20% DDH values with other known species of *Tetrasphaera*, possessed 3-OH m-A_2pm in the peptidoglycan, and 10-methyl fatty acids as cellular fatty acids, features absent in other *Tetrasphaera* species.

In 2018, Nouioui et al. (2018) recommended the reclassification of *T. duodecadis*, *T. remsis*, and *T. elongata* to *Phycicoccus duodecadis*, *Knoellia remsis* and *Phycicoccus elongatus*, respectively, based on whole genome sequence analysis. Specifically, they constructed phylogenetic trees using whole genome sequences and found that *T. elongata* and *T. duodecadis* formed a well-supported clade with *Phyciococcus jejuensis* the type species of the genus *Phyciococcus* while *T. remsis* formed a clade with species within the genus *Knoellia*. In addition, they noted that although the genera: *Knoellia*, *Phycicoccus* and *Tetrasphaera* have similar chemotaxonomic and morphological characteristics; their mergence into a single taxon was premature thus proposing the reclassification of these three species (Nouioui et al. 2018). Later, these new taxonomic positions were validated and became effective (Oren and Garrity 2018).

Even though *T. elongata* has been removed from the genus *Tetrasphaera* (Nouioui et al. 2018), some studies have continued to use it to understand the physiology of the genus *Tetrasphaera*. For instance, Herbst et al. (2019) explored the metabolomic and proteomic characteristics of *T. elongata* under changing environmental conditions, while Fernando et al. (2019) used *T. elongata* as a representative species for quantitative assessment of intracellular storage polymers in the genus *Tetrasphaera*. Since *T. elongata* has been reclassified, an assessment on its appropriateness as a representative species in *Tetrasphaera* studies needs to be evaluated.

2.4. Methods used to identify *Tetrasphaera* abundance and physiology in activated sludge systems

EBPR processes like other engineered ecosystems contain complex communities of interacting microorganisms that influence the overall performance of the system. A key challenge in evaluating the impact of communities on performance/function lies in the use of appropriate molecular tools to quantify the abundance and physiological function of bacteria. In this section, we provide an overview of molecular methods currently used in the determination of abundance and physiology of *Tetrasphaera* in activated sludge systems. A summary of these molecular tools and their advantages and disadvantages is provided in Table 2.2.

Table 2.2. Methods for the identification of *Tetrasphaera* abundance and physiology in activated sludge systems

Category	Methods	Advantages	Disadvantages	Key findings	Reference
Identify of *Tetrasphaera* abundance in activated sludge	16S rRNA gene sequencing	Provide ample information about the whole microbial community	Can only be regarded as an estimation of the true biomass abundance	The read abundance of *Tetrasphaera* ranged from 1.3% to 11.9% in EBPR processes across 12 countries	(Albertsen et al. 2015; Nielsen et al. 2019; Stokholm-Bjerregaard et al. 2017)
	qFISH	Provide direct quantification of biomass abundance	• Lack of consistent FISH probes for *Tetrasphaera* in different studies • Lack of specific coverage of current FISH probes	*Tetrasphaera* constituted up to 30% of the total biomass in six Danish WWTPs	(Nguyen et al. 2011; Onnis-Hayden et al. 2020)
Identification of *Tetrasphaera* physiology in activated sludge	FISH-staining	Provide direct and easy identification of intracellular polymers	Only yield qualitative information	Confirmed that *Tetrasphaera* could accumulate poly-P but not PHA under in situ conditions	(Kong et al. 2005; Nguyen et al. 2011)
	FISH-Raman	Provide quantitative measurements of intracellular polymers at the single-cell level	High instrument cost	Monitored the dynamics of poly-P content in *Tetrasphaera* and confirmed its contribution to P removal	(Fernando et al. 2019)
	FISH-MAR	Provide information about substrate utilization patterns	Only yield qualitative information	Confirmed that *Tetrasphaera* could utilize glucose and various amino acids for phosphorus removal	(Marques et al. 2017; Nguyen et al. 2011, 2015)

2.4.1. Identification of *Tetrasphaera* abundance

16S rRNA gene amplicon sequencing and quantitative fluorescence in situ hybridization (qFISH) have been used to quantify the abundance of *Tetrasphaera* in EBPR processes (Onnis-Hayden et al. 2020; Qiu et al. 2019; Rey-Martínez et al. 2019). In 16S rRNA gene analysis, polymerase chain reaction (PCR) first amplifies different hypervariable regions of the 16S rRNA gene, and then the PCR amplicons are compared against a known database to determine the identity of a microorganism in a sample (Kim and Chun 2014). *Tetrasphaera* abundance is calculated as the number of reads belonging to *Tetrasphaera* divided by the total amplicon reads. While 16s rRNA gene sequencing provides ample information about the whole microbial community (Aaron M. Saunders et al. 2016), its accuracy has been questioned. For instance, this read abundance can only be regarded as an estimation of the true biomass abundance due to DNA extraction and PCR biases and 16S rRNA gene copy number variance between different microorganisms (Albertsen et al. 2015; Stokholm-Bjerregaard et al. 2017). In contrast, qFISH analysis relies on fluorescent rRNA-targeted probes and quantifies microbial abundance according to the area covered by the targeted microbial group relative to the total microbial community (Nielsen, Daims, and Lemmer 2009). The key advantage to qFISH analysis is that it avoids PCR biases and provides direct measurements of the biovolume fraction of a specific microbial group in a sample (Nielsen et al. 2009). Nguyen et al. (2011) designed FISH probes Tetmix (Tet1–266, Tet2–174, Tet2–892 and Tet3–654) for the genus *Tetrasphaera* based on three distinctive phylogenetic clades. Specifically, Clade 1 related clones are *T. elongata* and *T. duodecadis*; Clade 2 includes *T. jenkinsii*, *T. australiensis*, *T. veronensis* and the filamentous "*Candidatus* Nostocoida limicola"; and Clade 3 contains uncultured clones. Since then, these FISH probes have been used in several studies to

quantify the biovolume of *Tetrasphaera* in EBPR processes (Marques et al. 2017; Onnis-Hayden et al. 2020; Qiu et al. 2019).

Unfortunately, the application of the FISH probes designed by Nguyen et al. (2011) can lead to significant overestimation of *Tetrasphaera* in activated sludge. Onnis-Hayden et al. (2020) noticed a huge discrepancy between the results of 16S rRNA gene amplicon analysis and FISH analysis when measuring the abundance of *Tetrasphaera* in full-scale EBPR plants. While FISH analysis showed a high abundance of *Tetrasphaera* (>15%) in all the surveyed EBPR plants in North America, 16S rRNA gene analysis revealed an average abundance of only 1.81%. They concluded that these FISH probes could be biased because some species covered by the probes have a filamentous morphology, which could lead to an overestimation of abundance. In addition, not all *Tetrasphaera* species covered by the probes can accumulate poly-P (see Table 2.1). Besides the probes designed by Nguyen et al. (2011), there are other FISH probes available for target groups within the *Tetrasphaera* genus (Kong et al. 2005; Liu et al. 2001). For instance, probe Actino-1011 was designed to specifically target *T. elongata* strain LP2 and *T. japonica* (Liu et al. 2001), and probe Actino-658 and Actino-221 were designed to target clones closely related to the genus *Tetrasphaera* (Kong et al. 2005). However, currently there is no consensus on which FISH probes should be used, and some studies have chosen the one that best fits the scope of their experiments. For instance, Fernando et al. (2019) chose to use Actino-658 in their study as it provided the most specific coverage of the dominant member of *Tetrasphaera* in Danish WWTPs.

2.4.2. Identification of *Tetrasphaera* physiology

Microscopy-based methods such as FISH-staining, FISH-Raman and FISH-MAR (microautoradiography) have been used to study the physiology of *Tetrasphaera* in activated sludge systems (Fernando et al. 2019; Kong et al. 2005; Marques et al. 2017; Nguyen et al. 2011,

2015). In FISH-staining analysis, chemical stains are applied after FISH to confirm whether the probe-defined cells store a specific polymer such as poly-P and PHA. These staining methods provide qualitative information. For instance, FISH combined with 4′,6-diamidino-2- phenylindole (DAPI) or Neisser stain confirmed that members of *Tetrasphaera* could accumulate poly-P in full-scale plants, while Nile blue and Sudan black staining did not detect PHA in *Tetrasphaera* under in situ conditions (Kong et al. 2005; Nguyen et al. 2011). Recently, the new FISH-Raman technique has been applied in activated sludge for the absolute quantification of intracellular poly-P, glycogen, and PHA (Fernando et al. 2019; Majed et al. 2012). The FISH-Raman technique identifies these intracellular polymers based on their unique Raman spectrum and quantifies their concentration according to a linear correlation between the Raman signal and the amount of analyte per unit surface area (Fernando et al. 2019; Majed et al. 2012). This new technique not only provides quantitative measurements of intracellular polymers, but also allows for in situ monitoring of the dynamics of storage polymers at the single-cell level. FISH-MAR is a third technique that has been used to test *Tetrasphaera*'s ability for phosphorus and carbon uptake (Kong et al. 2005; Marques et al. 2017; Nguyen et al. 2011, 2015). In FISH-MAR analysis, biomass samples are first incubated with a radioactively labeled compound (e.g., $^{33}P_i$ and ^{13}C-labeled glycine), and then hybridized with a specific FISH probe (Nguyen et al. 2011, 2015). MAR positive probe-defined cells suggest that the microbial group has the ability to utilize a specific compound. Through the use of FISH-MAR, prior studies have confirmed that members of *Tetrasphaera* have the capacity to utilize glucose and various amino acids (e.g., glycine, glutamate and aspartate) for phosphorus removal (Marques et al. 2017; Nguyen et al. 2011, 2015). However, the FISH-MAR technique yields qualitative information. This is not sufficient for developing mass balance equations and limits the quantitative comparison between different microorganisms.

2.5. Opportunities and challenges

As mentioned previously, one of the main benefits for potential application of the *Tetrasphaera* genus in the EBPR process lies in its ability to utilize diverse carbon sources and produce VFAs. This potential is significant because full-scale EBPR processes often face performance instability attributed to insufficient carbon (i.e., chemical oxygen demand; COD) in the influent (Barnard and Abraham 2006; Muszyński et al. 2013; Ucisik and Henze 2008). Modified EBPR processes such as side stream return activated sludge (RAS) fermentation and the addition of external carbon sources (e.g., acetate and propionate) have been applied to maximize the availability of COD (Barnard et al. 2017; Onnis-Hayden et al. 2020). However, these practices incur significant capital and operational costs and add to the carbon footprint of the process (Onnis-Hayden et al. 2020; Puig et al. 2008). In addition, RAS fermentation does not always improve EBPR performance if the retention time in the RAS line is insufficient (< 4 hours) and may selectively enrich for glycogen accumulating organisms (GAOs) in the process (Coats et al. 2018).

A potential solution to improve performance stability may lie in the PAO community structure. Prior studies on ecology have shown that species richness within a microbial community affects the functional redundancy of an ecosystem. Redundancy is an ecological phenomenon where certain species within an ecosystem perform the same function and one species may be a substitute for another thus strengthening the community (Konopka 2009; Muszyński et al. 2013). The application of this concept, i.e., increasing species richness through application of *Tetrasphaera* in an EBPR process has three potential benefits. First, a diverse PAO community with adequate *Tetrasphaera* proliferation may have the ability to utilize a variety of carbon sources to achieve the same goal; phosphorus removal. This confers functional overlap that may increase process resiliency. Specifically, process upsets such as insufficient COD as VFAs which may

otherwise eliminate or decrease the activity of one group of PAOs may have less of an impact to the overall performance if the system has a functional *Tetrasphaera* community with the ability to utilize other carbon sources and produce VFAs (Barnard et al. 2017; Nguyen et al. 2011). Second, this diversification of carbon sources may reduce chemical costs for the treatment facility by increasing their choice of external carbon sources. This means the utilization of a variety of waste carbon such as starch, sucrose and crude glycerol (Shen and Zhou 2016). Lastly, utilization of carbon sources other than VFAs has the potential to reduce substrate competition between VFA consuming microorganisms, for example competition between *Candidatus* Accumulibacter, the archetypical PAO and GAOs (Puig et al. 2008; Zengin et al. 2011).

Yet, this review has identified fundamental challenges that limit our understanding of *Tetrasphaera* species, their function and physiology, consequently limiting widespread application. Although, eight *Tetrasphaera* species have been proposed in the literature (Table 2.1), only five species currently remain in the *Tetrasphaera* genus: *T. japonica*, *T. australiensis*, *T. vanveenii*, *T. veronensis*, and *T. jenkinsii*. While these five species have the ability to accumulate poly-P, it is still inconclusive whether they fit into the classical definition of a PAO because their phosphorus release and uptake patterns as well as their storage compounds for energy remain unexplored or unidentified (Table 2.1). Additionally, prior studies investigating the *Tetrasphaera* genus utilized *T. elongata*, as the representative species, yet this species, has been reclassified as *Phycicoccus elongatus*. Therefore, to have a better understanding of the genus and its application in EBPR processes, future research should focus on the individual five *Tetrasphaera* species and elucidate their phosphorus removal ability as well as develop their metabolic energy balance under cyclic anaerobic and aerobic/anoxic conditions.

Lastly, the application of molecular tools such as FISH and 16S rRNA gene sequencing has greatly improved our understanding of *Tetrasphaera* in activated sludge systems but a lack of standardized molecular methods limits the design, testing, and optimization of wastewater treatment systems with this promising putative PAO. For example, the use of different FISH probes in different studies and a lack of specific coverage of current FISH probes has led to overestimation of *Tetrasphaera* abundance in full-scale treatment processes. This lack of consensus in molecular tools may lead to incompatible results and inconclusive knowledge of their true abundance and contribution to phosphorus removal. Therefore, it is necessary to design standardized FISH probes with more specificity and utilize consistent molecular methods for future studies.

Chapter 3. Biokinetic Study of *Tetrasphaera elongata* for Wastewater Treatment

3.1. Introduction

Tetrasphaera is an emerging PAO that has been identified at high abundance (e.g., >10% biovolume) in many enhanced biological phosphorus removal (EBPR) systems worldwide and is considered to be an important microorganism for phosphorus (P) removal (Liu et al. 2019; Marques et al. 2017; Nielsen et al. 2019; Singleton et al. 2022; Zhang and Kinyua 2020). Prior studies have shown that *Tetrasphaera* exhibits a non-classical PAO phenotype compared to the model PAO *Candidatus Accumulibacter* (hereafter *Accumulibacter*). Unlike *Accumulibacter* that solely relies on volatile fatty acids (VFAs) such as acetate and propionate for growth (Oehmen et al. 2007, 2010), *Tetrasphaera* is able to utilize a wide range of carbon sources including amino acids (glycine, glutamate, aspartate, etc.), glucose and lactate (Herbst et al. 2019; Kristiansen et al. 2013; Marques et al. 2017; Nguyen et al. 2015). This versatility on carbon sources is a major benefit to the EBPR process as it reduces or even eliminates the need for VFA addition. *Tetrasphaera* has also been shown to have a different metabolism compared to *Accumulibacter* (Kristiansen et al. 2013; Marques et al. 2017). While *Accumulibacter* stores polyhydroxyalkanoates (PHA) and glycogen as intracellular polymers to support polyphosphate (poly-P) cycling (He and McMahon 2011), most species of *Tetrasphaera* (e.g., *T. jenkinsii*, *T. australiensis* and *T. elongata*) lack the genetic potential to store PHA (Kristiansen et al. 2013).

Instead, several studies have proposed that *Tetrasphaera* may store free intracellular amino acids and glycogen as the internal carbon sources (Kristiansen et al. 2013; Marques et al. 2017; Nguyen et al. 2015). However, a recent study failed to detect glycogen in the pure culture of *Tetrasphaera in situ* by Raman microspectroscopy (Fernando et al. 2019).

Understanding the metabolism and kinetics of *Tetrasphaera* is important to the modeling of EBPR processes. In wastewater treatment, process modeling is an important tool for the design, upgrade, and operational control of a treatment system (Serdarevic, Amra and Dzubur, Alma 2016). The modeling of activated sludge systems is based on the kinetic and stoichiometric parameters of complex bioreactions involving different microorganisms (Hauduc et al. 2013). However, current process modeling tools have not considered the contribution of *Tetrasphaera* to the EBPR process, partially due to the lack of knowledge about the kinetics of this important PAO. Process models involved with EBPR process such as Activated Sludge Model No.2 (Henze et al. 1999) are developed based on stoichiometric and kinetic coefficients obtained from PAO cultures enriched with acetate or propionate where *Accumulibacter* is typically the dominant PAO in these systems (Brdjanovic et al. 1997; Oehmen et al. 2005; Smolders et al. 1994). Yet, knowing that *Tetrasphaera* has functional importance to the EBPR process, these process modeling tools could be improved or recalibrated by incorporating the kinetic features of emerging PAOs such as *Tetrasphaera*. As the application of process modeling has become a standard practice in wastewater design and optimization, an improved mathematical model could greatly increase the accuracy of model prediction and eventually lead to better design and performance of EBPR systems.

The first step to achieve model accuracy is to investigate essential kinetic parameters such as growth and substrate uptake rates required for EBPR process modeling. A few studies have

been performed to determine the growth rate of various *Tetrasphaera* species. For example, Herbst et al. (2019) investigated the anoxic growth of *Tetrasphaera elongata* in R2A medium with different compositions from wastewater but was not able to obtain sufficient data to determine the growth rate. Arroyo et al. (2014) measured the growth rate of *Tetrasphaera duodecadis* but utilized rich medium containing tryptone and yeast extract which are not typical in wastewater (Nielsen et al. 2010). In addition, it is still not clear whether *T. duodecadis* contributes to phosphorus removal as no intracellular poly-P has been identified in pure culture (Stackebrandt et al. 2014). Other studies have investigated the anaerobic-aerobic carbon consumption profile of *Tetrasphaera* to confirm its PAO phenotype but these lab-scale studies have only used glucose as a carbon source (Fernando et al. 2019; Kristiansen et al. 2013). Thus the activity of *Tetrasphaera* utilizing other commonly available carbon substrates remains unknown.

The objective of this study was to determine the specific growth rate (μ) and the specific anaerobic carbon uptake rate (q^{max}) of *Tetrasphaera* utilizing different carbon sources. *T. elongata* was selected as a representative species for investigation as it has been used as a model *Tetrasphaera sp.* in prior studies (Herbst et al. 2019; Kristiansen et al. 2013; Nguyen et al. 2015). This would allow direct comparisons between the results obtained from the present study and previous studies to assess potential bias. Kinetic parameters were measured in pure cultures under defined laboratory conditions to avoid the interference from other microorganisms. Results from this study provided a supporting framework for future EBPR design and modeling.

3.2. Materials and Methods

3.2.1. Bacteria strain and culture media

T. elongata str. Lp2 (JCM 11141) (Hanada 2002) was obtained from Japan Collection of Microorganisms in freeze-dried form. The bacteria strain was revived in R medium which contained the following constituents per liter: 10.0 g peptone, 5.0 g yeast extract, 5.0 g malt extract, 5.0 g casamino acids, 2.0 g beef extract, 2.0 g glycerol, 50.0 mg Tween 80 and 1.0 g $MgSO_4 \cdot 7H_2O$. The kinetic tests - specific growth rate and the anaerobic carbon uptake test were performed in modified mineral salts-vitamin (MSV) medium (Williams and Unz 1989) amended with different carbon sources. The modified MSV medium contained the following constituents per liter: $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.05g; K_2HPO_4, 0.11 g; KH_2PO_4, 0.085 g; $FeCl_3 \cdot 6H_2O$, 0.002 g; $Na_2 \cdot$ EDTA, 0.003 g; vitamin mix solution, 1 mL. The MSV medium has defined compositions of only essential minerals and therefore can best represent wastewater compositions compared to other rich media such as R2A medium (Kristiansen et al. 2013). All media were adjusted to pH=7±0.01 and autoclaved prior to use. Vitamin solution was filter-sterilized through a 0.22 μm filter membrane and added to cooled media right before each batch test.

3.2.2. Specific growth rate (μ) determination

The measurement of the specific growth rate (μ) took three steps: (1) grow primary bacterial culture in R medium to obtain sufficient biomass; (2) develop a growth curve at 100 and 200 mg-COD$\cdot L^{-1}$ acetate; and (3) determine the growth rate from the exponential portion of the growth curve. Acetate was selected as the carbon source because it is a common carbon substrate in wastewater (Nielsen et al. 2010). To obtain primary culture, a single colony of *T. elongata* was

inoculated into R medium in an Erlenmeyer flask equipped with a vented cap (Corning, USA). The vented cap was equipped with a 0.22 μm hydrophobic membrane for sterile air exchange. The primary culture was incubated at 28°C and 125 rpm for 3-4 days until sufficient biomass was obtained. To determine the growth curve, the primary culture was centrifuged at 2000 rpm for 20 min, washed three times, and diluted with sterile MSV medium to an initial OD_{600} value of around 0.04. Sodium acetate was spiked into the culture to an initial concentration of 100 or 200 mg-$COD \cdot L^{-1}$. These concentrations were chosen as they are typical in wastewater influent. Cell cultures were incubated aerobically at 28°C with agitation and OD_{600} values were monitored continuously until a plateau was reached. Experiments were conducted in four replicates. OD_{600} was measured using a visible light spectrophotometer (HACH DR3900).

The specific growth rate of cell cultures was determined using the exponential phase of the growth curve according to the following equation (Das et al. 2011; Hall et al. 2014):

$$\ln \frac{OD_t}{OD_0} = \mu(t - t_0)$$

where μ is the specific growth rate at a given substrate concentration (h^{-1}), which is the slope of ln OD vs t. The exponential portion of the growth curve was determined using R package "growthrates" (https://cran.r-project.org/web/packages/growthrates/index.html).

3.2.3. Anaerobic carbon removal efficiency test

The anaerobic carbon removal efficiency test was conducted to obtain a preliminary understanding about which carbon source (s) can be utilized by *T. elongata*. The result of this test was used to guide the anaerobic carbon uptake rate test. A single colony of *T. elongata* was selected from an agar plate and cultured aerobically in R medium at 28°C and 125 rpm for 3-4 days to generate adequate biomass. Cells were then harvested by centrifugation and washed three times with MSV medium. Cell pellets were diluted with MSV medium to an OD_{600} of 0.6 and then

incubated aerobically for 1 h to exhaust any intracellularly stored carbons. Cell cultures were then purged with N_2 gas for 1 h to ensure an anaerobic condition before experiments were initiated. At the beginning of the test, cell cultures were spiked with four different carbon sources: glucose, formate, acetate and gluconate, to a final concentration of 75 mg-C·L^{-1}. Then the cultures were incubated anaerobically with agitation for 8 h. A cell culture with no carbon supplement was included as the negative control. Samples were taken at the beginning and the end of the experiment for the determination of carbon and phosphorus concentrations. All batch experiments were conducted in triplicates.

3.2.4. Specific anaerobic carbon uptake rate (q^{max}) test

Batch activity tests were conducted to evaluate the anaerobic carbon uptake rate and phosphorus release rate of *T. elongata* with acetate, glucose, and gluconate as carbons sources. These three carbon sources were selected based on the result of the carbon removal efficiency test (see section 3.2.3). The experimental procedure was similar to that described in section 3.2.3 except the cultures were diluted to an initial OD_{600} of 0.8 and samples were taken at every 100-min interval for a total of 500 min. Experiments were conducted in triplicates with N_2 gas purged throughout the whole test. Active biomass concentration was determined in the form of volatile suspended solids (VSS) at the end of the experiment.

3.2.5. Analytical methods

Samples from batch activity tests were centrifuged at 3,000 rpm for 30-min to remove biomass solids. Supernatant was analyzed for acetate, gluconate, formate and PO_4^{3-}-P using Ion Chromatography (Metrohm IC System, Switzerland). Specifically, anions were measured using an Eco IC equipped with a Metrosep A Supp 7 analytical column. Chromatograms were acquired with 3.2 mM Na_2CO_3/1.0 mM $NaHCO_3$ as eluent at a flow of 0.7 mL·min^{-1}. Glucose concentration

was determined using the phenol-sulfuric acid method (Dubois et al. 1956). VSS concentration was determined according to standard methods (APHA 2005).

3.3. Results

3.3.1. The specific growth rate (μ) of *T. elongata*

Figure 3.1 shows the aerobic growth curves of *T.elongata* with 100 and 200 mg-COD·L^{-1} sodium acetate. Three phases of growth were observed: (1) a lag phase when no bacterial growth occurred; (2) an exponential phase when population increased significantly; and (3) a stationary phase when population reached a plateau, and no further growth was observed. The specific growth rate was calculated based on the exponential phase of the growth curve. Bacterial cultures took around 20- and 30-h to reach the plateau in 100 and 200 mg-COD·L^{-1} acetate, respectively. The specific growth rates were calculated to be μ=0.057 h^{-1} at 100 mg-COD·L^{-1} and μ=0.059 h^{-1} at 200 mg-COD·L^{-1}, which were deemed comparable at the two given carbon concentrations.

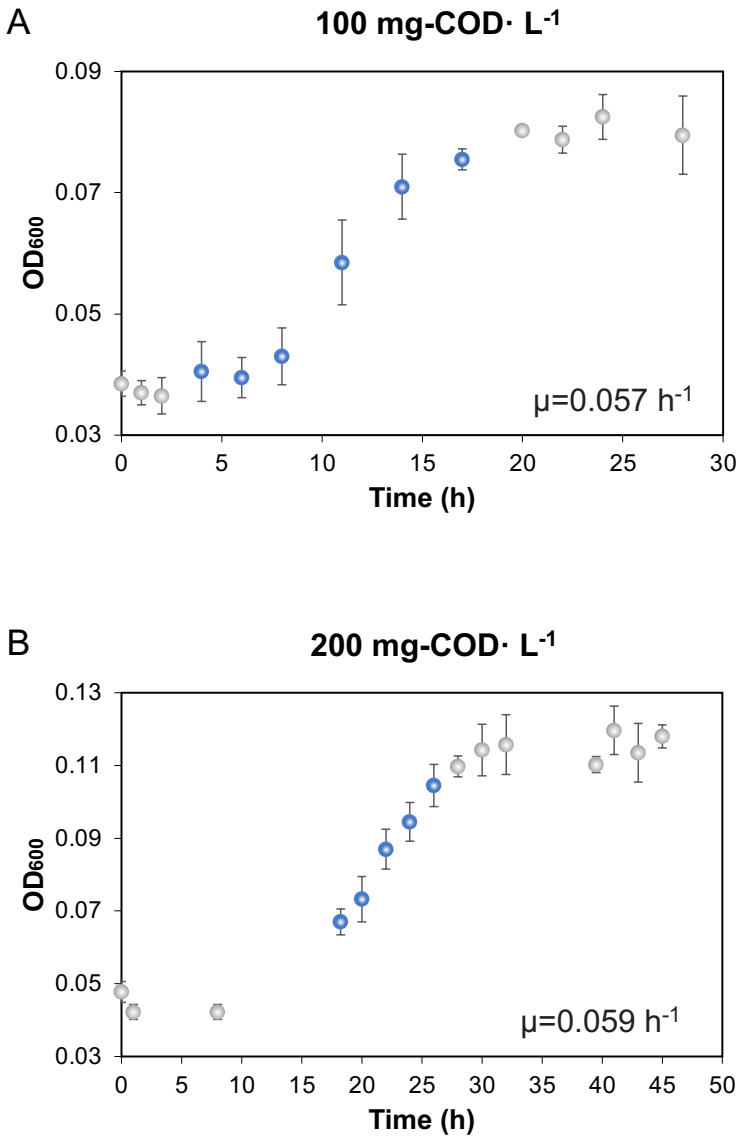


Figure 3.1. Growth curves of *T. elongata* with (A) 100 mg-COD·L^{-1} and (B) 200 mg-COD·L^{-1} acetate. Results are determined in four replicates and reported as mean ± standard deviation. Exponential portion of the growth curve is shown as blue dots.

3.3.2. Anaerobic carbon removal efficiency

To determine which carbon source(s) could be effectively utilized by *T. elongata*, formate, gluconate, acetate, and glucose were provided at an initial concentration of 75 mg-C·L^{-1}. Figure 3.2 shows the removal efficiency for each carbon source after 8-hour anaerobic incubation. The highest carbon removal was observed for glucose (42%), followed by acetate (36%) and gluconate (28%). The removal for formate was only 7%. Surprisingly, while carbon substrate was consumed during the anaerobic incubation, no anaerobic P-release was observed for any of the carbon sources tested.

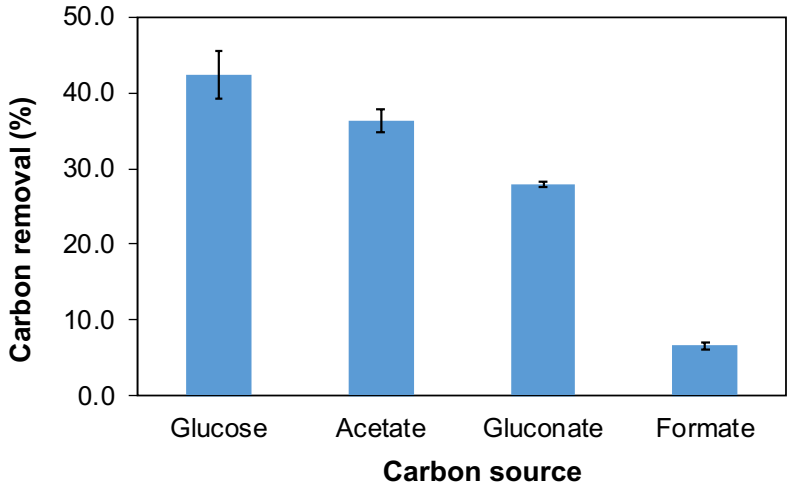


Figure 3.2. Removal of 75 mg-C·L^{-1} formate, gluconate, acetate and glucose after 8-h anaerobic incubation. Data represents mean of triplicate experiments and error bars indicate standard deviation.

3.3.3. The specific anaerobic carbon uptake rate (q^{max})

Anaerobic batch tests were conducted to determine the anaerobic carbon uptake rate (q^{max}) of *T. elongata* at 75 mg-C·L^{-1} gluconate, acetate, or glucose as the carbon source. Formate was not included for evaluation as little formate was removed in the anaerobic carbon removal test (see section 3.3.2). Figure 3.3 shows the specific anaerobic carbon uptake profile of *T.elongata* with

the selected carbon sources. Active biomass concentration was expressed in C-mol unit using the theoretical PAO biomass composition $CH_{2.09}O_{0.54}N_{0.20}P_{0.015}$ (Lopez-Vazquez et al. 2006). After 400-min of incubation (~7 h), glucose was completely consumed, and gluconate concentration reached a constant value (Figure 3.3). Based on this observation, the carbon uptake rate of glucose and gluconate was calculated based on the data from the first 400-min of incubation. Results showed that glucose yielded the highest carbon uptake rate (0.0421 C-mol·C-mol biomass^{-1}·h^{-1}), followed by acetate (0.0181 C-mol·C-mol biomass^{-1}·h^{-1}) and gluconate (0.0159 C-mol·C-mol biomass^{-1}·h^{-1}) (Figure 3.3). Similar to the result obtained from the anaerobic carbon removal efficiency test in section 3.3.2, no P-release was observed during the whole test.

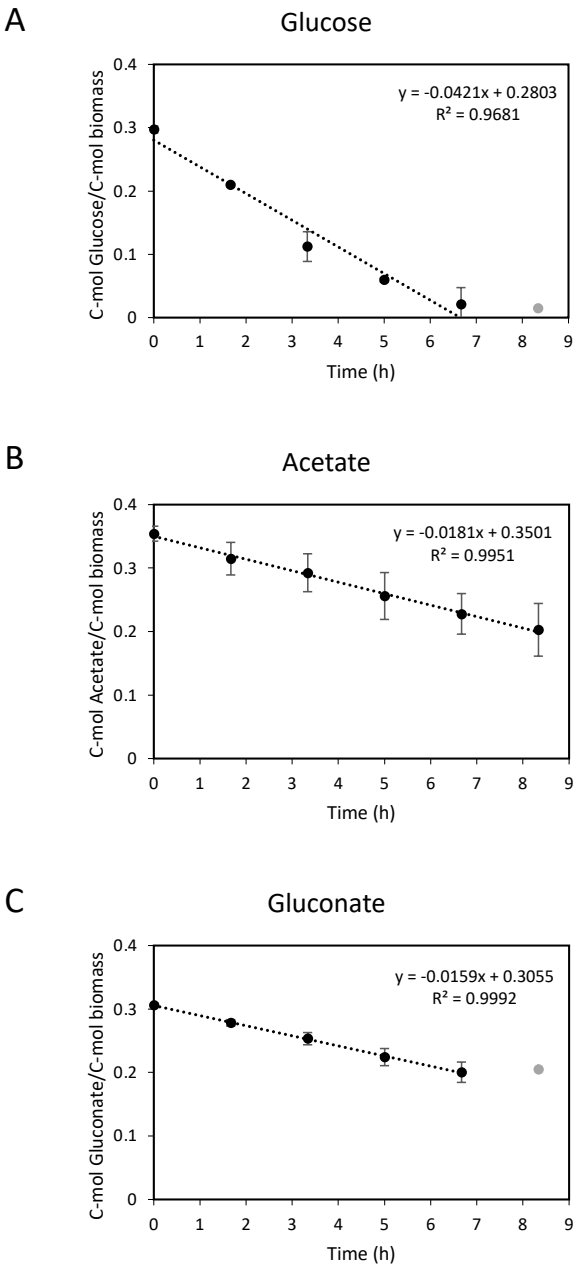


Figure 3.3. Anaerobic carbon uptake profile of *T. elongata* when supplied with 75 mg-C·L^{-1} (A) glucose, (B) acetate and (C) gluconate as the carbon source. Data represents mean of triplicate experiments; error bars indicate standard deviation and, in some cases, may be smaller than symbols. Data points labeled in grey are not included in rate calculation as concentration has not changed significantly from the previous time point.

3.4. Discussion

3.4.1. The specific growth rate of *T. elongata*

The measured specific growth rates of *T. elongata* were nearly identical at 100 and 200 mg-COD·$L^{-1}$of acetate [μ=0.057 h^{-1} (1.37 d^{-1}) at 100 mg-COD·L^{-1} vs μ=0.059 h^{-1} (1.42 d^{-1}) at 200 mg-COD·L^{-1})] (Figure 3.1), indicating that the bacterial culture may have reached its maximum growth rate at the given substrate concentrations. Although *Tetrasphaera* has been reported to be a slow-growing bacterium (Kataoka et al., 1996), the specific growth rate determined in this study (1.37-1.42 d^{-1}) appeared to be slightly higher than typical values reported in previous studies on PAOs (Table 3.1). Unlike this study that used a pure culture, previous studies have relied on PAO cultures enriched with acetate or glucose in lab-scale settings because PAO groups such as *Accumulibacter* cannot be isolated in pure culture (Lu et al. 2006). In ASM No.2, μ_{PAO} is set at 1.0 $d^{-1}$and 0.67 d^{-1} at 20°C and 10°C, respectively (Henze et al. 1999). One possible reason for the high growth rate observed in this study could be the lack of competition for substrate in pure culture compared to enriched culture. The higher specific growth rate observed for *T. elongata* is beneficial to wastewater treatment due to a smaller SRT required to treat the same volume of waste stream (Serdarevic, Amra and Dzubur, Alma 2016), thus leading to footprint reductions and energy savings. Future studies might measure the specific growth rates at different substrate concentrations <100 mg-COD·L^{-1} to determine the substrate half-saturation coefficient of *T. elongata*. This will allow direct prediction of microbial competition as organisms with a lower substrate half-saturation coefficient will outcompete others at low substrate concentrations (Arnaldos et al. 2015).

Table 3.1. The specific growth rate of *T. elongata* compared to typical values of PAOs

PAO culture	Carbon	Specific growth rate (μ)	SRT (d)	Reference
Enriched	Acetate	1.0 d^{-1}	5	(Smolders et al. 1995)
Enriched	Acetate	0.336 d^{-1}	20	
Enriched	Acetate	1.0 d^{-1}	10	(Yagci et al. 2004)
Enriched	Acetate	0.7 d^{-1}	No data	(Whang et al. 2007)
Enriched	Acetate	0.122 d^{-1}	20	(Wang, Park, and Whang 2001)
Enriched	Glucose	0.161 d^{-1}	20	
T. elongata pure culture	Acetate	1.37-1.42 d^{-1}	NA	This study

3.4.2. The anaerobic carbon uptake capacity of *T. elongata*

Anaerobic carbon uptake tests showed that *T. elongata* can efficiently utilize glucose, acetate and gluconate as carbon sources (Figure 3.2 and Figure 3.3). This versatility to utilize different carbon sources is a major benefit to wastewater treatment as *Tetrasphaera* may thrive under different wastewater conditions.

In EBPR modeling, q^{max} is an important parameter that is directly linked to the rate of carbon uptake (Filipe, Daigger, and Grady 2001). Organisms with a higher q^{max} generally have an advantage in competition especially at low substrate concentrations. Results of batch activity tests showed that the q^{max} of *T. elongata* was dependent on the type of carbon substrate provided (Figure 3.3). The highest q^{max} was observed for glucose which agreed with prior studies (Kristiansen et al. 2013; Nguyen et al. 2011). The q^{max} of *T. elongata* determined in this study was further compared to values reported in the literature (Table 3.2). The q^{max} of enriched PAO cultures was reported to range from 0.17 to 0.43 C-mol·C-mol $biomass^{-1}·h^{-1}$ with VFAs such as acetate and propionate as the carbon source (Table 3.2) (Brdjanovic et al. 1997; Filipe et al. 2001; Lopez-Vazquez et al. 2006; Oehmen et al. 2005; Smolders et al. 1994). These values were significantly different from

the values measured in this study. For instance, when acetate was provided, the q^{max} of enriched PAO cultures (0.17-0.43 C-mol·C-mol biomass^{-1}·h^{-1}) was much higher than that of *T. elongata* (Table 3.2). Overall, results from this study showed that *T. elongata* had very different substrate uptake capacity compared to typical PAO cultures. Current EBPR modeling tools such as ASM2d and UCTPHO+ (Zuthi et al. 2013) utilize VFA as the only source of carbon for PAOs. However, results from this study suggest that broadening the substrate parameter in future EBPR process modeling may be necessary.

Table 3.2. The specific anaerobic carbon uptake rate (q^{max}) of *Tetrasphaera* compared to typical values of PAOs

PAO	Carbon	q^{max} (C-mol·C-mol biomass^{-1}·h^{-1})	Reference
Enriched	Acetate	0.43	(Smolders et al. 1994)
Enriched	Acetate	0.17-0.19	(Brdjanovic et al. 1997)
Enriched	Acetate	0.185	(Filipe et al. 2001)
Enriched	Acetate	0.17	(Lopez-Vazquez et al. 2006)
Enriched	Propionate	0.18	(Oehmen et al. 2005)
T. elongata pure culture	Glucose	0.042	This study
T. elongata pure culture	Acetate	0.018	This study
T. elongata pure culture	Gluconate	0.016	This study

3.4.3. Anaerobic phosphorus release of *T. elongata*

No anaerobic P-release was observed with any of the carbon sources tested in this study (glucose, acetate, gluconate and formate). This result was particularly interesting as previous studies have shown that *T. elongata* exhibits an anaerobic P-release and aerobic P-uptake pattern when supplied with glucose or glycine in batch tests (Fernando et al. 2019; Kristiansen et al. 2013;

Nguyen et al. 2015). One possibility could be that *Tetrasphaera* has a versatile phosphorus metabolism. For instance, when grown in a mixed culture, anaerobic P-uptake instead of release was observed in an enriched *Tetrasphaera* culture supplied with glucose, aspartate, glutamate or glycine as the carbon source (Marques et al. 2017). Other environmental factors such as pH and temperature may also impact the P-removal capability of *Tetrasphaera*. More studies are needed to illustrate the anaerobic P-release capacity of *T. elongata* and to confirm whether *T. elongata* is truly a PAO.

3.5. Conclusion

In this study, batch tests were conducted to investigate the anaerobic carbon utilization capability and the aerobic growth kinetics of *T. elongata*. The specific growth rate was determined to be 1.37–1.42 d^{-1}, which was slightly higher than values of typical PAOs. *T. elongata* showed versatility on carbon sources. Glucose yielded the highest specific anaerobic carbon uptake rate, followed by acetate and gluconate. The anaerobic carbon uptake rates of *Tetrasphaera* measured in this study were significantly lower than the values of typical PAOs reported in the literature. Results of this study could be useful for future EBPR design and modeling.

Chapter 4. An Investigation of Nutrient Removal and Microbial Community in Facilities with Unintended Phosphorus Removal in the San Francisco Bay Area

4.1. Introduction

Coastal eutrophication, caused by excessive nutrient additions to estuaries and coastal waters, has become a global environmental concern (Conley et al. 2009; Le Moal et al. 2019; Scavia and Bricker 2006; Smith and Schindler 2009; Wang et al. 2018). While nutrients loading to estuaries is a natural process, recent population growth and related human-induced nutrient sources such as agriculture, industry, urban runoff and wastewater have drastically accelerated this process (Bricker et al. 2008; Paerl et al. 2014). In California, the San Francisco (SF) Bay has become one of the most nutrient-enriched estuaries in the world due to high nutrient discharges into the watershed (Cloern and Jassby 2012). While severe eutrophication has not been reported in the SF bay, recent research shows that the SF Bay has been gradually losing its historic resiliency to nutrients pollution and may suffer from water quality impairment in the near future (Cloern et al. 2020; Novick and Senn 2014).

While wastewater discharges are considered a major contributor of nutrient point-source pollution to receiving waters (Carey and Migliaccio 2009; A. Drolc and Zagorc Koncan 2002), most municipal wastewater treatment plants (WWTPs) in the SF Bay are not designed as systems

for biological nutrient removal (BNR). In wastewater treatment, the BNR process refers to the biological removal of nitrogen (N) and phosphorus (P) from wastewater. It is typically achieved through activated sludge systems such as the Modified Ludzak-Ettinger (MLE) process and the A2O process (Ong 2014). Specifically, configurations uniquely designed to realize high performance of P removal are named enhanced biological phosphorus removal (EBPR) process. These systems are characterized by the enrichment of polyphosphate-accumulating organisms (PAOs) under alternating anaerobic-aerobic conditions (Acevedo et al. 2012; Mino et al. 1998; Nielsen et al. 2010). The EBPR process has been implemented worldwide as an efficient, sustainable, and economic approach for P removal from municipal wastewater (Coats, Brinkman, and Lee 2017; Lanham et al. 2013; Onnis-Hayden et al. 2020; Qiu et al. 2019). The successful operation of an EBPR process relies on many different factors, among which the microbial community compositions and influent carbon characteristics play a key role (Coats et al. 2017; Lopez-Vazquez et al. 2009; Nielsen et al. 2010; Puig et al. 2008; Zhang and Kinyua 2020). There are a variety of PAOs in the EBPR process, among which *Candidatus* Accumulibacter (hereafter *Accumulibacter*) is the model PAO that utilizes volatile fatty acids (VFAs) as the carbon source (He and McMahon 2011). Previous research has shown that the abundance of *Accumulibacter* is associated with EBPR performance in full-scale WWTPs (López-Vázquez et al. 2008; Zilles et al. 2002). Other putative PAOs including *Tetrasphaera*, *Thiothrix*, *Thauera,* and *Dechloromonas* may also have significant contributions to the EBPR process, and they thrive on other carbon sources such as glucose, lactate and amino acids (Marques et al. 2017, 2018; Rey-Martínez et al. 2019; Stokholm-Bjerregaard et al. 2017; Terashima et al. 2016; Wang and He 2020). Glycogen-accumulating organisms (GAOs) are believed to be detrimental to the EBPR process as they compete with PAOs for VFAs in wastewater (Mielczarek et al. 2013).

In an effort to protect water quality and meet future nutrient discharge limits, 37 WWTPs in the SF Bay have participated in nutrient reduction studies led by the Bay Area Clean Water Agency (BACWA) (Falk et al. 2018). The short-term objective of these studies is to evaluate the current treatment performance and to seek opportunities for total nitrogen (TN) reduction via treatment optimization or upgrading. The long-term objective is to incorporate total phosphorus (TP) reduction into the treatment process. Among these 37 WWTPs, seven of them already have an anaerobic selector before the aerobic zone in the secondary treatment process. Although the original intent for this design was for filamentous organism control, this configuration has an anaerobic-aerobic (A/O) configuration similar to that common in EBPR systems. In addition, these anaerobic selector facilities have occasionally observed unintended P removal activities. However, because these systems are not specifically designed for nutrient removal and currently do not have a discharge limit for P or N, their nutrient removal capacity has been overlooked. Little is known about the microbial community and kinetic rates in these unintended P removal systems. These facilities may have the potential to be optimized for nutrient removal and meet future nutrient discharge limits. Increasing our knowledge and understanding about how these systems function is a step towards that goal.

Therefore, the objective of this study was to investigate the nutrient removal rates and microbial community structure of 7 WWTPs with unintended P removal in the SF Bay and compare the results to typical EBPR processes. Specific tasks of this study include: (1) conduct a field survey to obtain nutrient removal rates and influent characteristics (i.e., COD fractions) at each facility; (2) investigate the impact of different operating factors (i.e., pure oxygen) on the overall microbial community compositions and genetic potentials of these facilities; and (3)

quantify the relative abundance of the PAOs and GAOs within the microbial community and investigate their impact on P removal.

4.2. Material and Methods

4.2.1. Field investigation

Field investigation was conducted at 7 municipal WWTPs in the SF Bay area from September 2020 to January 2021 (Table 4.1). These WWTPs were selected because they have an anaerobic selector before the aerobic tank, suggesting a potential for unintended nutrient removal (emphasis on phosphorus removal). For simplicity, P1, P2, etc. will be used to describe each facility hereafter. The sludge retention time (SRT) of these facilities varied significantly from 1 to 57 days during the period of investigation. Some unique features of these facilities include: (1) P1 and P2 use pure oxygen activated sludge processes to facilitate oxygen transfer and organic removal; (2) P2, P3, and P4 receive a proportion of industry wastewater (e.g., dairy products and beverage) in addition to municipal wastewater; and (3) severe sludge bulking issues have been observed in P5.

Table 4.1. Summary of operational factors in 7 surveyed facilities in the SF Bay area

Parameter	Unit	Surveyed Facilities						
		P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**
Pure oxygen	-	Yes	Yes	No	No	No	No	No
Industrial wastewater loading	-	No	Yes	Yes	Yes	No	No	No
Primary treatment	-	Yes	Yes	Yes	No	Yes	Yes	No
SRT	Day	1.3	2.1	5.3	11	1.2	4	57
HRT	Hour	3	4	10.9	32	4.2	14	91
SVI	mL/g	120	97	No data	130	171	225	86

Field sampling was performed at each facility to investigate treatment performance, kinetic rates, and microbial community compositions. Specifically, 24-hour composite raw influent, primary effluent (where applicable), and secondary effluent were collected from each facility for TP, PO_4^{3-}, TN, NH_4^+, NO_2^-, NO_3^- and carbon fractionation analysis. Mixed liquor suspended solids (MLSS) samples were collected at the end of the aerobic zone for batch activity tests and microbial community analysis. Once collected, all samples were immediately transported on ice to the Kinyua Wastewater Research Lab at UC Davis. For microbial community analysis, 1.5 mL of MLSS samples were centrifuged at 12,000 rpm for 10 min, then the supernatant was removed, and cell pellets were stored at -80 °C before DNA extraction. All analyses and batch activity tests were performed on the day of sampling.

4.2.2. Carbon fractionation analysis

The COD fractionation of raw influent and secondary effluent was evaluated using the particle size distribution (PSD) analysis. Specifically, raw influent and secondary effluent were sequentially filtered through 1.5-µm (Whatman, glass fiber), 0.45-µm (Millipore, cellulose

membrane), and 0.22-µm (Millipore, cellulose membrane) pore size filters, and the COD concentration of each fraction was determined.

4.2.3. Batch activity tests

To examine N and P removal activities at each facility, batch activity tests were performed in a 2-L double-jacketed reactor connected to a recirculating water bath with temperature maintained at 20°C. Before the start of the experiment, 1,000 mL of fresh MLSS collected at each facility was transferred to the batch reactor and purged with N_2 gas for 30-min to achieve initial anaerobic conditions. At the beginning of the test, 250 mL of primary effluent was added to the batch reactor. The batch test was composed of a 2-h anaerobic phase followed by a 2-h aerobic phase. N_2 gas and air were purged continuously to provide the anaerobic and aerobic conditions, respectively. Samples were taken at 10-min intervals during the first hour of the test and then were reduced to every 30-min intervals. pH was maintained at 7.5 ± 0.1 with the addition of 0.1 M NaOH or 0.1 M HCl. Oxygen concentration was monitored using a Hach HQ30D Meter connected with a LDO101 DO probe. Samples were immediately filtered through 0.45-µm membrane filters for the determination of PO_4^{3-}-P, NH_4^+-N, NO_2^--N and NO_3^--N concentrations. Additional samples were taken at the beginning and the end of the experiment for the determination of MLSS, mixed liquor volatile suspended solids (MLVSS) and COD concentrations. The ammonia-oxidizing bacteria (AOB) rates and nitrite-oxidizing bacteria (NOB) rates were calculated as the slope of NO_x-N production and NO_3-N production, respectively (Regmi et al. 2014).

4.2.4. DNA extraction, PCR amplification and bioinformatic analysis

DNA extraction of MLSS was performed using FastDNA Spin kit for soil (MP Biomedicals, USA) following the manufacturer's protocol. The extracted DNA was sent to the UC Davis Genome Center for DNA quantification, PCR amplification and sequencing. Specifically,

DNA concentration was determined using the Qubit DNA Assay and quality was checked using gel electrophoresis. The V3-V4 region of bacterial 16S rRNA gene was amplified using primer sets 341F (5'- CCTACGGGNGGCWGCAG-3') and 806R (5'- GGACTACNVGGGTATCTAAT-3'). The amplicons were sequenced on an Illumina Miseq platform. Raw sequences were imported into QIIME 2 platform (Bolyen et al. 2019) for downstream analyses. More specifically, paired-end sequences were first processed using DADA2 platform (Callahan et al. 2016) for quality filtering, sequence denoising, sequence merging and chimera removal. The output of DADA2 is amplicon sequence variants (ASVs), which distinguishes sequence variation to the level of single-nucleotide differences and is equivalent to 100% OTU (Callahan, McMurdie, and Holmes 2017). The processed sequences were compared to MiDAS v3.7 database for bacterial taxonomy assignment (Callahan et al. 2017).

The abundances of functional genes related to N and P metabolisms (Table 4.2) were predicted based on 16S rRNA gene sequencing data using the PICRUSt2 platform (Douglas et al. 2020) following the method described by Samaddar et al. (2021). Analysis of functional genes provides direct predictions on system performance and stability as the removal of substrate is directly associated with the functional enzymes in a community (Douglas et al. 2020). Specifically, ASVs from the DADA2 platform were aligned and placed in a reference tree using HMMER (http://hmmer.org) and SEPP (Mirarab, Nguyen, and Warnow 2011), respectively. A new phylogenetic tree incorporating both reference genome and ASV replacements was generated using GAPPA (Czech, Barbera, and Stamatakis 2020). The gene abundance was predicted using the hidden-state prediction function in the castor R package (Louca and Doebeli 2018). ASVs with a nearest-sequenced taxon index (NSTI) score above 2 were excluded from the analysis as they

were usually off-target or noise. Finally, pathway level abundance was predicted using MinPath (Ye and Doak 2009) and functional descriptions were added to the output abundance table.

Table 4.2. Key metabolic pathways related to N and P activities

Pathway	Enzyme Commission No.	Protein
Nitrification	1.14.99.39	Ammonia monooxygenase (amoA)
Denitrification	1.7.99.4	Nitrate reductase
	1.7.2.1	NO-forming Nitrite reductase
	1.7.2.5	Nitric oxide reductase
	1.7.2.4	Nitrous oxide reductase
Phosphorus removal	2.7.4.1	Polyphosphate kinase

4.2.5. Analytical method

TP, PO_4^{3-}-P, TN, NH_4^+-N, NO_2^--N, NO_3^--N, COD, MLSS and MLVSS were measured according to Standard Methods (APHA 2005). ffCOD was determined according to Kinyua et al. (2017). Briefly, 1 mL of $ZnSO_4$ solution was added to 100 mL of liquid sample and pH was adjusted to 10.5 for flocculation. The solution was allowed to settle, and the supernatant was filtered through a 0.45-μm cellulose membrane filter, which was soaked in DI water for at least 24-h prior to use.

4.2.6. Data analysis

The abundance of functional genes was analyzed using STAMP software (Parks et al. 2014). Other analyses were conducted in RStudio. Specifically, Pearson correlation coefficients were calculated using the package Hmisc (https://cran.r-project.org/web/packages/Hmisc/index.html); the impact of different operational parameters on microbial community structure was evaluated using ANOSIM test with the package vegan (https://cran.r-project.org/web/packages/vegan/index.html); and bacterial groups with differential abundance in different systems were identified using the package indicspecies (https://cran.r-

project.org/web/packages/indicspecies/index.html). For all statistical analyses, a *p*-value of <0.05 was considered significant.

4.3. Results

4.3.1. Batch activity tests for nutrient removal rates

Nutrient removal rates of the surveyed facilities were calculated based on batch activity tests and the results are summarized in Table 4.3. Although these facilities have many similarities in process configuration, different levels of nutrient removal activity were seen. For P removal kinetics, no P release or uptake was observed in P3 and P7, while for other facilities a large variation in P release and uptake rates was seen. For those five facilities with P removal, their specific P-release rates ranged from 2.1 to 9.6 $mgP \cdot gVSS^{-1} \cdot hr^{-1}$and the specific P-uptake rates ranged from 0.4 to 4 $mgP \cdot gVSS^{-1} \cdot hr^{-1}$. Interestingly, for P5, only aerobic P-uptake (0.6 $mgP \cdot gVSS^{-1} \cdot hr^{-1}$) was seen but no anaerobic P-release was observed.

For N removal kinetics, nitrification and denitrification activities were observed in all facilities except P1. Similar to P removal rates, a wide variation in nitrification and denitrification rates was seen (Table 4.3). For the six facilities where N removal activities were seen, AOB, NOB and denitrification rates were in the range of 0.1-3.0 $mgNO_X\text{-}N \cdot gVSS^{-1} \cdot hr^{-1}$, 0.02-2.1 $mgNO_3\text{-}N \cdot gVSS^{-1} \cdot hr^{-1}$, and 0.02-3.4 $mgNO_X\text{-}N \cdot gVSS^{-1} \cdot hr^{-1}$, respectively. Low AOB and NOB rates were observed in P2 and P5.

Table 4.3. Comparisons between nutrient removal rates in the surveyed facilities and typical BNR process

Parameter	Unit	Surveyed Facilities							Typical BNR process	
		P1	P2	P3	P4	P5	P6	P7	Rate	Reference
AOB rate	$mgNO_X$-N·$gVSS^{-1}$·hr^{-1}	0	0.1	2.2	1.8	0.6	3.0	1.1	2.7-3.8	(Yao and Peng 2017)
NOB rate	$mgNO_3$-N·$gVSS^{-1}$·hr^{-1}	0	0.02	2.1	1.8	0.02	1.4	1.1	4.0-5.0	(Yao and Peng 2017)
Denitrification rate	$mgNO_X$-N·$gVSS^{-1}$·hr^{-1}	0	0.02	1.1	2.5	0.7	3.4	0.2	3.4-5.5	(Onnis-Hayden et al. 2007)
P release rate	mgP·$gVSS^{-1}$·hr^{-1}	3.6	2.1	0	9.6	0	6.1	0	11-22	(He, Gu, and McMahon 2008; Lee and Yun 2014)
P uptake rate	mgP·$gVSS^{-1}$·hr^{-1}	4	1.5	0	2.2	0.6	0.4	0	1.9-11	(He et al. 2008)

4.3.2. Relating carbon fractionations with nutrient removal rates

COD concentrations for each facility were evaluated as the (1) cumulative COD, defined as the total COD concentration below the selected filter size and (2) differential COD, defined as the COD concentration at a certain particle size range. The cumulative COD concentrations of influent and secondary effluent of the surveyed facility are summarized in Table 4.4 below. The raw influent differential COD percent distribution is shown in Figure 4.1. The total COD concentration of raw influent and secondary effluent averaged 715.8 ± 230.5 mg-COD·L^{-1} and 38.9 ± 19.1 mg-COD·L^{-1}, respectively, with an average total COD removal of 94.0 ± 4.0%. Particulate COD (pCOD) (>1.5 μm) was the major fraction (62.8 ± 10.4%) in the raw influent, followed by the <0.22 μm (23.6 ± 7.5%) fraction and the fraction between 0.45-1.5 μm (10.5 ± 4.6%) (Figure 4.1). These results are similar to Dulekgurgen et al. (2006) who reported 65% pCOD fraction and 19.32% soluble fraction (<0.22 μm) in domestic wastewater. For secondary effluent, there was no significant difference between COD fractions in the size bins of 1.5 μm, 0.45 μm and 0.22 μm.

Table 4.4. Cumulative COD concentrations of influent and secondary effluent from the surveyed WWTPs (n=3)

Facilities	Total	1.5 µm	0.45 µm	0.22 µm	ffCOD
Influent (mg-COD·L^{-1})					
P1	461.8	255.6	171.0	151.5	104.8
P2	1131.0	292.6	256.6	243.0	231.3
P3	724.4	205.0	118.4	100.9	63.0
P4	777.0	361.6	294.5	270.2	243.0
P5	574.6	195.3	146.7	123.3	91.2
P6	835.3	304.2	201.1	184.6	159.3
P7	506.6	170.0	116.5	96.1	94.1
Secondary Effluent (mg-COD·L^{-1})					
P1	60.6	38.7	36.8	35.0	16.7
P2	56.0	45.1	37.8	31.4	28.6
P3	28.6	31.4	40.5	25.0	12.1
P4	20.4	17.6	18.5	22.2	13.1
P5	58.8	40.5	33.2	35.9	25.9
P6	32.3	23.1	18.5	26.8	23.1
P7	15.8	15.8	15.8	17.6	8.5

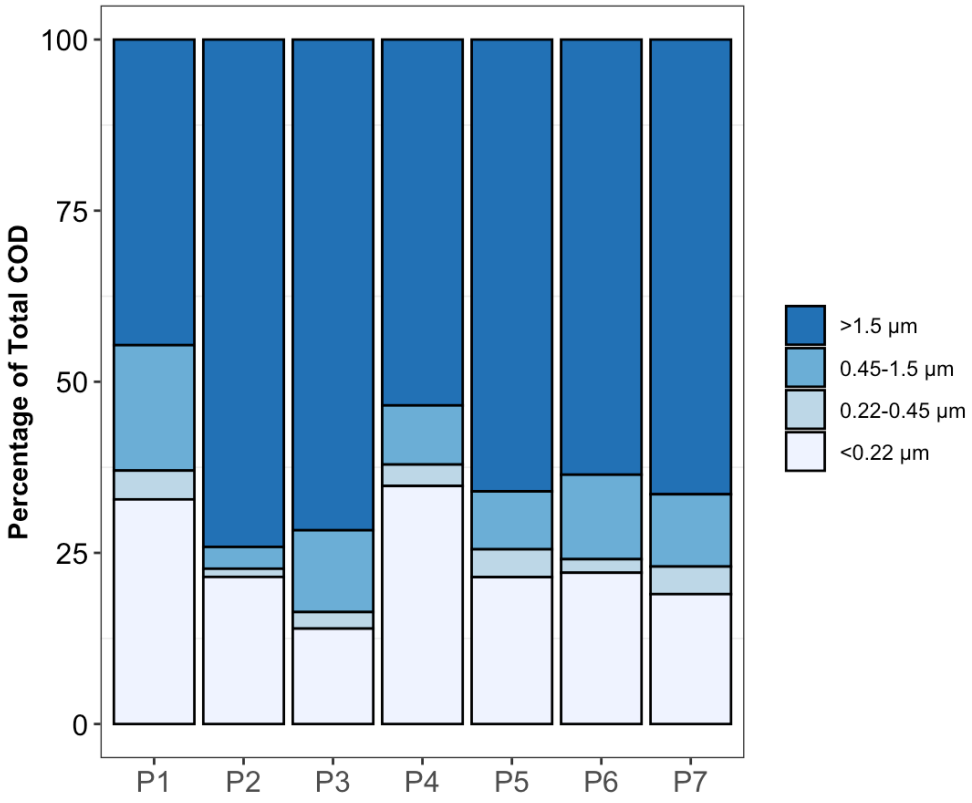


Figure 4.1. Percent distribution of influent COD fractions from the surveyed WWTPs.

A Pearson correlation analysis was conducted to evaluate the relationship between nutrient removal rates, influent carbon fractions and operational parameters (see Figure B.1). Some of the correlations were obvious, for instance: (1) total inorganic nitrogen (TIN) removal rate was positively correlated with AOB rate and denitrification rate; (2) a strong positive correlation was observed between P-release rate and the COD fraction <1.5-µm; and (3) a strong negative correlation was found between TP removal rate and NOB rate. Interestingly, a strong positive correlation was observed between the nitrite accumulation ratio and pCOD concentration (>1.5-µm) in the influent.

4.3.3. Overall bacterial communities based on alpha and beta diversity

The overall bacterial community composition was evaluated based on the result of alpha and beta diversity analysis. Beta diversity analysis was conducted to quantify the extent of similarities and differences among the microbial communities in different facilities, and the result was visualized in Principal Coordinate Analysis (PCoA) plots. PCoA plots based on non-phylogenetic Bray-Curtis dissimilarity distance (p=0.043*, PERMANOVA; Figure 4.2A) and phylogenetic weighted UniFrac distance (p=0.048*, PERMANOVA; Figure 4.2B) both showed that facilities with pure oxygen processes (P1 and P2) clearly separated from others on the first axis, indicating that pure oxygen played a key role in differentiating microbial community compositions. SRT also appeared to impact microbial community structure, as facilities with similar SRT tended to cluster together, and this trend was especially obvious in the PCoA plot of Bray-Curtis dissimilarity distance (Figure 4.2A). Alpha diversity evaluates microbial diversity of a single sample and results showed that facilities with pure oxygen process appeared to have lower Faith's phylogenetic diversity compared to other facilities (Figure 4.2C), although this difference was not statistically significant (p=0.121, Kruskal–Wallis test).

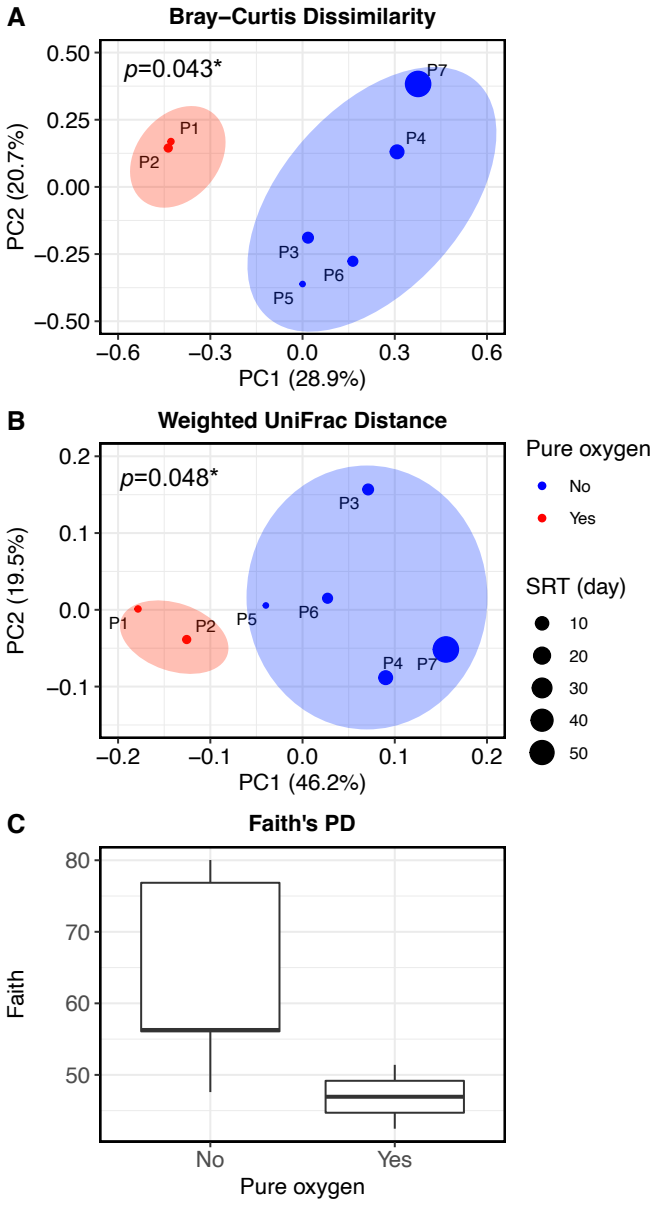


Figure 4.2. Bacterial community analysis based on alpha and beta diversity analysis. (A) PCoA plot of Bray-Curtis dissimilarity distance; (B) PCoA plot of weighed UniFrac distance; and (C) Faith's phylogenetic diversity between facilities with and without pure oxygen process. Statistical significance values show the impact of pure oxygen process on overall bacterial community diversity.

4.3.4. Microbial community analysis at the functional gene level

To further understand the microbial community difference at the functional gene level, the functional profile of the surveyed facilities was investigated using PICRUST2. PICRUST2 predicts the functional potential of a bacterial community based on marker gene sequence (Douglas et al. 2020). Functional enzymes related to nitrification, denitrification and P removal activities were of specific interest in this study (Table 4.2). Principal component analysis (PCA) based on the overall community functional enzyme abundance showed that the first axis and the second axis explained 37.1% and 27.0% of variation, respectively. Facilities with pure oxygen process also clearly separated from others on the first axis, suggesting an impact of pure oxygen on the functional genes of the community (Figure 4.3). Based on this observation, the impact of pure oxygen on the key metabolic pathways (see Table 4.2) was further tested using Welch's t-test which is used to test if two populations have equal means. Results showed that facilities with pure oxygen processes had significantly higher abundance in polyphosphate kinase (p=0.034*) but significantly lower abundances in nitrite reductase (p=0.010*) and nitrous-oxide reductase (p=0.045*) (Figure 4.4). The abundances of ammonia monooxygenase, nitrate reductase and nitric-oxide reductase showed no significant differences between facilities with and without pure oxygen process.

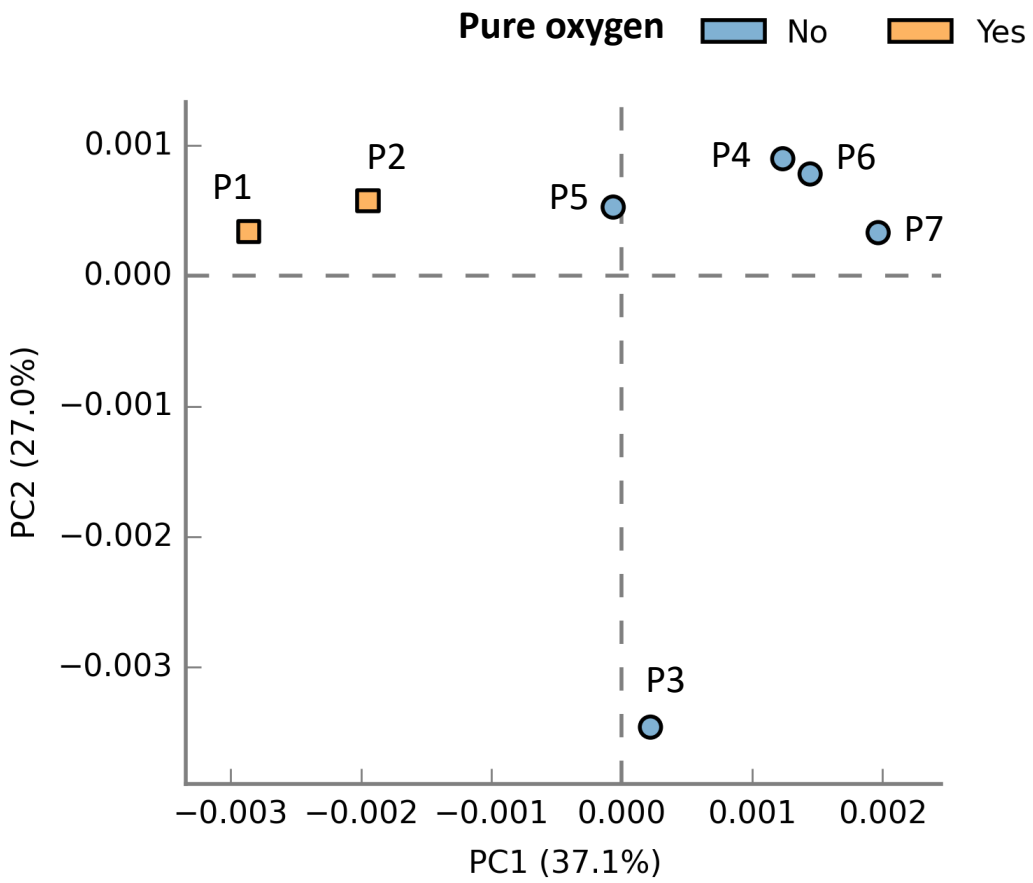


Figure 4.3. Principal component analysis (PCA) plot based on functional enzyme abundances encoded by predicted sequence.

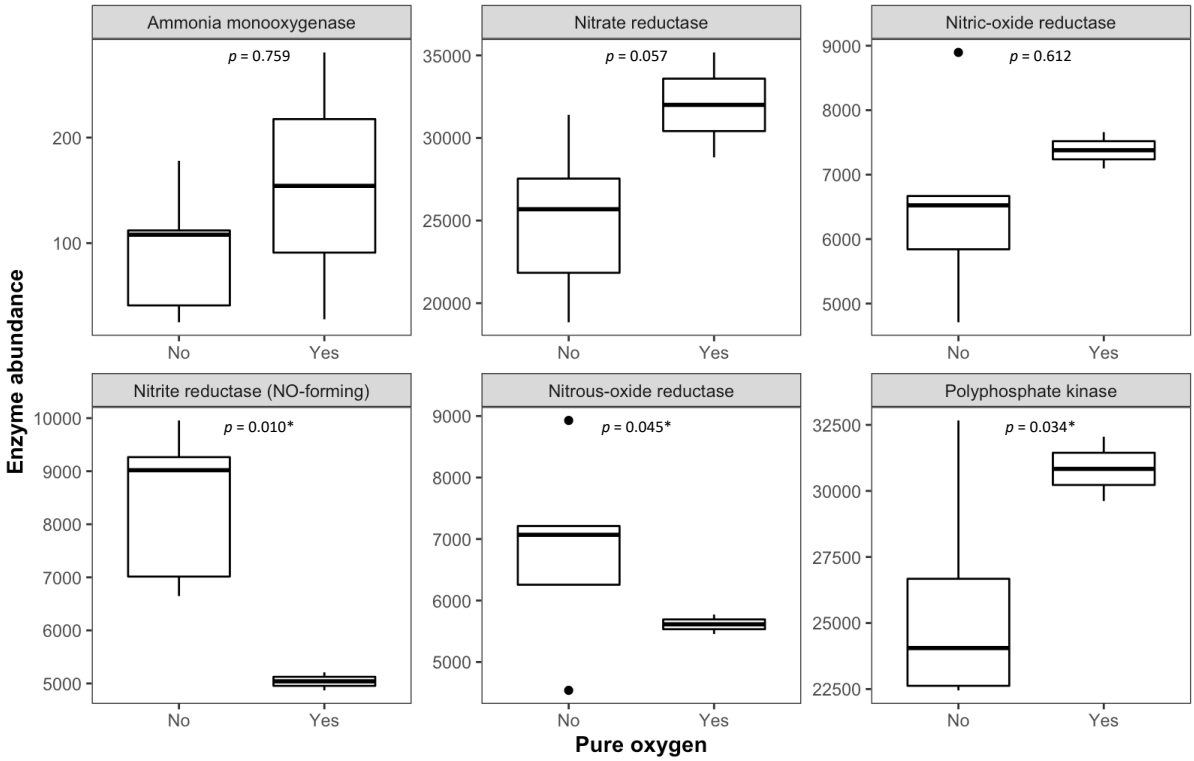


Figure 4.4. Boxplot showing the enzyme abundance profiles between facilities with and without pure oxygen processes. Significance levels are calculated based on Welch's t-test.

4.3.5. The core community and the abundant community

In addition to the overall community structure discussed in section 4.3.3 and 4.3.4, the individual bacterial abundance at the genus level was also investigated. Among the total of 921 bacterial genera identified in all systems, a list of 54 genera were found to be present in all 7 facilities, even though some of them appeared with low abundance (e.g., <0.01%). This list of only 54 genera together, accounted for 26-52% of the overall community abundance at the different facilities. Because this group of 54 genera were common to all systems and they together accounted for a high overall abundance, they were defined as the "core community" in this study. Many members of the core community appeared with high abundance in the surveyed systems. For

instance, as shown in Figure 4.5, 15 genera among the core community belonged to the top 20 abundant genera and half of the core community (27) belonged to the top 50 abundant genera.

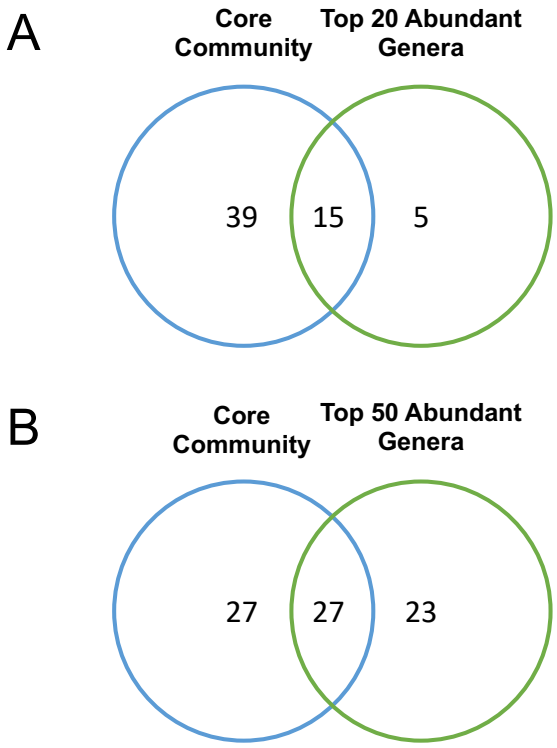


Figure 4.5. Venn diagram showing the number of core genera that belong to (A) the top 20 abundant genera and (B) the top 50 abundant genera. The abundant genera are determined based on their average abundance among different facilities.

An ANOSIM test was conducted to investigate the potential impact of different operational factors on the structure of the core community and the abundant community. Results showed that facilities with pure oxygen process were significantly different from other facilities in the bacterial compositions of both the core community (p=0.042*) and the top 50 abundant community (p=0.048*). Differences in bacterial compositions were further explored by identifying the key bacterial groups with differential abundances between the two systems (pure oxygen vs. non pure

oxygen). Among the top 50 abundant genera, 8 bacterial groups were found to be significantly more enriched (p<0.05) in the pure oxygen process (Figure 4.6). These bacteria groups included *Agitococcus lubricus group*, *Ca. Competibacter, midas_g_558*, *midas_g_3838*, *midas_g_33*, *Gordonia*, *Acinetobacter* and *Tetrasphaera*. Most of them were also more enriched in the core community.

Other operational factors such as primary treatment and industrial wastewater loading showed no significant impact on the microbial structure of the abundant community or the core community.

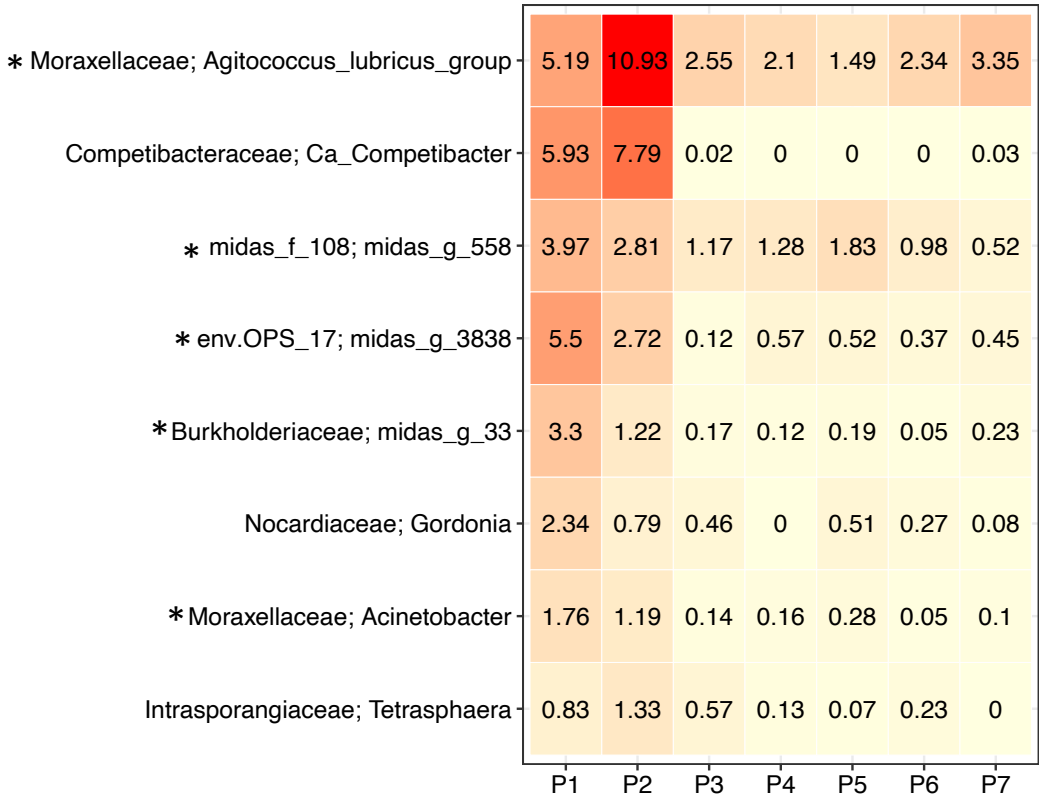


Figure 4.6. Heatmap showing bacterial groups among the top 50 abundant genera with significantly more enriched abundances in the pure oxygen process (P1 and P2). The ones labeled with asterisks are also more enriched in the core community.

4.3.6. Abundances of PAO and GAO communities

Analysis of the PAO community is a direct way to evaluate whether a system has P-removal potential. A wide variety of PAOs were observed in the surveyed facilities (Figure 4.7). Interestingly, these PAOs were also widely identified in P3 and P7 where no P-removal activities were seen (see Table 4.1). *Accumulibacter* was the most abundant PAO appearing in all systems with abundance ranging from 0.1%-5.4%. Other PAO groups including *Dechloromonas*, *Thauera, Halomonas* and *Pseudomonas* were also observed in all systems. *Thiothrix*, a putative filamentous PAO (Mardanov et al. 2020), appeared with a high abundance of 4.2% in P5 where severe sludge settling issues were frequently reported (Table 4.1). *Tetrasphaera* appeared with low abundance in the surveyed systems (0-1.33%). No statistically significant difference was observed in the overall microbial structure of PAOs between facilities with and without P removal activity based on ANOSIM test.

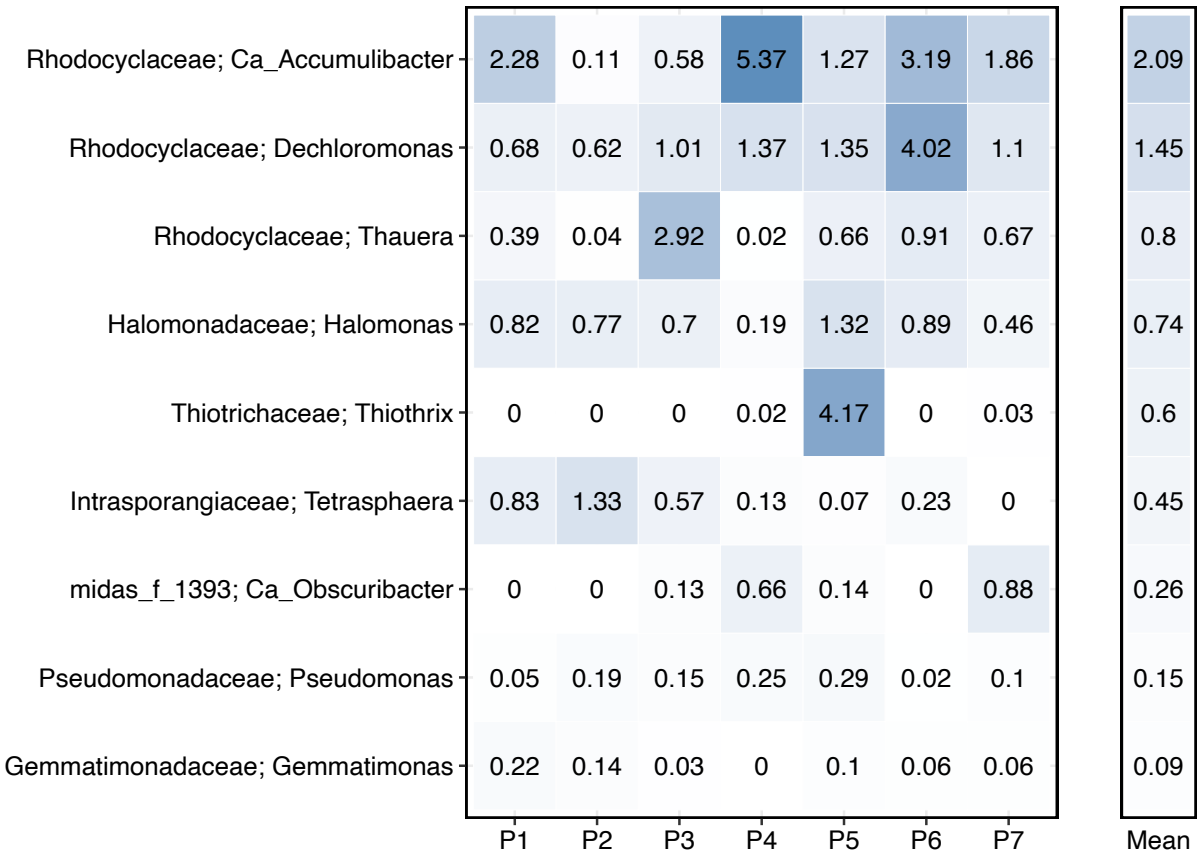


Figure 4.7. Heatmap showing the relative abundance of putative PAOs at the surveyed WWTPs. The numbers represent the read abundance at the given WWTP. Family and genus are shown on the y-axis.

The GAO community was much less diverse compared to the PAO community. In fact, only four groups of GAOs including *Ca. Competibacter*, *Propionivibrio*, *Defluviicoccus*, and *Ca. Contendobacter* were observed in the surveyed facilities. *Propionivibrio* was found in six facilities (except P6) with abundances ranging from 0.03-0.34%. *Defluviicoccus* (0.04%) and *Ca. Contendobacter* (0.03%) only appeared in P3 and P1, respectively. While the abundance of most GAOs was low, *Ca. Competibacter* showed high abundance, 5.9% and 7.8% in the pure oxygen processes P1 and P2, respectively. The abundance of *Ca. Competibacter* in these facilities was not

only significantly higher than the non-pure oxygen facilities (p<0.05) surveyed in this study, but also much higher than its typical abundance in many EBPR systems: 0.1-0.5% (Nielsen et al. 2019).

A Pearson correlation analysis was conducted to investigate the potential correlation between the PAO and GAO community and the P release and uptake rates. The following significant correlations were observed: (1) P release rate was positively correlated with the abundance of *Accumulibacter* and (2) the abundance of *Tetrasphaera* was positively correlated with *Ca. Competibacter* and the total abundance of GAOs.

4.4. Discussion

4.4.1. Nutrient removal rates in unintended P removal process

Although all seven surveyed facilities have a process configuration similar to the A/O process, P-removal activity was only observed in five facilities. The specific P-release rates (2.1-9.6 $mgP \cdot gVSS^{-1} \cdot hr^{-1}$) and P-uptake rates (0.4-4 $mgP \cdot gVSS^{-1} \cdot hr^{-1}$) measured in this study were lower than typical values reported in purpose-built EBPR processes (Table 4.3). In lab-scale or full-scale EBPR processes, the specific P-release and P-uptake rates are usually in the range of 11-22 and 2-19 $mgP \cdot gVSS^{-1} \cdot hr^{-1}$, respectively (He et al. 2008; Lee and Yun 2014; López-Vázquez et al. 2008). Compared to typical EBPR processes, the impact of factors such as lack of suitable substrate, competition between PAOs and other organisms for substrate (i.e.., GAOs), and the impact of pH and temperature (Bond, Keller, and Blackall 1999; Puig et al. 2008; Saunders et al. 2003; Whang and Park 2006) remain relevant. However, results show that even though these systems are not designed and operated as EBPR systems, the average unintended P release/uptake ratio of 4.4, was within the range of 1.6-5.0 reported in typical EBPR systems by Onnis-Hayden

et al. (2020). Another interesting observation in P5 was that only aerobic P-uptake was observed but no anaerobic P-release (Table 4.3). One possible explanation could be that the PAOs in this system utilized other sources of energy, such as energy generated through anaerobic fermentation, to support subsequent aerobic P-uptake, thus avoilding the need for anerobic P-release for energy production (Marques et al. 2017). Since the energy obtained from anaerobic fermentation is usually too low to support high aerobic P-uptake rates (Marques et al. 2017), a low aerobic P-uptake rate (0.6 $mgP \cdot gVSS^{-1} \cdot hr^{-1}$) was observed in P5. For N removal kinetics, the AOB, NOB and denitrification rates measured in this study were also lower than typical values in BNR processes (Table 4.3). In P1, P2 and P5, AOB and NOB activities were either low or not observed, likely due to the low SRTs ($\leq$ 2.1 day) in these systems. AOB and NOB typically require a minimum SRT of 5.7 and 7.7 days, respectively, to avoid being washed out from the system (Liu and Wang, 2014).

4.4.2. The impact of pure oxygen on community diversity, functional gene profile and the core community structure

The pure oxygen process showed a significant impact on both beta diversity and alpha diversity of the bacterial community. Facilities with pure oxygen processes clearly separately from others on the PCoA plots (Figure 4.2A and 4.2B), indicating a distinct microbial structure difference between pure oxygen facilities and others. Facilities with pure oxygen also showed a lower Faith's phylogenetic diversity (Figure 4.2C). Although pure oxygen has been commonly used as an efficient way to treat high strength wastewater (Rodríguez et al. 2010; Skouteris et al. 2020), its impact on microbial communities has rarely been investigated. Only a few studies have looked into the impact of pure oxygen on microbial communities in bench-scale or pilot-scale activated sludge systems and similar results to this study have been reported (Calderón et al. 2012; Wang et al. 2021; Zhuang et al. 2016). These studies also show that, compared to ambient air

aeration, the pure oxygen process leads to a microbial structure shift and promotes the abundances of certain groups of bacteria, which eventually results in a decrease of richness and diversity of the overall community.

In addition to the alpha and beta diversity, pure oxygen also showed an impact on the functional gene profile of the community (Figure 4.3). The analysis of functional genes is a useful way to predict the functional potentials of the community and may provide more direct information on system performance since the removal of nutrient and organic matter is directly linked to the functional enzymes in a community (Douglas et al. 2020). Polyphosphate kinase, an essential enzyme involved in poly-P synthesis and phosphorus removal (McMahon et al. 2002), appeared with significantly higher abundance in the pure oxygen process (Figure 4.4). This indicates that pure oxygen may promote the gene expression of polyphosphate kinase and lead to higher phosphorus removal potentials (Du et al. 2012; Li et al. 2022). Regarding N metabolism, pure oxygen facilities showed significantly lower abundances in nitrite reductase and nitrous-oxide reductase (Figure 4.4), which are both important to the denitrification process. The complete denitrification reaction sequentially converts nitrate → nitrite → nitric-oxide → nitrous-oxide → dinitrogen (Besson, Almeida, and Silveira 2022). Nitrite reductase is involved in the convention of nitrite to nitric-oxide while nitrous-oxide reductase is involved in the final step of the reaction that reduces nitrous-oxide to molecular nitrogen. The low denitrification rates observed in the two pure oxygen facilities (P1 and P2; Table 4.3) might be attributed to the low genetic abundances of nitrite reductase and nitrous-oxide reductase in these systems.

The structure of the core community and abundant community also appeared to be impacted by pure oxygen process. A core community is typically defined as organisms shared among microbial consortia from a particular habitat (Mielczarek et al. 2013; Shade and

Handelsman 2012) and may play a key role to the stability of the system. In this study, a core community composed of only 54 bacteria genera was found to be common to all surveyed facilities and together they accounted for a high overall abundance (26-52%). Many members of the core community also belonged to the abundant community (Figure 4.5). This showed that a limited number of microorganisms constituted the majority of the microbial community in these activated sludge systems. This finding agrees with other recent studies that also identify a core community in activated sludge (Mielczarek et al. 2013; Qiu et al. 2019; Aaron M Saunders et al. 2016). Particularly in this study, the pure oxygen process was found to have a significant impact on the structure of both the abundant community and the core community (Figure 4.6). Bacterial groups such as *Agitococcus lubricus group*, *Ca. Competibacter, midas_g_558*, *midas_g_3838*, *midas_g_33*, *Gordonia*, *Acinetobacter* were selectively more enriched in the pure oxygen process. This agrees with previous studies that the pure oxygen process could promote the abundance of certain groups of organisms in activated sludge (Wang et al. 2021; Zhuang et al. 2016).

Overall, the pure oxygen process is likely to impact the abundances of metabolic pathways and overall community diversity by selectively stressing and enriching certain microbial groups, as elevated concentrations of oxygen can be toxic to many organisms (Baez and Shiloach, 2014).

4.4.3. The PAO-GAO community and nutrient removal

Although not every surveyed facility showed P-removal activity (Table 4.3), a diverse group of PAOs were identified in all systems (Figure 4.7), and many of them were members of the core community. *Accumulibacter* was the most abundant PAO and appeared in all unintended P removal facilities, at an average abundance of 2.1% , similar to levels found in EBPR systems (e.g., 0.5-3%) (Nielsen et al. 2019; Onnis-Hayden et al. 2020). Other putative PAOs including *Dechloromonas*, *Thauera, Halomonas*, *Pseudomonas* and *Gemmatimonas* were also observed in

all systems and some (e.g., *Dechloromonas*) had abundances comparable to those of full-scale EBPR systems (Nielsen et al. 2019; Aaron M Saunders et al. 2016). Interestingly, while *Tetrasphaera* has been identified as one of the most abundant PAOs (e.g., 10% read abundance) in EBPR systems worldwide including several plants in the US (Mielczarek et al. 2013; Nielsen et al. 2019; Onnis-Hayden et al. 2020), its abundance was relatively low in the surveyed systems (e.g., 0.1-1.3%). It is also interesting that in facility P3 and P7 where no P-removal activity occurred, a diverse group of PAOs were seen. A similar observation was reported by Mielczarek et al. (2013) that PAOs were detected in many non-EBPR plants in Denmark. One possibility of this wide distribution of PAOs could be that many PAOs such as *Tetrasphaera* (Marques et al. 2018), *Thauera* (Wang and He 2020), and *Accumulibacter* (Lanham et al. 2011) contain denitrifying strains and may contribute to denitrification instead of P removal in non-EBPR systems (Table 4.3) (Mielczarek et al. 2013). It is also possible that PAOs such as *Accumulibacter* may have metabolic flexibility under different environments (Guedes da Silva et al. 2020).

The GAO community was much less diverse than the PAO community, and most of them appeared with low abundance in the surveyed facilities. Again, a high abundance of *Ca. Competibacter* was seen in the two pure oxygen facilities P1 and P2 ($p<0.05$). It is likely that pure oxygen processes impose a selective pressure and preferentially selects the outgrowth of *Ca. Competibacter* in the system. However, the impact of pure oxygen on *Ca. Competibacter* has not been reported in previous studies.

Pearson correlation analysis revealed a strong positive correlation between P-release rate and *Accumulibacter* abundance, indicating that *Accumulibacter* was the major contributor to P removal in these unintended P removal facilities. This result agrees with previous studies that *Accumulibacter* abundance is associated with P-removal performance in full-scale EBPR plants

(López-Vázquez et al. 2008; Zilles et al. 2002). *Tetrasphaera* was found to have a strong positive correlation with *Ca. Competibacter* abundance ($p<0.05$) and the total abundance of GAOs ($p<0.05$), suggesting a mutual dependance of these two groups of bacteria. One possibility could be that the fermentation capability of *Tetrasphaera* provides additional VFAs that creates a favorable condition to the growth of GAOs. Future studies might investigate this mutual dependence between *Tetrasphaera* and GAOs.

4.5. Conclusion

This study investigated the nutrient removal potential and microbial community structure in seven unintended P removal facilities in the San Francisco Bay area. The surveyed WWTPs showed large variations in nutrient removal rates, and the observed P and N removal rates were both lower than the rates in observed in BNR processes. The two pure oxygen facilities showed distinct differences in microbial diversity, functional gene profile, and the core community structure compared to the non-pure oxygen facilities. PAOs were widely present in all systems and many of them belonged to the core community. *Accumulibacter* was the most abundant PAO and its abundance correlated with P removal. Overall, these unintended P removal systems have the potential to be optimized for full nutrient removal with better process control and design.

Chapter 5. Conclusion

The Enhanced Biological Phosphorus Removal (EBPR) process is an efficient, economic, and sustainable approach for phosphorus (P) removal from municipal wastewater. For an EBPR process to meet wastewater effluent discharge limits, the kinetics and characteristics of different polyphosphate-accumulating organisms (PAOs) in EBPR have to be thoroughly understood for proper system design and operation. However, previous studies on EBPR microbiology have mainly focused on the model PAO *Candidatus* Accumulibacter while overlooking the importance of other putative PAOs such as *Tetrasphaera*.

This dissertation sought to understand the diversity and kinetics of one putative PAO in both lab-scale and full-scale wastewater treatment process with a focus on *Tetrasphaera*. Specifically, this dissertation (1) systematically reviewed the identification history of *Tetrasphaera* and summarized our current knowledge about this important PAO; (2) conducted a lab-scale study to evaluate the biokinetic parameters of *Tetrasphaera elongata* in pure cultures; and (3) investigated PAO communities in full-scale wastewater treatment plants (WWTPs) in the San Francisco (SF) Bay area. Results from the lab-scale pure culture study showed that the specific growth rate and the specific anaerobic carbon uptake rate of *Tetrasphaera* were significantly different from values of typical PAOs. In addition, the specific carbon uptake rate of *Tetrasphaera* was highly dependent on substrate type. These findings encourage future wastewater process design to incorporate the biokinetic parameters of *Tetrasphaera* into EBPR modeling. Results from full-scale investigation showed that a diverse group of PAOs were widely present in all seven surveyed facilities with unintended phosphorus removal in the SF Bay area. However, the observed

nutrient removal rates in the surveyed facilities were lower than typical values in biological nutrient removal (BNR) process. This suggests that these unintended P removal systems have the potential to be optimized for full nutrient removal with better process control and design.

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Appendices

Appendix A. Developing Occupational and Health Susceptibility Personas for Wastewater Personnel in the United States in the Age of COVID-19[2]

Abstract

Compared to the general public, wastewater personnel, are at an increased risk of infection and illness from wastewater pathogens due to work-related tasks. Unfortunately, current risk assessment approaches do not consider individual personnel factors (e.g., age and health conditions) that may influence their susceptibility to a health effect. The objective of this study is to establish a baseline level of occupational and health factors among the wastewater personnel population, quantify these factors using a susceptibility evaluation scoring system and examine relevant susceptibility features using the concept of "Personas". Using survey data from 246 respondents and risk ratios for SARS-CoV-2 as a surrogate virus, personnel clustered into three persona groups: "low susceptibility", "high occupational susceptibility" and "high health susceptibility". Results highlight the intersectionality between gender, age, underlying health conditions, job tasks and level of exposure to wastewater and provide context for incorporating individual variables into risk assessment methodologies with the goal of protecting this essential workforce.

[2]Adapted from a manuscript accepted by *Water Environment Research*. Zhang, Y., Ha, J., Kinyua, M.N. "Developing Occupational and Health Susceptibility Personas for Wastewater Personnel in the United States in the Age of COVID-19" (2022).

Appendix B. The relationship between carbon fractionation, nutrient removal, and operational parameter

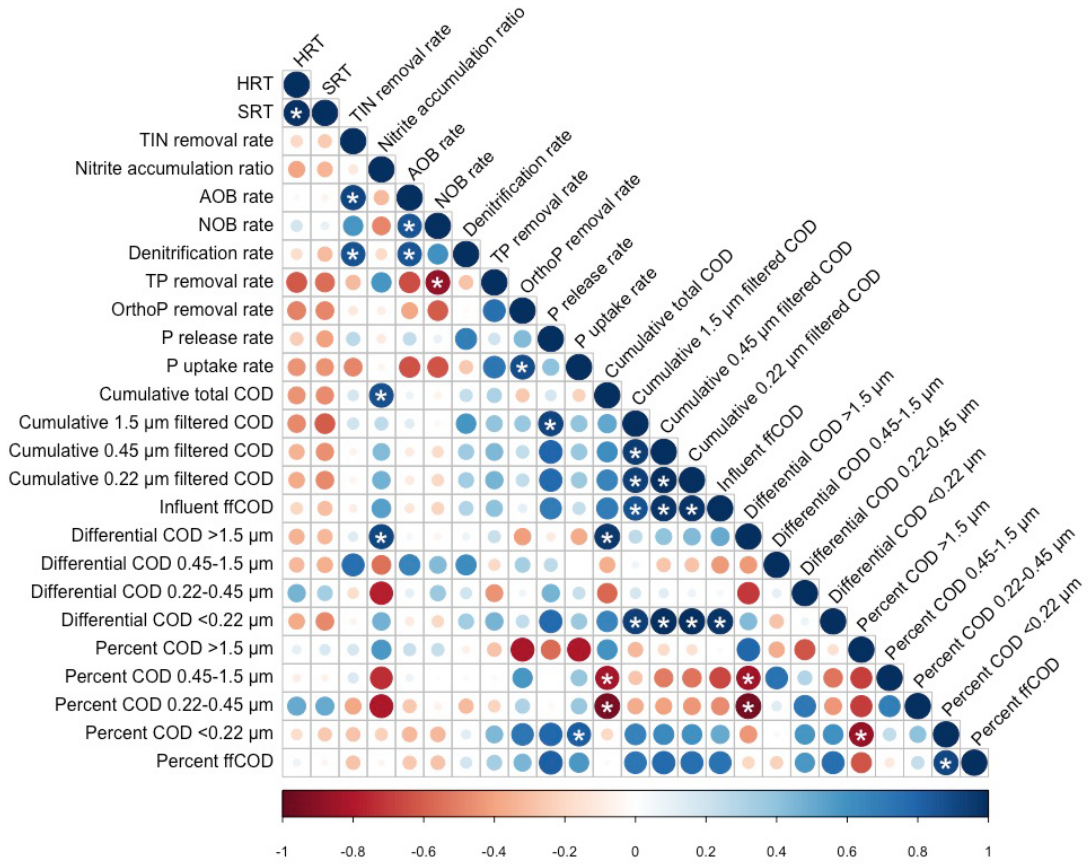

Figure B.1. Pearson correlation matrix showing the relationship between nutrient removal rates, influent COD fractionation and operational parameters. Blue, positive correlation; red, negative correlation; asterisk (*), P-value of < 0.05. Cumulative COD is defined as the total COD below the selected filter size, and differential COD is defined as the COD concentration at a certain particle size category.