Identity and physiology of glycogen accumulating organisms in activated sludge

A thesis presented in fulfilment of the requirements for the degree of

Doctor of Philosophy

Cristobal Andres Onetto Carvallo

The University of Adelaide

School of Agriculture, Food & Wine

May 2018

Supervisors:

Assoc. Prof. Paul Grbin
Dr. Kathryn Eales

Table of Contents

Summary

Glycogen accumulating organisms (GAOs) form part of the core microbial component of the activated sludge community of both industrial and domestic wastewater treatment plants. A plethora of research has focused on the activity of GAOs in enhanced biological phosphorus removal (EBPR) domestic wastewater treatment plants due to their competition for carbon with the polyphosphate accumulating organisms (PAOs) at the expense of EBPR efficiency. However, in industrial systems such as winery wastewater (WWW), where GAOs are known to proliferate and cause settling problems, limited research has focused on their identification and ecophysiology.

Initial metagenomics and fluorescence *in situ* hybridization (FISH) surveys of WWW treatment plants revealed that *Defluviicoccus*-related GAOs belonging to cluster II (DF2) and III (DF3) appeared as highly dominant with abundances reaching up to 52% of the bacterial population. GAOs belonging to the Competibacteraceae (CPB_S18 and CPB_S60) were also observed in notable numbers along with putative GAOs belonging to the Actinobacteria (*Micropruina* spp. and *Nakamurella* spp.) and Gammaproteobacteria CCM19a.

Previous investigations have suggested that high carbon loads along with low nitrogen levels are a combination of factors that enhance the proliferation of GAOs. In order to elucidate if the low nitrogen levels of WWW are the underlying cause of DF2 proliferation, incubations under different COD:N ratios using ^{13}C - acetate and ^{15}N - urea were performed. Cell substrate assimilation was quantified using an innovative technique, FISH-NanoSIMS, revealing that low (100:1) or null nitrogen concentrations enhanced DF2 carbon uptake while ratios of 60:1 and 20:1 reduced carbon uptake. Nitrogen dosing at COD:N ratios of 60:1 or higher was demonstrated as a feasible strategy for controlling the excessive DF2 growth in WWW treatment plants.

The unique filamentous morphology of DF3 is important as its proliferation leads to severe bulking issues. Although previously reported as abundant in industrial and domestic activated sludge, limited research has focused on understanding the physiology of these organisms. The first genome of a filamentous DF3 was extracted from a WWW activated sludge metagenome. Annotation revealed interesting metabolic features that help to understand the competitiveness and abundance of this microorganism in WWW activated sludge. The genetic potential to cycle trehalose through glycogen, nitrogen fixation, hydrogenase activity and urea uptake appear as adaptive strategies of DF3 to the WWW nitrogen limited environment.

In a recent 16S rRNA survey of EBPR plants, *Micropruina* spp. were identified as the most abundant GAO, yet little is known about their ecophysiology. To further elucidate the ecophysiology of this putative GAO observed in WWW and EBPR treatment plants, genomic and metabolomic studies were made in pure culture *Micropruina glycogenica* str. Lg2^T and compared to the *in situ* physiology of the

genus using state-of-the-art single cell techniques. *Micropruina* spp. were observed to take up carbon substrates under anaerobic conditions, which were partly fermented to lactate, acetate, propionate and ethanol, and partly stored as glycogen for aerobic use. This physiology is markedly different from the classical GAO model, suggesting a need to reconsider current understanding of the GAO phenotype.

Metagenomics analyses revealed a codominance of filamentous *Thiothrix* spp. and GAOs in a particular WWW treatment plant with bulking issues. In an attempt to understand this problem, a further experiment based on raw wastewater feeding was performed. Raw feeding reduced the *Thiothrix* spp. population and improved settling, therefore, direct feeding is proposed as a control method for industrial plants with surge/anaerobic lagoons in order to manage the bulking problems caused by *Thiothrix* spp..

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Cristobal A. Onetto   Date   15/05/2018

Acknowledgements

Chapter 1

1. Review of literature

1.1. Winery wastewater

1.1.1. Winery wastewater characteristics

Winery wastewater (WWW) is composed primarily of grape juice, wine, suspended solids and cleaning agents (Mosse et al. 2011). Organic compounds represent approximately 80 to 85% of the total waste generated (not including water) (Ruggieri et al. 2009), with the inorganic component dependent on the types of cleaning agents and winemaking additives used.

The composition of WWW differs considerably from that of domestic wastewater (**Table 1.1**), as it contains high organic loads, low pH and variable nutrient concentrations. Due to its characteristics, WWW requires treatment before reuse or discharge, creating a significant challenge for biological treatment.

The major organic constituents in WWW are typically ethanol, sugars and organic acids (**Table 1.2**), generally measured in wastewater according to two different parameters; biological oxygen demand (BOD) and chemical oxygen demand (COD).

The inorganic composition of WWW can vary markedly depending on the chemical characteristics of the cleaning agent used in the winery and diverse winemaking practices (Mosse et al. 2011). The main inorganic ions are potassium, sodium and calcium.

Table 1.1. Typical domestic and winery wastewater composition. Adapted from (Conradie, et al. 2014; Gray 2010)

Parameter	Unit	Domestic		Winery
		US	UK	
pH	–	7.0	7.0	4.9
BOD	mg/L	250	350	9574
COD	mg/L	500	700	14426
Suspended solids	mg/L	250	400	2845

BOD: biochemical oxygen demand; COD: chemical oxygen demand.

Table 1.2. Winery wastewater summary of carbon composition (g/L). Data obtained from AGWA project (Project UA1301)

Parameter	Max	Mean
Citrate	0.39	0.06
Tartrate	5.80	1.20
Malate	1.23	0.06
Succinate	0.50	0.09
Lactate	1.52	0.31
Acetate	4.20	0.40
Glucose	1.30	0.18
Fructose	2.87	0.45
Glycerol	0.51	0.06
Ethanol	7.39	1.36

1.1.2. Treatment options

One of the major issues associated with WWW treatment are the large fluctuations in flow volumes throughout the year, due to the seasonal nature of the product with the majority of WWW generated in a 8 week period. This results in high variations of BOD/COD, pH, ion and nutrient concentrations (Brito et al. 2007), consequently affecting the stability and efficiency of the water treatment process.

Despite the availability of multiple physicochemical and biological ways to treat WWW, the high concentrations of readily biodegradable organic compounds make aerobic biological treatment one of the most effective options as well as the most commonly used for WWW in Australia (Kumar et al. 2009).

Aerobic treatment relies on oxygen to facilitate the breakdown of organic compounds by heterotrophic microorganisms (Seviour & Nielsen 2010). The microbial community composition of heterotrophic microorganisms will be affected by the fluctuations in volume and unique chemical composition of WWW, which can lead to unbalanced microbial populations and operational problems (McIlroy et al. 2011). Despite this, limited research has focused on understanding the WWW activated sludge community (Jobbgy et al. 2002; Kiss et al. 2011; McIlroy et al. 2011) and specific microbial populations associated to operational problems.

1.1.3. Problems of solid separation

The activated sludge process will ultimately depend on the rapid and complete separation of the biomass from the liquid supernatant. However, this is not always achieved and poor flocculation and settling of the biomass occurs, an event that is defined as bulking. Much of the activated sludge biomass is organised in flocs. Flocs are structures composed from aggregates of bacteria and other organisms embedded

in a polymeric matrix. Bulking is usually associated with filamentous bacteria growing away from the floc (Seviour & Nielsen 2010), yet previous WWW activated sludge investigations have associated the proliferation of tetrad forming organisms (TFOs) to non-filamentous bulking events (McIlroy et al. 2011). These TFOs were identified as alphaproteobacterial GAOs using fluorescence *in situ* hybridization (FISH). High abundance of TFOs have also been observed in samples taken from sequencing batch reactors (SBR) treating WWW in Australia (Project UA1301[1]). These samples have often been taken from plants with poor sludge settleability. They are present as a part of the floc and in free suspension, increasing the turbidity of the treated water, therefore affecting effluent quality.

Evidence suggests that GAOs may play a fundamental role in the major problems observed in WWW treatment plants. In order to manage these issues, appropriate control strategies are required. To effectively design these control strategies, the precise identification and physiology of the dominating organisms has to be elucidated. This methodology has been previously performed for other problematic microbial communities, leading to the development of control tools to manage their excessive proliferation (e.g. nutrient addition, manipulation of SRT/HRT and aeration) (Nielsen et al. 2000; Seviour & Nielsen 2010).

1.2. Who are the GAOs?

The term glycogen accumulating organism was proposed by Mino et al. (1995) and is described as the phenotype of organisms that store glycogen aerobically and consume it anaerobically as the primary source of energy for taking up carbon sources and storing them as polyhydroxyalkanoates (PHAs) granules.

Cech and Hartman (1993) first recorded the presence of gram-negative cocci in clumps and packages of tetrads in a laboratory scale enhanced biological phosphorus removal (EBPR) treatment system. This culture could take up substrates anaerobically without any polyphosphate accumulation, and so they were considered to compete with the polyphosphate accumulating organisms (PAOs) for the uptake of carbon substrates. Liu et al. (1994) proposed that the use of stored glycogen through glycolysis was giving these organisms the energy to assimilate substrates anaerobically. This was based on the observations that acetate uptake and PHA granules decreased after the inhibition of glycolysis in a sample dominated TFOs.

GAOs are considered competitors of PAOs because they can proliferate under similar conditions of anaerobic and aerobic cycling without contributing to the phosphorus removal of the EBPR system.

The current knowledge related to GAOs is based on EBPR systems. Due to the lack of studies focused on GAOs in WWW, the next sections will review the reported research of GAOs associated to EBPR systems, making references to the available WWW reports when suitable.

---

[1] Developing a fundamental understanding of the microbiological treatment of winery wastewater. Wine Australia (2014-2016)

1.2.1. Identification of GAOs

Extensive research has focused on the identification of potential GAOs in wastewater environments (Seviour et al. 2000; Oehmen et al. 2007). However, this is not the case for WWW, where only one study has profiled the microbial community of WWW treatment plants (McIlroy et al. 2011).

The ability to study GAOs primarily relies on *in situ* molecular methods to identify and quantify them. The following is a review of the putative GAOs discovered so far, including their most recent phylogenetic classification (**Table 1.3**).

Table 1.3. Putative GAOs (Adapted from Stockholm-Bjerregaard et al. 2017)

Phylum/class	Family	Genus
Alphaproteobacteria	Rhodospirillaceae	*Defluvicoccus*
Betaproteobacteria	Rhodocyclaceae	*Propionivibrio*
	Comamondaceae	spb280
Gammaproteobacteria	Competibacteraceae	Competibacter- lineage (13 clades)
	CCM19a	CCM19a
Actinobacteria	Propionibacteriaceae	*Micropruina*
		sbr-gs28
	Nakamurellaceae	*Nakamurella*
	Dermatophilaceae	*Kineosphaera*

Alphaproteobacteria

Defluvicoccus vanus - related.

Alphaproteobacterial GAOs have been reported in multiple laboratory scale and full-scale treatment plants. Wong et al. (2004) observed a highly enriched culture of TFOs in a laboratory scale EBPR with deteriorated phosphorus removal. Using 16S rRNA clone libraries, these bacteria were identified as closely related to the isolate *Defluvicoccus vanus* (Maszenan et al. 2005), and two FISH probes were designed for their identification (TFO_DF218 and TFO_DF618). Meyer et al. (2006) observed TFOs not binding with these probes therefore phylogenetic analyses were performed revealing a second cluster of *D. vanus* related organisms. FISH probes DF988 and DF1020 were designed to target them. Two more clusters have subsequently been identified, including cluster III, with bacteria displaying a filamentous morphology (Wong & Liu 2007; McIlroy & Seviour 2009). The GAO physiology for the *D. vanus* related has been confirmed *in situ* (Wong & Liu 2007) and through metagenomics (Nobu et al. 2014; Wang et al. 2014). *D. vanus* - related bacteria belonging to cluster I and II appear to be highly relevant to WWW treatment plants (McIlroy et al. 2012) yet, larger surveys using different techniques such as 16S rRNA gene profiling have to be performed to confirm their actual abundance.

<u>Betaproteobacteria</u>

Propionivibrio spp.

Closely related to the Accumulibacter PAOs, GAOs belonging to the *Propionivibrio* genus have been recently reported in full-scale EBPR treatment plants (Albertsen et al. 2016). Their GAO metabolism was confirmed with *in situ* techniques as well as genome annotation. They were recently reported as abundant in Danish wastewater treatment plants (Stokholm-Bjerregaard et al. 2017).

spb280

Kong et al. (2007) investigated the ecophysiology of the microbial community of an EBPR treatment plant using 16S rRNA sequencing, FISH probe design and microautoradiography (MAR). Betaproteobacteria binding to the Bet65 probe displayed anaerobic uptake of volatile fatty acids (VFAs), positive PHAs and negative polyP storage and were therefore proposed as putative GAO. The family and genus name spb280 are based on the MiDAS taxonomy database (McIlroy et al. 2015).

<u>Gammaproteobacteria</u>

'*Candidatus* Competibacter phosphatis' lineage

Crocetti et al. 2002 originally investigated these group of GAOs using FISH and staining procedures, observing positive PHAs and negative polyP storage. Since then, the Competibacter - lineage has substantially expanded into thirteen subgroups and FISH probes have been designed to target them at different hierarchical levels (Crocetti et al. 2002; Kong et al. 2002; Kim et al. 2011; McIlroy et al. 2015). Although these group of GAOs are phylogenetically diverse, they share a similar GAO phenotype (Kong et al. 2006).
Bacteria belonging to the Competibacter - lineage are routinely observed proliferating in numerous full and lab-scale EBPR treatment plants (Crocetti et al. 2002; McIlroy et al. 2013), however there is no report of the presence of these microorganisms in WWW.

CCM19a

Kong et al. (2007) investigated the ecophysiology of the microbial community of an EBPR treatment plant using 16S rRNA sequencing, FISH probe design and microautoradiography (MAR). Gammaproteobacteria binding to the Gam445 probe displayed anaerobic uptake of VFA, positive PHAs and negative polyP storage and therefore were proposed as putative GAO. The family and genus CCM19a name are based on the MiDAS taxonomy database (McIlroy et al. 2015).

Actinobacteria

Micropruina spp.

Micropruina glycogenica was isolated from an EBPR system. The pure culture displayed anaerobic carbohydrate accumulation without polyP storage (Shintani et al. 2000). A recent study revealed them as the most abundant putative GAO in Danish wastewater treatment plants (Stokholm-Bjerregaard et al. 2017). Despite this, little is known about their physiology and role in wastewater treatment plants.

sbr-gs28

Kong et al. 2001 observed small cocci in an aerobic-anaerobic non-P removing SBR binding to the probe designed to target the clone sbr-gs28. They showed *in situ* storage of both acetate and glucose as PHAs without polyP storage, consistent with the GAO phenotype. Sbr-gs28 has been recently reported as one of the most abundant GAOs in Danish wastewater treatment plants (Stokholm-Bjerregaard et al. 2017).

Nakamurella multipartita

Strain Y-104^T was isolated from activated sludge initially acclimated with sugar-containing synthetic wastewater (Yoshimi et al. 1996). This strain showed an obligately aerobic metabolism along with the ability to uptake a broad range of sugars (no acetate uptake) and accumulation of large amounts of polysaccharides with no polyP storage. Its GAO metabolism has not been confirmed *in situ*.

Kinoesphaera limosa

Kinoesphaera limosa was isolated by Liu et al. (2002) from an inefficient EBPR system. With the use of staining methods, the storage of PHAs and not polyP was observed indicating a putative GAO metabolism. However, their GAO metabolism has not been confirmed *in situ*.

1.2.2. Metabolism of the GAOs

Several metabolic models have been proposed for the uptake of acetate and propionate in some of the known GAOs related to EBPR systems (Oehmen et al. 2007). However, it is still unknown if these models work in the same way for the aerated systems commonly used in the wine industry (Mosse et al. 2011). During the anaerobic phase of the EBPR treatment plants, GAOs can compete with PAOs for the uptake of carbon substrates that are stored as PHAs. Then in the aerobic phase, PHAs are used for biomass growth, glycogen replenishment and cell maintenance (Oehmen et al. 2007). There is no universal biochemical model for GAOs, mainly because of

the lack of characterisation of the populations present and the diversity of putative GAOs.

As detailed below, under the anaerobic/aerobic cycling of EBPR systems, GAOs present several metabolic traits that allow them to outcompete other microorganisms in the activated sludge. These metabolic traits are well understood under EBPR conditions, however GAOs observed in SBRs treating WWW are exposed to completely different conditions. The mechanisms that GAOs use to proliferate and outcompete other microorganisms under SBR conditions are still unknown.

1.2.3. Glycogen and PHA metabolism

Hydrolysis of glycogen by GAOs during the anaerobic phase was originally postulated by Satoh et al. (1994) to occur through the Embden–Meyerhof–Parnas (EMP) pathway. This was later supported by Filipe et al. (2001) observations on the inactivity of the enzyme glucose 6 – phosphate (Entner–Doudoroff (ED) pathway enzyme) on a GAO enriched sludge. However, using *in vivo* nuclear magnetic resonance, Lemos et al. (2007) demonstrated that an enriched culture of GAOs containing 46% Competibacter and 42% *D. vanus* – related, consumed glycogen through the ED pathway. This was later supported by the presence of genes belonging to the ED pathway in one of the two Competibacter - lineage genomes investigated by McIlroy et al. (2013). Metagenomic characterisation of two *D. vanus* – related genomes belonging to cluster I (Nobu et al. 2014) and cluster II (Wang et al. 2014) showed absence of genes encoding for ED pathway enzymes. These differences support the idea that phylogenetically different GAOs possess different glycogen degradation pathways, and it is most likely that some GAOs belonging to the Competibacter - lineage can shift their dependence on the EMP to the ED pathway as a response to certain environmental conditions such as high temperatures (Lemos et al. 2007).

Under anaerobic conditions, GAOs are able to synthesise PHAs from organic acids derived from glycogen degradation and/or from uptake of external substrates including acetate and propionate (Wong & Liu 2007). The energy required for synthesis of PHAs is derived from the consumption of glycogen. During this process, an excess of reducing equivalents are produced that need to be balanced (see later). Assimilated acetate and propionate are reduced to acetyl-CoA and propionyl-CoA (**Figure 1.1**) (Nobu et al. 2014; Satoh et al. 1994) and then polymerised into polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) as storage compounds (Filipe et al. 2001). All the reported draft genomes of putative GAOs encode genes for the activation of acetate and propionate along with their polymerization to PHAs as well as glycogen synthesis and hydrolysis (McIlroy et al. 2013; Nobu et al. 2014; Wang et al. 2014; Albertsen et al. 2016).

The synthesis of propionyl-CoA through the reductive branch of the TCA cycle and the methylmalonyl-CoA pathway (**Figure 1.1**) may help to consume the excess of reducing equivalents produced from glycogen consumption and also may be

indispensable to create a proton motive force (PMF) for the anaerobic intake of carbon substrates through the membrane (Burow et al. 2008). Hydrogenase activity (Albertsen et al. 2016) and fermentation of sugars (McIlroy et al. 2013) have also been recently proposed as mechanism to balance the excess of reducing equivalents. During the aerobic phase, PHAs are oxidised to obtain energy for cell growth, maintenance and glycogen replenishment (Zeng et al. 2003).

1.2.4. Mechanisms for VFAs uptake

Saunders et al. (2007) postulated that VFAs uptake in the Competibacter - lineage GAOs is energised by a PMF generated by the export of protons through an F_1F_0-ATPase, based on the observed reduction of acetate uptake with addition of NN'-dicyclohexylcarboiimide, an ATPase inhibitor. The export of protons by the activity of the fumarate reductase complex was predicted to contribute to the remaining PMF. McIlroy et al. (2013) reported the presence of genes corresponding to these two membrane associated complexes, however, they also suggested the involvement of a sodium potential related to the methylmalonyl-CoA decarboxylase complex (**Figure 1.1**) as reported for the *D. vanus* related – GAOs (Burow et al. 2008). For these GAOs, no VFA uptake reduction was observed in the presence of NN' - dicyclohexylcarboiimide suggesting no involvement of an ATPase in the generation of a PMF. However, part of the acetate uptake was inhibited with oxantel (selective inhibitor of fumarate reductase activity) suggesting that a PMF is generated through the fumarate reductase complex. An observed uptake inhibition in the presence of monesin also indicated the possible involvement of a sodium potential in the substrate uptake mechanism (Burow et al. 2008)
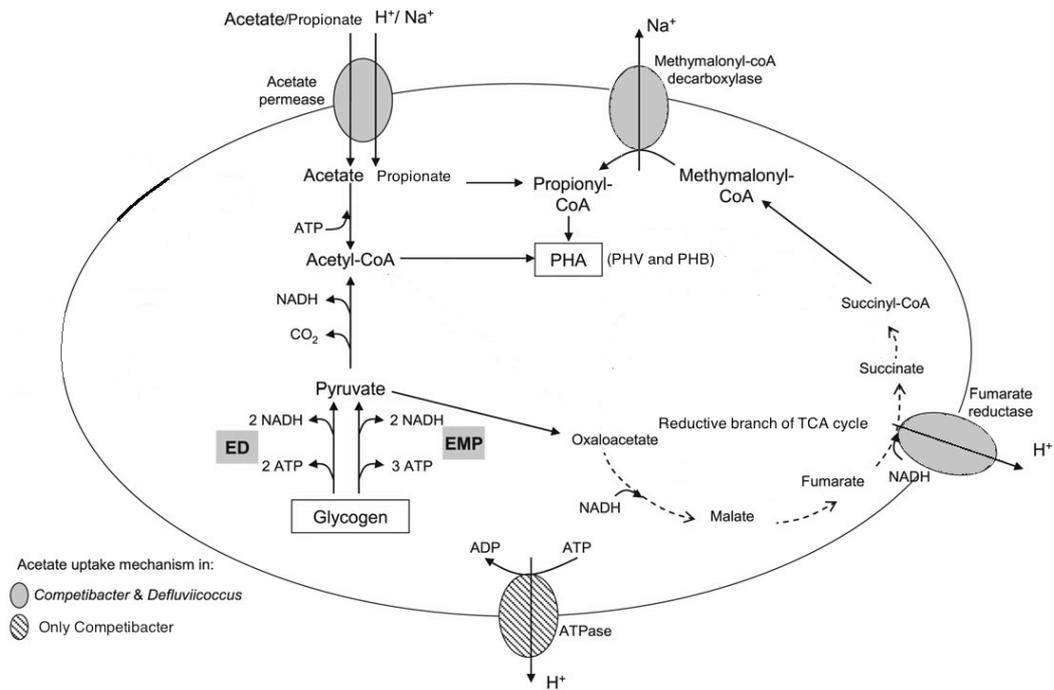
Figure 1.1. Proposed anaerobic metabolic pathways and VFA uptake mechanism for Competibacter - lineage and *Defluvicoccus vanus* - related GAOs. GAOs take up carbon substrates anaerobically through the membrane with energy generated by a proton or a sodium efflux. ATP and NADH production and consumption processes from GAOs are shown, including glycolysis and the reductive branch of the TCA cycle. Adapted from Oehmen et al. (2010).

1.2.5. Nitrogen metabolism

Differences in regard to nitrogen metabolisms have been reported between the putative GAOs. *In situ* observations indicate that Competibacter species exhibit different denitrification abilities (Kong et al. 2006; McIlroy et al. 2013) (**Table 1.4**), with only some subgroups being able to carry out full denitrification while others are only able to reduce nitrate to nitrite. This correlates with McIlroy et al. (2013) observations in which only a Competibacter GAO from subgroup 2 presented the genetic potential for full denitrification from nitrate to nitrogen.

Wang et al. (2008) investigated the denitrification capability of cluster I *D. vanus* - related GAOs. It was observed that in a highly enriched culture, cluster I GAOs were only able to reduce nitrate to nitrite, even in treatments in which GAOs were previously adapted to anoxic conditions. This is consistent with other metagenomic studies where neither members of cluster I or II showed a complete set of genes encoding for denitrification (Nobu et al. 2014; Wang et al. 2014)(**Table 1.4**). The absence of these metabolic pathways has been postulated as a control method for the proliferation of *D. vanus* - related GAOs in EBPR treatment plants by the addition of nitrite (Tayà et al. 2013).

The recent characterisation of a *Propionivibrio* GAO also revealed the absence of genes for full denitrification (Albertsen et al. 2016).

Table 1.4 Summary of the nitrogen metabolisms reported for GAOs. Adapted from (Albertsen et al. 2016)

Nitrogen metabolism	"*Ca.* P. aalborgensis"	*Competibacteraceae*	*D. vanus* – related
Nitrate reduction to nitrite	+	+	+
Nitrate reduction to N_2 (denitrification)	-	±	-
Nitrite reduction (respiratory)	+	±	-
Nitrite reduction to ammonia (assimilatory)	+	±	+
Nitrogen fixation	+	±	+

1.2.6. Ecophysiology

Microautoradiography (MAR) in combination with FISH is a powerful technique that illustrates *in situ* substrate uptake of specific microorganisms in the activated sludge. Multiple studies have been conducted to understand the substrate competition between PAOs and GAOs in the EBPR process along with genomic analyses to confirm the genetic potential for utilisation of these substrates. **Table 1.5** summarises the MAR ecophysiology studies focused on the Competibacter – lineage and *D. vanus* – related GAOs.

Table 1.5. Uptake of carbon substrates by Cluster I and Cluster II *Defluvicoccus* and Competibacter - lineage under anaerobic and aerobic conditions determined by MAR and metagenomics. Adapted from Kong et al. (2006); Burow et al. (2007); Wong & Liu (2007); Schroeder et al. (2008); McIlroy et al. (2013).

Labelled substrate	*Defluvicoccus - related*				Competibacter - lineage	
	Cluster I		Cluster II			
	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic
Aspartate	-	- (+)	-	- (+)	-	- (+)
Glutamate	-	-	-	- (+)	-	-
Glycerol	n/a	-	n/a	+	n/a	-
Oleate	-	-	-	- (+)	-	-
Palmitate	n/a	-	n/a	-	n/a	-
Propionate	+	+	+	+	+	+
Glucose	- (+)	- (+)	+	- (+)	+	- (+)
Acetate	+	+	+	+	+	+
Formate	-	-	-	-	- *	- *
Butyrate	-	-	-	-	-	-
Lactate	+	n/a	n/a	n/a	+	-
Pyruvate	+	+	+	+	+	+
Ethanol	-	-	-	-	-	-
Mannose	-	-	-	-	-	-
Leucine	-	-	-	-	-	-
Glycine	-	-	-	-	-	-
Thymidine	-	-	-	-	- **	- **
Amino acids	n/a	n/a	+	+	+	+

* Only sub group 6 showed formic acid uptake.

** Only sub group 1 and 6 showed thymidine uptake.

() Discrepancies between different studies.

+ Bacteria took up substrate.

- Bacteria did not take up substrate.

1.2.7. Effect of environmental factors

The effect of pH in the competition between GAOs and PAOs has been extensively studied for EBPR systems (Oehmen et al. 2007). The literature suggests that changes in pH have an effect on the growth of GAOs. It has been reported that high pH decreases the acetate uptake rates, anaerobic use of glycogen and PHV accumulation in GAOs (Filipe et al. 2001). Filipe et al. (2001) postulated that pH 7.25 was the shifting point for the GAOs and PAOs competition based on the observation that GAOs had a faster acetate uptake rate under this pH and on the contrary, PAOs could take up acetate faster above this pH.

Temperature has also been suggested as an environmental factor affecting the proliferation of GAOs, where in most cases GAOs tend to be more competitive at higher temperatures (Oehmen et al. 2007). With the application of FISH, Ong et al. (2014) could observe a clear shift of population from PAOs to Competibacter GAOs as temperature increased in a SBR, along with a high abundance of GAOs at 32°C.

Dissolved Oxygen (DO) is an important factor to consider when managing an SBR, mainly due to the energetic costs associated to aerating and because it is one of the few parameters that can be controlled. The literature suggests that higher levels of DO would favour GAOs proliferation (Carvalheira et al. 2014).

1.3. Research aims

Evidence suggests that GAOs form part of the core microbial component of WWW activated sludge (McIlroy et al. 2012). Their proliferation is associated with operational problems such as bulking and turbid supernatants. The plethora of research focused on these microorganism (Oehmen et al. 2007; Stokholm-Bjerregaard et al. 2017) has helped to elucidate their phylogeny, abundance and metabolism in EBPR treatment plants. Despite being phylogenetically diverse, GAOs share unique metabolic traits including anaerobic uptake of VFAs and storage of glycogen. These traits make them competitive and potentially detrimental to EBPR systems due to their competition with the PAOs. In contrast to EBPR systems, WWW treatment relies on the activated sludge aerobic consumption of high carbon loads. The limited research available indicates phylogenetic similarities between the GAOs dominating in WWW and EBPR activated sludge (McIlroy et al. 2012). Yet, the underlying causes of their proliferation are still unknown.

The primary aim of this research is to expand the current knowledge associated to GAOs in WWW. One priority will be to identify and quantify the main GAOs present in WWW in order to specifically investigate the potential factors promoting their proliferation.

The nutrient deficiency of WWW has been hypothesised as the main factor positively influencing the proliferations of GAOs (McIlroy et al. 2012), however, there is no scientific background to support this statement. *In situ* experiments will be performed to investigate the effect of nitrogen supplementation over the carbon metabolism of

the main GAO observed in WWW activated sludge. Quantifying this effect will potentially help to develop tools to control the growth of these microorganisms.

The genome of a dominant *Defluviicoccus* - related will be extracted and annotated to investigate the genetic features that allows it to compete and proliferate under nitrogen limited conditions.

The ecophysiology of a rare and less studied GAO will be also investigated using a suite of state-of-the-art techniques in order to expand the current general GAO knowledge.

Finally, based on an understanding of the identity and ecophysiology of the microbial community in a full-scale wastewater treatment plant, a three-month trial will be conducted to improve bulking problems.

References

Albertsen, M, McIlroy, SJ, Stokholm-Bjerregaard, M, Karst, SM & Nielsen, PH 2016, ' "*Candidatus* Propionivibrio aalborgensis": a novel glycogen accumulating organism abundant in full-scale enhanced biological phosphorus removal plants', Frontiers in Microbiology, vol. 7, doi: 10.3389/fmicb.2016.01033.

Albertsen, M, Karst, S, Ziegler, AS, Kirkegaard, RH & Nielsen, PH 2015, 'Back to Basics ? The influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities', PLoS ONE, vol. 10, e0132783.

Albertsen, M, Hugenholtz, P, Skarshewski, A, Nielsen, KL., Tyson, GW & Nielsen, PH 2013, 'Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes', Nature Biotechnology, vol. 31, p. 533.

Amann, RI, Binder, BJ, Olson, RJ, Chisholm, SW, Devereux, R & Stahl, DA 1990, 'Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations', Applied and Environmental Microbiology, vol. 56, pp. 1919-1925.

Bolger, AM, Lohse, M & Usadel, B 2014, 'Trimmomatic: a flexible trimmer for Illumina sequence data', Bioinformatics, vol. 30, pp. 2114-2120.

Brito, AG, Peixoto, J, Oliveira, JM, Oliveira, JA, Costa, C, Nogueira, R & Rodrigues, A 2007, 'Brewery and winery wastewater treatment: some focal points of design and operation', Utilization of by-products and treatment of waste in the food industry, Springer, Boston, MA, pp. 109-131.

Burow, LC, Kong, Y, Nielsen, JL, Blackall, LL & Nielsen, PH 2007, 'Abundance and ecophysiology of *Defluviicoccus* spp., glycogen-accumulating organisms in full-scale wastewater treatment processes', Microbiology, vol. 153, pp. 178-185.

Burow, LC, Mabbett, AN, McEwan, AG, Bond, PL & Blackall, LL 2008, 'Bioenergetic models for acetate and phosphate transport in bacteria important in enhanced biological phosphorus removal', Environmental Microbiology, vol. 10, pp. 87-98.

Caporaso, JG, Kuczynski, J, Stombaugh, J, Bittinger, K, Bushman, FD, Costello, EK, Fierer, N, Pena, AG, Goodrich, JK & Gordon, JI 2010, 'QIIME allows analysis of high-throughput community sequencing data', Nature Methods, vol. 7, pp. 335-336.

Carvalheira, M, Oehmen, A, Carvalho, G, Eusébio, M & Reis, MAM 2014, 'The impact of aeration on the competition between polyphosphate accumulating organisms and glycogen accumulating organisms', Water Research, vol. 66, pp. 296-307.

Cech, JS & Hartman, P 1993, 'Competition between polyphosphate and polysaccharide accumulating bacteria in enhanced biological phosphate removal systems', Water Research, vol. 27, pp. 1219-1225.

Conradie, A, Sigge, G & Cloete, T 2014, 'Influence of winemaking practices on the characteristics of winery wastewater and water usage of wineries', South African Journal of Enology and Viticulture, vol. 35, pp. 10-19.

Crocetti, GR, Banfield, JF, Keller, J, Bond, PL & Blackall, LL 2002, 'Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes', Microbiology, vol. 148, pp. 3353-3364.

Daims, H, Brühl, A, Amann, R, Schleifer, K-H & Wagner, M 1999, 'The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set', Systematic and Applied Microbiology, vol. 22, pp. 434-444.

Daims, H, Stoecker, K & Wagner, M 2005, 'Fluorescence *in situ* hybridization for the detection of prokaryotes', Molecular Microbial Ecology, vol. 213, p. 239.

Edgar, RC 2013, 'UPARSE: highly accurate OTU sequences from microbial amplicon reads', Nature Methods, vol. 10, pp. 996-998.

Falvo, A, Levantesi, C, Rossetti, S, Seviour, R & Tandoi, V 2001, 'Synthesis of intracellular storage polymers by *Amaricoccus kaplicensis*, a tetrad forming bacterium present in activated sludge', Journal of Applied Microbiology, vol. 91, pp. 299-305.

Filipe, CD, Daigger, GT & Grady, C 2001, 'A metabolic model for acetate uptake under anaerobic conditions by glycogen accumulating organisms: stoichiometry, kinetics, and the effect of pH', Biotechnology and Bioengineering, vol. 76, pp. 17-31.

Filipe, CDM, Daigger, GT & Grady, CPL 2001, 'pH as a key factor in the competition between glycogen-accumulating organisms and phosphorus-accumulating organisms', Water Environment Research, vol. 73, pp. 223-232.

Fillaudeau, L, Bories, A, Decloux, M, Klemeš, J, Smith, R & Kim, J 2008, 'Brewing, winemaking and distilling: an overview of wastewater treatment and utilisation schemes', Handbook of water and energy management in food processing, Woodhead Publishing, Cambridge, England, pp. 929-995.

Gray, NF 2010, 'Chapter 13 - Nature of wastewater', in Water technology (third edition), Butterworth-Heinemann, Oxford, pp. 403-424.

Jobbgy, A, Literthy, B & Tardy, G 2002, 'Implementation of glycogen accumulating bacteria in treating nutrient-deficient wastewater', Water Science & Technology, vol. 46, pp. 185-190.

Kim, JM, Lee, HJ, Lee, DS, Lee, K & Jeon, CO 2011, 'Identification of a novel subgroup of uncultured gammaproteobacterial glycogen-accumulating organisms in enhanced biological phosphorus removal sludge', Microbiology, vol. 157, pp. 2287-2296.

Kiss, B, Bakos, V, Liu, W-T & Jobbágy, A 2011, 'Full-scale use of glycogen-accumulating organisms for excess biological carbon removal', Water Environment Research, vol. 83, pp. 855-864.

Kong, Y, Ong, SL, Ng, WJ & Liu, WT 2002, 'Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic–aerobic activated sludge processes', Environmental Microbiology, vol. 4, pp. 753-757.

Kong, Y, Xia, Y, Nielsen, JL & Nielsen, PH 2006, 'Ecophysiology of a group of uncultured gammaproteobacterial glycogen-accumulating organisms in full-scale enhanced biological phosphorus removal wastewater treatment plants', Environmental Microbiology, vol. 8, pp. 479-489.

Kong, Y, Xia, Y, Nielsen, JL & Nielsen, PH 2007, 'Structure and function of the microbial community in a full-scale enhanced biological phosphorus removal plant', Microbiology, vol. 153, pp. 4061-4073.

Kong, YH, Beer, M, Seviour, RJ, Lindrea, KC & Rees, GN 2001, 'Structure and functional analysis of the microbial community in an aerobic: anaerobic sequencing batch reactor (SBR) with no phosphorus removal', Systematic and Applied Microbiology, vol. 24, pp. 597-609.

Kumar, A, Frost, P, Correll, R, & Oemcke, D 2009, 'Winery wastewater generation, treatment and disposal: A survey of Australian practice'. CSIRO Land and Water Report, 17 March 2009, Glen Osmond, South Australia.

Lemos, PC, Dai, Y, Yuan, Z, Keller, J, Santos, H & Reis, MA 2007, 'Elucidation of metabolic pathways in glycogen-accumulating organisms with in vivo ^{13}C nuclear magnetic resonance', Environmental Microbiology, vol. 9, pp. 2694-2706.

Li, T, Wu, TD, Mazéas, L, Toffin, L, Guerquin-Kern, JL, Leblon, G & Bouchez, T 2008, 'Simultaneous analysis of microbial identity and function using NanoSIMS', Environmental Microbiology, vol. 10, pp. 580-588.

Liu, W-T, Hanada, S, Marsh, TL, Kamagata, Y & Nakamura, K 2002, '*Kineosphaera limosa* gen. nov., sp. nov., a novel gram-positive polyhydroxyalkanoate-accumulating coccus isolated from activated sludge', International Journal of Systematic and Evolutionary Microbiology, vol. 52, pp. 1845-1849.

Liu, W-T, Mino, T, Nakamura, K & Matsuo, T 1994, 'Role of glycogen in acetate uptake and polyhydroxyalkanoate synthesis in anaerobic-aerobic activated sludge with a minimized polyphosphate content', Journal of Fermentation and Bioengineering, vol. 77, pp. 535-540.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G & Förster, W 2004, 'ARB: a software environment for sequence data', Nucleic Acids Research, vol. 32, pp. 1363-71.

Ma, SJ, Ding, LL, Huang, H, Geng, JJ, Xu, K, Zhang, Y & Ren, HQ 2016, 'Effects of DO levels on surface force, cell membrane properties and microbial community dynamics of activated sludge', Bioresource Technology, vol. 214, pp. 645-652.

Magoč, T & Salzberg, SL 2011, 'FLASH: Fast length adjustment of short reads to improve genome assemblies', Bioinformatics, vol. 27, pp. 2957-2963.

Manz, W, Amann, R, Ludwig, W, Wagner, M & Schleifer, K-H 1992, 'Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions', Systematic and Applied Microbiology, vol. 15, pp. 593-600.

Maszenan, AM, Seviour, R, Patel, B, Janssen, P & Wanner, J 2005, '*Defluvicoccus vanus* gen. nov., sp. nov., a novel gram-negative coccus/coccobacillus in the 'Alphaproteobacteria' from activated sludge', International Journal of Systematic and Evolutionary Microbiology, vol. 55, pp. 2105-2111.

McIlroy, S & Seviour, RJ 2009, 'Elucidating further phylogenetic diversity among the *Defluviicoccus*-related glycogen-accumulating organisms in activated sludge', Environmental Microbiology Reports, vol. 1, pp. 563-568.

McIlroy, S, Speirs, L, Tucci, J & Seviour, RJ 2011, 'In situ profiling of microbial communities in full-scale aerobic sequencing batch reactors treating winery waste in Australia', Environmental Science & Technology, vol. 45, pp. 8794-8803.

McIlroy, SJ, Albertsen, M, Andresen, EK, Saunders, AM, Kristiansen, R, Stokholm-Bjerregaard, M, Nielsen, KL & Nielsen, PH 2013, "Candidatus Competibacter'-lineage genomes retrieved from metagenomes reveal functional metabolic diversity', The ISME journal, vol. 8, pp. 613-624.

McIlroy, SJ, Albertsen, M, Andresen, EK, Saunders, AM, Kristiansen, R, Stokholm-Bjerregaard, M, Nielsen, KL & Nielsen, PH 2013, "Candidatus Competibacter'-lineage genomes retrieved from metagenomes reveal functional metabolic diversity', The ISME journal, vol. 8, pp. 613-624.

McIlroy, SJ, Nittami, T, Kanai, E, Fukuda, J, Saunders, AM & Nielsen, PH 2015, 'Re-appraisal of the phylogeny and fluorescence in situ hybridization probes for the analysis of the Competibacteraceae in wastewater treatment systems', Environmental Microbiology Reports, vol.7, pp.166-174.

McIlroy, SJ, Saunders, AM, Albertsen, M, Nierychlo, M, McIlroy, B, Hansen, AA, Karst, SM, Nielsen, JL & Nielsen, PH 2015, 'MiDAS: the field guide to the microbes of activated sludge', Database, vol. 2015, doi:10.1093/database/bav062.

Meyer, RL, Saunders, AM & Blackall, LL 2006, 'Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing', Microbiology, vol. 152, pp. 419-429.

Mino, T, Liu, W-T, Kurisu, F & Matsuo, T 1995, 'Modelling glycogen storage and denitrification capability of microorganisms in enhanced biological phosphate removal processes', Water Science and Technology, vol. 31, pp. 25-34.

Mosse, KPM, Patti, AF, Christen, EW & Cavagnaro, TR 2011, 'Review: Winery wastewater quality and treatment options in Australia', Australian Journal of Grape and Wine Research, vol. 17, pp. 111-122.

Musat, N, Foster, R, Vagner, T, Adam, B & Kuypers, MM 2012, 'Detecting metabolic activities in single cells, with emphasis on NanoSIMS', FEMS microbiology reviews, vol. 36, no. 2, pp. 486-511.

Neef, A, Witzenberger, R & Kämpfer, P 1999, 'Detection of sphingomonads and in situ identification in activated sludge using 16S rRNA-targeted oligonucleotide probes', Journal of Industrial Microbiology and Biotechnology, vol. 23, pp. 261-267.

Nittami, T, McIlroy, S, Seviour, EM, Schroeder, S & Seviour, RJ 2009, 'Candidatus Monilibacter spp., common bulking filaments in activated sludge, are members of cluster III Defluviicoccus', Systematic and Applied Microbiology, vol. 32, pp. 480-489.

Nielsen, PH, De Muro, MA & Nielsen, JL 2000, 'Studies on the in situ physiology of Thiothrix spp. present in activated sludge', Environmental Microbiology, vol. 2, pp. 389-398.

Nobu, MK, Tamaki, H, Kubota, K & Liu, WT 2014, 'Metagenomic characterization of 'Candidatus Defluviicoccus tetraformis strain TFO71', a tetrad-forming organism, predominant in an anaerobic–aerobic membrane bioreactor with deteriorated

biological phosphorus removal', Environmental Microbiology, vol. 16, pp. 2739-2751.

Oehmen, A, Carvalho, G, Lopez-Vazquez, C, Van Loosdrecht, M & Reis, M 2010, 'Incorporating microbial ecology into the metabolic modelling of polyphosphate accumulating organisms and glycogen accumulating organisms', Water Research, vol. 44, pp. 4992-5004.

Oehmen, A, Lemos, PC, Carvalho, G, Yuan, Z, Keller, J, Blackall, LL & Reis, MA 2007, 'Advances in enhanced biological phosphorus removal: from micro to macro scale', Water Research, vol. 41, pp. 2271-2300.

Ong, YH, Chua, ASM, Fukushima, T, Ngoh, GC, Shoji, T & Michinaka, A 2014, 'High-temperature EBPR process: The performance, analysis of PAOs and GAOs and the fine-scale population study of *Candidatus* "Accumulibacter phosphatis"', Water Research, vol. 64, pp. 102-112.

Roller, C, Wagner, M, Amann, R, Ludwig, W & Schleifer, K-H 1994, '*In situ* probing of gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides', Microbiology, vol. 140, pp. 2849-2858.

Ruggieri, L, Cadena, E, Martínez-Blanco, J, Gasol, CM, Rieradevall, J, Gabarrell, X, Gea, T, Sort, X & Sánchez, A 2009, 'Recovery of organic wastes in the Spanish wine industry. Technical, economic and environmental analyses of the composting process', Journal of Cleaner Production, vol. 17, pp. 830-838.

Satoh, H, Mino, T & Matsuo, T 1994, 'Deterioration of enhanced biological phosphorus removal by the domination of microorganisms without polyphosphate accumulation', Water Science and Technology, vol. 30, pp. 203-211.

Saunders, AM, Mabbett, AN, McEwan, AG & Blackall, LL 2007, 'Proton motive force generation from stored polymers for the uptake of acetate under anaerobic conditions', FEMS Microbiology Letters, vol. 274, no. 2, pp. 245-251.

Schroeder, S, Ahn, J & Seviour, RJ 2008, 'Ecophysiology of polyphosphate-accumulating organisms and glycogen-accumulating organisms in a continuously aerated enhanced biological phosphorus removal process', Journal of Applied Microbiology, vol. 105, pp. 1412-1420.

Seviour, RJ, Maszenan, AM, Soddell, JA, Tandoi, V, Patel, BK, Kong, Y & Schumann, P 2000, 'Microbiology of the 'G-bacteria' in activated sludge', Environmental Microbiology, vol. 2, pp. 581-593.

Seviour, RJ & Nielsen, PH 2010, Microbial ecology of activated sludge, IWA Publishing, London, UK.

Shintani, T, Liu, W-T, Hanada, S, Kamagata, Y, Miyaoka, S, Suzuki, T & Nakamura, K 2000, '*Micropruina glycogenica* gen. nov., sp. nov., a new gram-positive glycogen-accumulating bacterium isolated from activated sludge', International Journal of Systematic and Evolutionary Microbiology, vol. 50, pp. 201-207.

Stokholm-Bjerregaard, M, McIlroy, SJ, Nierychlo, M, Karst, SM, Albertsen, M, & Nielsen, PH 2017, 'A critical assessment of the microorganisms proposed to be important to enhanced biological phosphorus removal in full-scale wastewater treatment systems, Frontiers in Microbiology, vol. 8, doi:10.3389/fmicb.2017.00718.

Tayà, C, Garlapati, VK, Guisasola, A & Baeza, JA 2013, 'The selective role of nitrite in the PAO/GAO competition', Chemosphere, vol. 93, pp. 612-618.

Wallner, G, Amann, R & Beisker, W 1993, 'Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms', Cytometry, vol. 14, pp. 136-143.

Wang, X, Zeng, RJ, Dai, Y, Peng, Y & Yuan, Z 2008, 'The denitrification capability of cluster 1 *Defluviicoccus vanus*-related glycogen-accumulating organisms', Biotechnology and Bioengineering, vol. 99, pp. 1329-1336.

Wang, Z, Guo, F, Mao, Y, Xia, Y & Zhang, T 2014, 'Metabolic characteristics of a glycogen-accumulating organism in *Defluviicoccus* Cluster II revealed by comparative genomics', Microbial Ecology, vol. 68, pp. 716-728.

Wong, M, Tan, FM, Ng, WJ & Liu, W-T 2004, 'Identification and occurrence of tetrad-forming Alphaproteobacteria in anaerobic–aerobic activated sludge processes', Microbiology, vol. 150, pp. 3741-3748.

Wong, MT & Liu, WT 2007, 'Ecophysiology of *Defluviicoccus*-related tetrad-forming organisms in an anaerobic–aerobic activated sludge process', Environmental Microbiology, vol. 9, pp. 1485-1496.

Yoshimi, Y, Hiraishi, A & Nakamura, K 1996, 'Isolation and characterization of *Microsphaera multipartita* gen. nov., sp. nov., a polysaccharide-accumulating Gram-positive bacterium from activated sludge', International Journal of Systematic and Evolutionary Microbiology, vol. 46, pp. 519–525, doi: 10.1099/00207713-46-2-519.

Zeng, RJ, van Loosdrecht, M, Yuan, Z & Keller, J 2003, 'Metabolic model for glycogen-accumulating organisms in anaerobic/aerobic activated sludge systems', Biotechnology and Bioengineering, vol. 81, pp. 92-105.

Zeng, RJ, Yuan, Z & Keller, J 2003, 'Enrichment of denitrifying glycogen-accumulating organisms in anaerobic/anoxic activated sludge system', Biotechnology and Bioengineering, vol. 81, pp. 397-404.

Chapter 2

Preliminary investigations: Survey of GAOs in winery wastewater activated sludge

2.1. Introduction

Previous reports have shown that GAOs can dominate the microbial component of WWW (Kiss et al. 2011; McIlroy et al. 2012), despite this, little is known about the phylogeny and abundance of this group of microorganisms. McIlroy et al. (2012) surveyed three WWW during two operational periods (vintage and non-vintage) using FISH microscopy. Biovolume investigations revealed that GAOs belonging to the *Defluviicoccus* cluster I and II were the only observed GAOs with abundances of 5-25% of the total bacterial population. It is most likely that many putative GAOs were overlooked due to the lack of fluorescence probes to target them. Furthermore, as previously described, SBRs treating WWW are exposed to considerable fluctuations in flow volume and chemical compositions of wastewater throughout the year, affecting the activated sludge microbial community composition. For these reasons, a more detailed and long-term survey of WWW using molecular techniques is required to identify the GAOs community.

The following chapter will focus on identifying and quantifying the GAOs present in WWW using 16S rRNA gene sequencing. The data obtained will serve as a starting point for the next chapters in which the underlying factors associated to the proliferation of the main GAOs will be investigated.

2.2. Materials and methods

2.2.1. Sampling, DNA extraction, bioinformatics analyses and fluorescence *in situ* hybridization (FISH).

Grab samples were taken for a period of three years (2014-2016) from four full-scale SBRs treating WWW in Australia. The characteristics of each treatment plant are described in **Table 2.1**. All samples were stored at 4 °C before processing. Biomass DNA was extracted using a FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA) following the standard protocol except for four times increased bead beating duration (Albertsen et al. 2015). Amplicon libraries and sequencing were performed by the Australian Centre for Ecogenomics (Brisbane, Australia) using primers targeting the V3-4 16S rRNA gene region and the Miseq (Illumina) platform. Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32. (Bolger et al. 2014). The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 (Magoč & Salzberg 2011) then dereplicated and formatted for use in the UPARSE workflow (Edgar 2013). Taxonomy was assigned with QIIME (Caporaso et al. 2010), using the MiDAS database v. 2.1 (McIlroy et al. 2015). The results were analysed

with R (http://www.r-project.org) using the Ampvis2 package v 2.3.0. (Albertsen et al. 2015).

Paraformaldehyde fixed cells were assessed by FISH using standard procedures (Daims et al. 2005).

Table 2.1. Treatment plant characteristics

	Plant A	Plant B	Plant C	Plant D
Location	Griffith, NSW	Barossa Valley, SA	Griffith, NSW	Barossa Valley, SA
CAL[a] (ML)	30	N/A	N/A	5
SBR (ML)	6	5	0.6	1.5
Aeration system	coarse/surfaces	surface	fine	coarse/surface

[a] Covered anaerobic lagoon.

2.3. Results and discussion

2.3.1. GAOs observed in WWW

Phylogenetic analyses of 16S rRNA gene sequences revealed operational taxonomic units (OTUs) classified as multiple putative GAOs (**Figure 2.1**). In contrast to the McIlroy et al. (2012) study, where bacteria belonging to the *Defluviicoccus* genus were the only reported GAO in WWW, OTUs belonging to the *Defluviicoccus* (Cluster I, II and III), Competibacteraceae (clades CPB_S18 and CPB_S60), Gammaproteobacteria phylotype CCM19a and Actinobacteria (*Nakamurella* sp. and *Micropruina* sp.) were all observed (**Figure 2.1** and **2.2**).
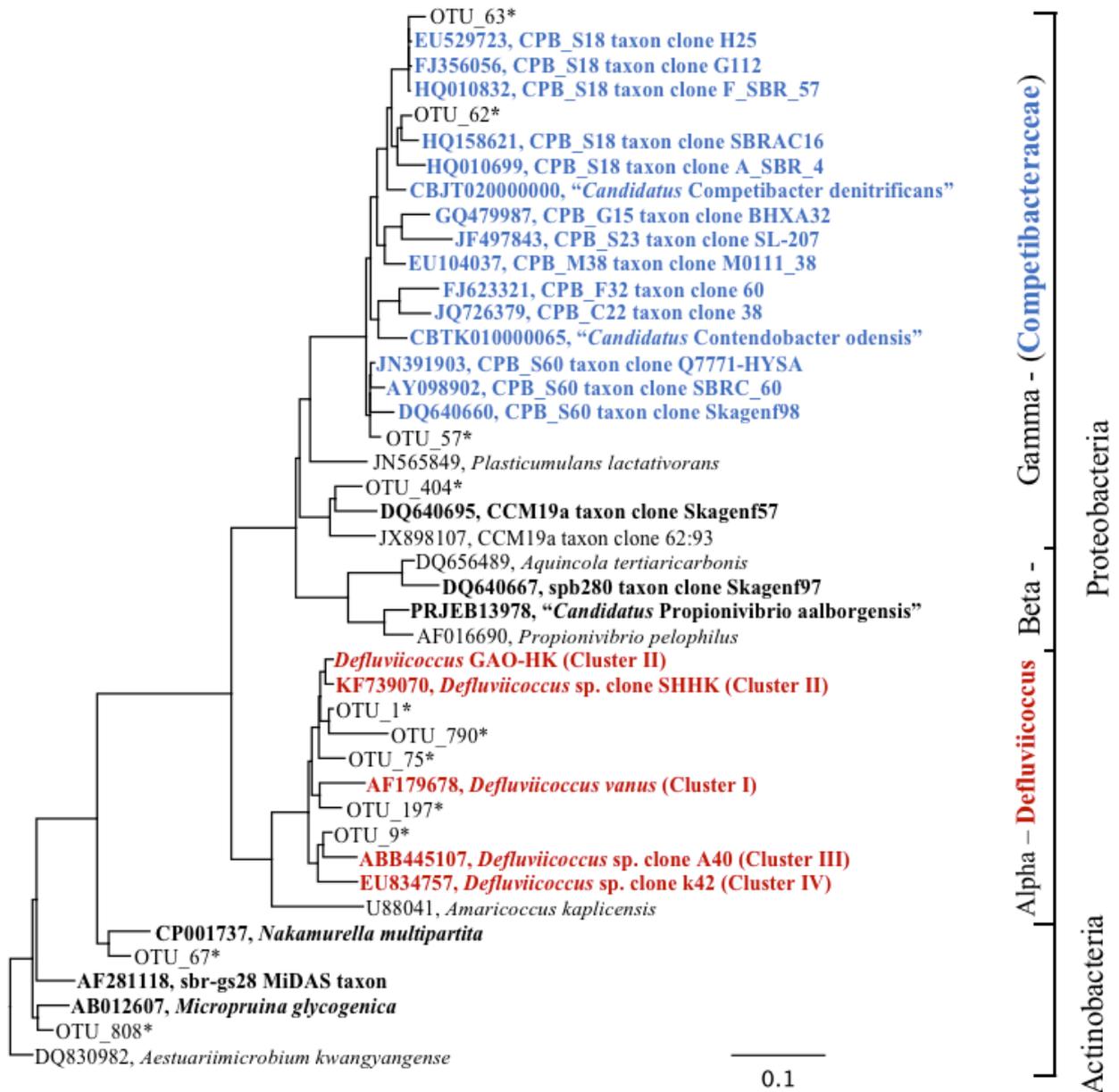
Figure 2.1. Maximum likelihood (PhyML) phylogenetic tree based on > 1200 bp 16S rRNA gene sequences for GAOs observed in activated sludge. Previously proposed GAOs are in bold. Subgroup classifications are taken from the MiDAS database (McIlroy et al. 2015). Brackets indicate phylogenetic classifications. The tree was performed using ARB (Ludwig et al. 2004). Sequences from this study (*) (approximately 450bp) were added after the calculation of the tree with the quick add function in ARB. Scale bar represents substitutions per nucleotide base.

Each of the four WWW treatment plants contained OTUs representing the *Defluviicoccus* at some sampling time. Abundance analysis shows that cluster II (DF2) and III (DF3) were the most abundant (**Figure 2.2**). Cluster IV of the *Defluviicoccus* were not observed. The abundance and identity of *Defluviicoccus* were confirmed with fluorescence microscopy and the application of specific probes targeting DF2 and DF3 (DF2mix and DF198 respectively) (Meyer et al. 2006; Nittami et al. 2009) (**Figure 2.3**). Competibacteraceae (clades CPB_S18 and CPB_S60) and Actinobacterial *Nakamurella* spp. were detected in relatively high abundances (**Figure 2.2**). While *Micropruina* spp. and OTUs belonging to the CCM19a phylotype were observed in low abundances.

Interestingly, a positive response to the DF3 probe was not observed in the McIlroy et al. (2012) study but they have been reported along with DF2 as the most abundant *Defluviicoccus* in EBPR configuration treatment plants (Stokholm-Bjerregaard et al. 2017). It can be speculated that the limited sampling points investigated in McIlroy et al. 2012 study overlooked the DF3, as high abundance fluctuations in time were observed for the *Defluviicoccus* community in this study (**Figure 2.4**).

The observed diversity and abundance of GAOs clearly confirms their relevance to WWW activated sludge. Interestingly, cluster II and III have a different morphology with the latter being filamentous (**Figure 2.3B**) and previously associated to bulking events (Nittami et al. 2009). DF2 has been well studied in EBPR systems (see Chapter 1). Their high affinity for VFAs under anaerobic conditions and glycogen storage abilities make them competitive under EBPR conditions, however, why they dominate in WWW purely aerobic systems is still unknown.
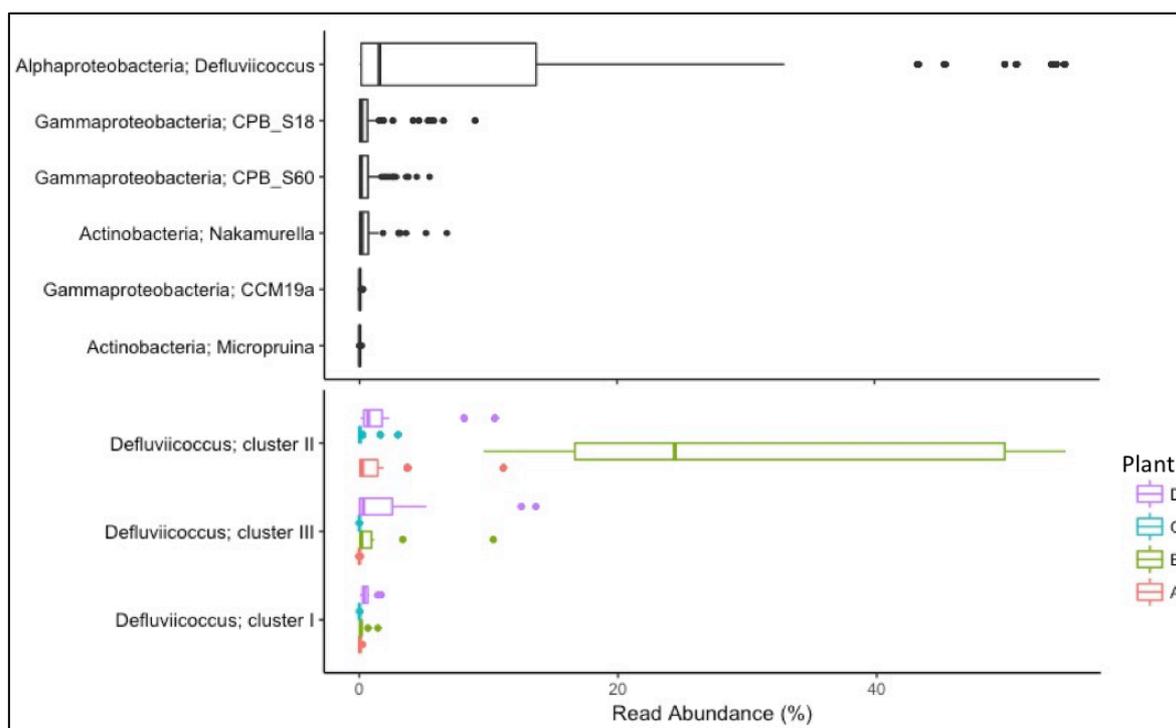
Figure 2.2. Box plots showing the abundances of putative GAOs for all samples collected from four WWW SBRs during the periods 2014-2016. Bottom box plot shows specific abundance of *Defluviicoccus* clusters for each treatment plant.
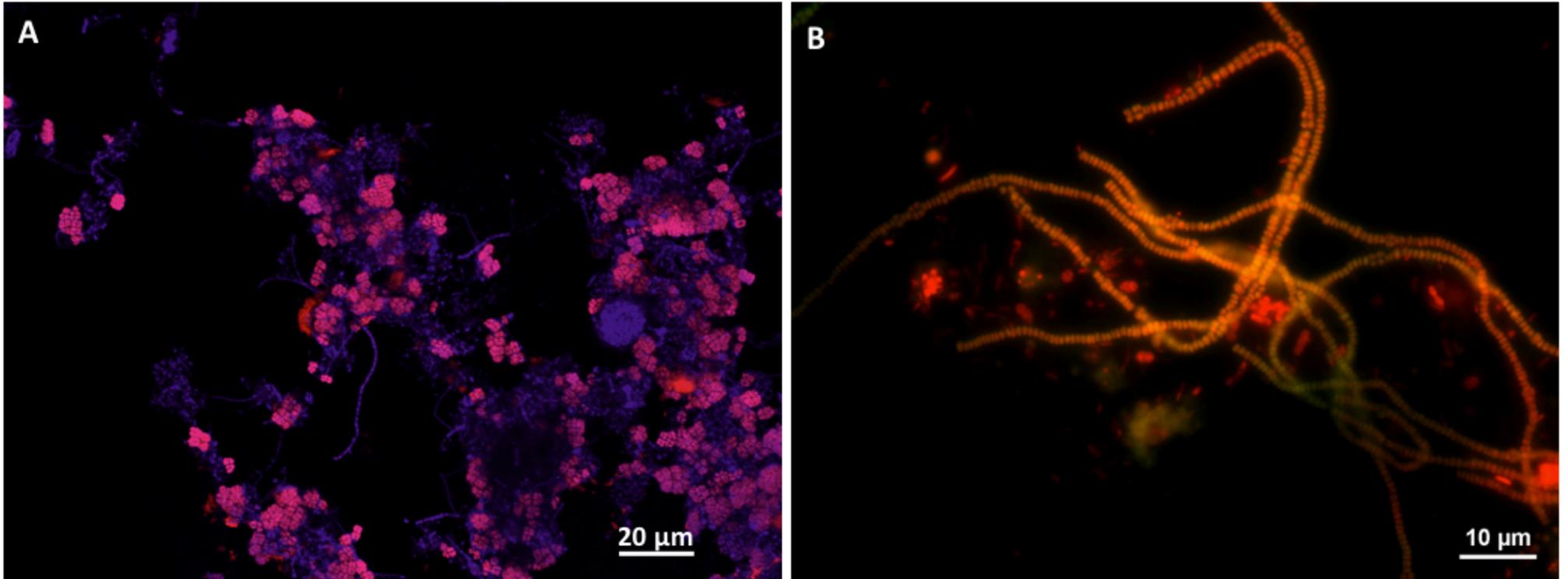
Figure 2.3. Composite FISH micrographs from WWW SBR samples. (A) Plant B SBR sample, FISH probes applied include DF2mix (Meyer et al. 2006) (DF988 and DF1020, Cy3-label red) and EUBmix (Cy5-label, blue). *Defluviicoccus* cluster II cells appear magenta. (B) Plant D SBR sample, FISH probes applied include DF198 (Nittami et al. 2009) and EUBmix (Cy3-label, red). *Defluviicoccus* cluster III cells appear yellow/orange.

The phylogenetic diversity observed through the different clades of the Competibacteraceae family is also reflected in diverse phylotypes (Kong et al. 2006; McIlroy et al. 2014) including substrate uptake types and fermentation abilities. The co-existence of *Defluviicoccus* GAOs and the Competibacteraceae observed in this study (**Figure 2.4**) might be explained as competition for a different niche.

Little is known about the ecophysiology of the *Nakamurella* genus in activated sludge communities. The isolated *Nakamurella multipartita* displayed a high uptake rate of multiple sugars under aerobic conditions with absence of nitrogen and phosphorus sources (Yoshimi et al. 1996). An increase of the *Nakamurella* spp. abundance was also reported after the increase of DO levels in activated sludge (Ma et al. 2016). From these reports, it can be speculated that the specific characteristics of WWW such as sugar contents, low nutrient levels and aerobic treatment (see Chapter 1) will promote the proliferation of bacteria belonging to this genus.

As previously described (see Chapter 1), WWW is highly seasonal, showing periods of high organic loads during vintage. Abundance time series suggest that these variations affect the proliferation of the GAO community with observed changes in their abundance in time (**Figure 2.4**).
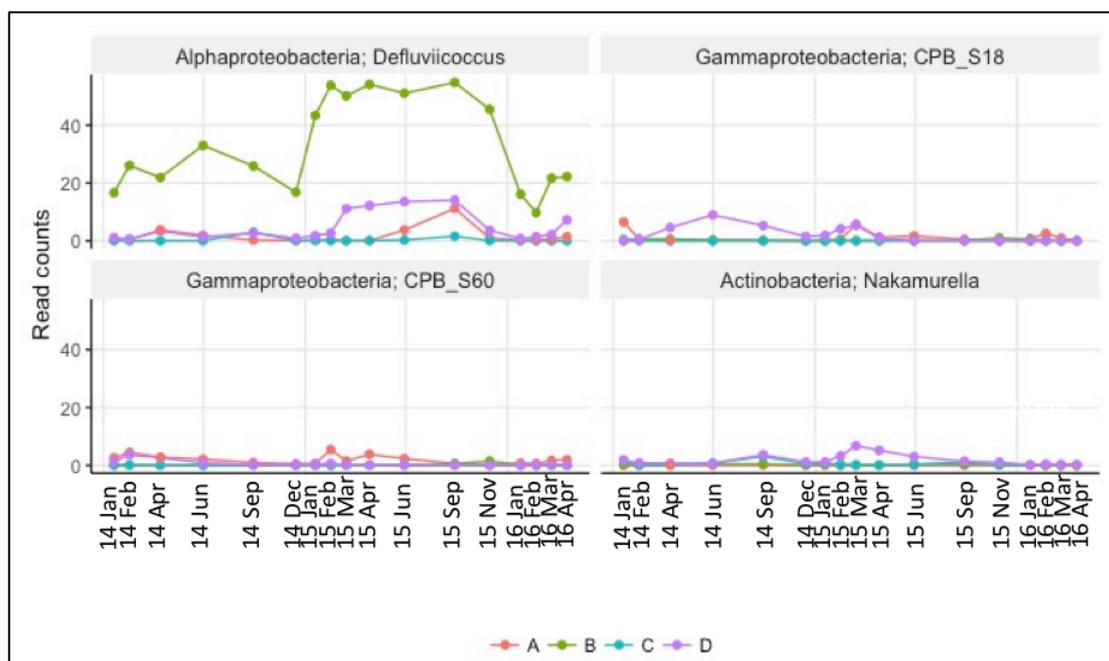
Figure 2.4. Time series plot showing the relative OTU abundance of the four most dominant genera of GAOs observed in all samples collected from four WWW SBRs. Each treatment plant is represented by a different color.

2.3.2. Plant A: A different microbial community

Metagenomics analyses of Plant A showed the presence of GAOs belonging to the Competibacteraceae clade CPB_S60. However, in contrast to the other treatment plants surveyed, this plant was highly dominated by OTUs classified as *Thiothrix* spp. (**Figure 2.5**). *Thiothrix* is a well-documented genus of filamentous bacteria commonly observed in activated sludge samples (Nielsen et al. 2000) in which their proliferation is associated with severe bulking issues. *Thiothrix* spp. reported metabolic plasticity helps to understand their observed abundance in several treatment plant configurations (Seviour & Nielsen 2010). Despite this, it is unknown why specifically Plant A displayed a high proliferation of this filamentous bacteria as well as a codominance with the Competibacteraceae clade CPB_S60. In terms of treatment plant configuration, the main difference of Plant A design is a large covered anaerobic lagoon (CAL) (**Table 2.1**) followed by an SBR. The chemical composition of the influent WWW could be affected by this configuration, consequently altering the microbial community composition. This will be further investigated in Chapter 6.
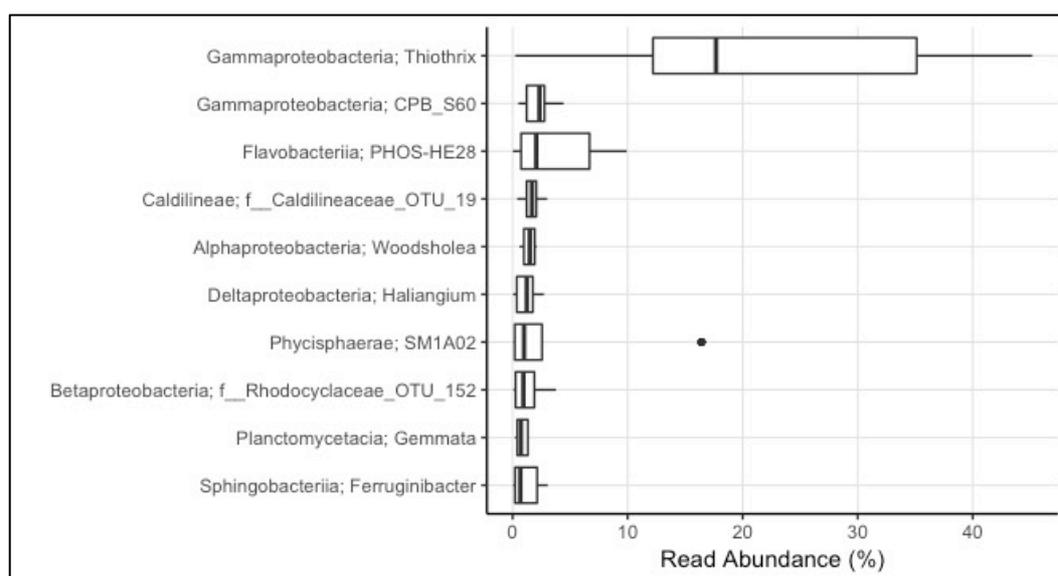
Figure 2.5. Box plot showing the 10 most abundant genera as revealed by 16S rRNA gene profiling in Plant A SBR samples collected during 2014.

2.4. Conclusions

Investigations on the activated sludge microbial community of four WWW treatment plants revealed the presence of multiple putative GAOs, including less characterised genera such as *Micropruina,* CCM19a and *Nakamurella*. As previously reported, *Defluviicoccus* GAOs belonging to cluster II and III are highly abundant and regularly observed, and therefore should be considered as a core component of the microbial community of WWW activated sludge. The underlying factors favouring the proliferation of these two clusters in WWW are unknown and will be investigated in Chapters 3 and 4.

Chapter 3

Managing the excessive proliferation of glycogen accumulating organisms in industrial activated sludge by nitrogen supplementation: A FISH-NanoSIMS approach

Contextual statement

The excessive proliferation of the main GAOs observed in WWW has been attributed to low nutrient levels, with nutrient supplementation suggested as a potential control strategy. The aim of this chapter was to provide scientific rigour to support this strategy. The effect of nitrogen addition on the carbon uptake of the main GAOs was observed at the single cell level through the application of FISH-NanoSIMS. Precise quantification of ^{13}C - acetate and ^{15}N - urea enabled the effect of different COD:N ratios on DF2 to be investigated.

Statement of Authorship

Title of Paper	Managing the excessive proliferation of glycogen accumulating organisms in industrial activated sludge by nitrogen supplementation: A FISH-NanoSIMS approach
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
on Details	Onetto, CA, Eales, KL, Guagliardo, P, Kilburn, MR, Gambetta, JM & Grbin, PR 2017, Mar the excessive proliferation of glycogen accumulating organisms in industrial activated slud nitrogen supplementation: A FISH-NanoSIMS approach. Systematic and applied microbiology, vol. 40, pp. 500-507.

Principal Author

Name of Principal Author (Candidate)	Cristobal Onetto
Contribution to the Paper	Designed experiments, performed incubations, chemical analyses, NanoSIMS analyses, bioinformatics, analysed and interpreted data and drafted the manuscript.
Overall percentage (%)	80
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature		Date	22/02/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i.   the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Kathryn Eales
Contribution to the Paper	Oversaw experimental design and aided in drafting of manuscript.
Signature		Date	21/02/2018

Name of Co-Author	Paul Guagliardo
Contribution to the Paper	Performed NanoSIMS analyses and revised manuscript.

Signature		Date	12/02/2018

Name of Co-Author	Matt Kilburn		
Contribution to the Paper	Performed NanoSIMS analyses and revised manuscript.		
Signature		Date	12/02/2018

Name of Co-Author	Joanna Gambetta		
Contribution to the Paper	Performed chemical analyses		
Signature		Date	12/02/2018

Name of Co-Author	Paul Grbin		
Contribution to the Paper	Oversaw experimental design and revised manuscript.		
Signature		Date	22/02/208

Managing the excessive proliferation of glycogen accumulating organisms in industrial activated sludge by nitrogen supplementation: A FISH-NanoSIMS approach

Cristobal A. Onetto[a], Kathryn L. Eales[a], Paul Guagliardo[b], Matt R. Kilburn[b], Joanna M. Gambetta[a], Paul R. Grbin[a,*]

[a] Department of Wine & Food Science, University of Adelaide, Australia
[b] Centre for Microscopy, Characterisation and Analysis, University of Western Australia, Australia

ARTICLE INFO

ABSTRACT

Defluviicoccus vanus-related glycogen accumulating organisms (GAO) regularly proliferate in industrial wastewater treatment plants handling high carbon but nitrogen deficient wastes. When GAO dominate, they are associated with poor performance, characterised by slow settling biomass and turbid effluents. Although their ecophysiology has been studied thoroughly in domestic waste treatment plants, little attention has been paid to them in aerobic industrial systems.

In this study, the effect of nitrogen addition on GAO carbon metabolism was investigated during an 8 h cycle. Activated sludge dominated by GAO from a winery wastewater sequencing batch reactor was incubated under different carbon to nitrogen (COD:N) ratios (100:1, 60:1 and 20:1) using ^{13}C — acetate and ^{15}N — urea. GAO cell assimilation was quantified using FISH-NanoSIMS. The activated sludge community was assessed by 16S rRNA gene profiling, DNA and storage polymer production. Carbon and nitrogen quantification at the cellular level by NanoSIMS revealed that low (COD:N of 100:1) or null nitrogen concentrations enhanced GAO carbon uptake. COD:N ratios of 60:1 and 20:1 reduced GAO carbon uptake and promoted whole microbial community DNA production. Nitrogen dosing at COD:N ratios of 60:1 or higher was demonstrated as feasible strategy for controlling the excessive GAO growth in high COD waste treatment plants.

© 2017 Elsevier GmbH. All rights reserved.

Introduction

Glycogen accumulating organisms (GAO) have been observed proliferating in activated sludge plants of various configurations treating high chemical oxygen demand (COD) industrial waste, such as paper mill waste, brewery wastes and winery wastewater [5,26–28,30,42]. These wastewater streams share similar characteristics (e.g. high COD:N:P ratios) suggesting that these conditions may enhance the growth of GAO. For successful aerobic biological treatment of wastewater an accepted rule of thumb is a C:N:P ratio of 100:5:1 [32], however industrial wastewater composition differs considerably, containing COD:N ratios as high as 1000:1 [7,34].

GAO is a generic name given to a phylogenetically diverse group of bacteria based on the similarity of their metabolism that leads to the accumulation of intracellular glycogen. The metabolism of

GAO has been well studied with regard to the two main groups of GAO commonly observed in activated sludge communities. These are the alphaproteobacterial *Defluviicoccus vanus*-related organisms [38,50] and the gammaproteobacterial Competibacter lineage [29]. It has been shown that GAO have the ability to assimilate carbon sources that can be stored as both polyhydroxyalkanoates (PHAs) and glycogen [40].

GAO have attracted much research interest as they are considered detrimental to domestic EBPR plants, outcompeting the polyphosphate accumulating organisms (PAO) with consequently low P removal capacity [40]. In sequencing batch reactors (SBR), where the main objective is the reduction of high organic loads some report GAO to be beneficial [19], while others regard them as problematic nonfilamentous bulking microorganisms [28].

The effect that different C:P ratios have on GAO proliferation has been previously addressed by qFISH [21], where lower C:P ratios induced a shift in the microbial community, decreasing the GAO population. Furthermore, GAO are enriched in laboratory scale reactors under high COD:P conditions [8,13,41], as are iso-

Table 1
Treatment description.

Treatment	^{13}C — acetate (mg L^{-1})	^{15}N — urea (mg L^{-1})
Control	–	–
Acetate control	200	–
Nitrogen control	–	22.3
100:1 (COD:N)	200	4.46
60:1 (COD:N)	200	7.44
20:1 (COD:N)	200	22.3

Table 2
List of FISH probes used in this study.

Probe name	Target	[FA][a] (%)	Ref.
EUB338-I[b]	Most *Bacteria*	20–50	[3]
EUB338-II[b]	*Planctomycetales*	20–50	[14]
EUB338-III[b]	*Verrucomicrobiales*	20–50	[14]
NON-EUB	Control probe complementary to EUB338	–	[49]
ALF968	*Alphaproteobacteria*, except of *Rickettsiales*	20	[37]
DF988[c]	Cluster II *Defluviicoccus*	35	[33]
DF1020[c]	Cluster II *Defluviicoccus*	35	[33]
Noli-644	*Candidatus* Alysiosphaera europaea	35	[46]

Competitor and helper probes were applied according to the original papers.
  [a] [FA] – formamide concentration in hybridization buffer.
  [b] EUBmix.
  [c] DF2mix.

lates obtained from rich carbohydrate wastewater treatment plants [26,28].

GAO have been shown to dominate winery wastewater (WWW) activated sludge [18] and they can cause operational problems. This waste stream was shown to have adequate phosphorus levels but was nitrogen limited, suggesting that phosphorus limitation was not the only limiting nutrient that would make this group of microorganisms thrive. In this study, the effect of nitrogen supplementation on the carbon metabolism of *Defluviicoccus* cluster II (DF2) GAO was investigated. WWW activated sludge samples dominated by DF2 were incubated under three different COD:N ratios.

NanoSIMS analysis was used to quantify carbon and nitrogen assimilation in these microorganisms. This highly sensitive technique has helped to further elucidate the metabolism of microorganisms in other fields [20,35,36], and was shown to be a valuable technique for the study of bacterial ecophysiologies in complex microbial populations [16]; however, it has never been applied to these microorganisms before. To complement the findings of the study, the effect that these COD:N ratios had on DNA production, glycogen and PHA accumulation was also explored.

Materials and methods

Sampling

A 5 L grab sample was taken 3 h after the standard aeration cycle from a full-scale SBR treating WWW in South Australia during the vintage period (April 2015) and it was stored at 4 °C. The COD and ammonia levels in the SBR sample taken after the aeration cycle were 13 and 0.1 mg L^{-1}, respectively. Analysis was carried out within 24 h of receiving the sample.

Incubations

Total suspended solids (TSS) of the initial SBR sample were corrected to 2 g L^{-1} using 0.2 μm filtered SBR water and they were then divided into 100 mL incubations. Six treatments (Table 1) in duplicate were carried out using ^{13}C — acetate (99 atom % ^{13}C) and ^{15}N — urea (98 atom % ^{15}N) (Sigma-Aldrich, Australia) during an 8 h aeration cycle. 4.5 mL samples were periodically taken after homogenization for cell fixation, chemical analysis and DNA extraction.

Chemical analyses

The production and/or consumption of acetate and glycogen was measured in duplicate during a time course of 8 h by HPLC using an ion-exchange Aminex HPX-87H HPLC column (Bio-Rad, USA) coupled to a refractive index detector (Agilent Technologies). Glycogen extraction was performed as described by Lanham et al. [23]. Briefly, 2 mL of 0.9 M HCL were added to freeze-dried biomass in screw-capped tubes and heated at 100 °C for 3 h. After cooling, the supernatant was transferred to 0.2 μm PVDF filter HPLC vials. PHA extraction and determination were performed as described by Oehmen et al. [39] using 3% (v/v) sulphuric acid for the acidified

methanol solution and a 6 h digestion time. A total of 3 μL of the extract was analysed with an Agilent 6890 gas chromatograph (Palo Alto, CA) coupled to an Agilent 5973N mass spectra detector (MSD), using a Gerstel MPS2 autosampler (LaserSan Australasia, Robina, Qld, Australia). Poly 3-hydroxybutyric acid and 3-hydroxyvaleric acid copolymer (Sigma-Aldrich, Australia) were used as authentic standards.

Cell fixation and fluorescence in situ hybridization (FISH)

The original SBR and incubation samples were fixed using standard procedures [3]. For the initial characterization of the SBR sample and determination of bio-volumes, standard FISH procedures were used [3]. The quantitative FISH (qFISH) bio-volume of the control sample was calculated using the DAIME software [15] in a set of 44 images using the DF2mix as a specific target and the EUB338mix probe for total bio-volume (Table 2). For NanoSIMS analyses, Wet-FISH was performed as previously described by Chew at al. [10] and resuspended in 50 μL of sterile MilliQ water. Prior to NanoSIMS analysis, 1 μL of the hybridized cells suspension was spread on a 7 × 7 mm silicon chip (Proscitech, Australia) and air-dried in the dark overnight. For relocation purposes based on unique floc structure, the complete 1 μL drop was imaged automatically using a motorized XY stage and auto-focus with a 20× objective (Nikon A1R laser scanning confocal microscope, Adelaide Microscopy). All images were automatically stitched together after the complete drop was imaged. Areas of interest containing positive FISH cells were inspected and 400× magnification images were recorded for subsequent NanoSIMS analyses (Fig. S1 in the Supplementary material). These areas were subsequently marked in the stitched image of the complete 1 μL drop for relocation purposes.

Nanoscale secondary ion mass spectrometry (NanoSIMS) analyses of cells

Isotopic mapping was carried out using the Cameca NanoSIMS 50 at the Centre for Microscopy, Characterisation and Analysis (CMCA), University of Western Australia. The samples were coated with 10 nm of gold to provide conductivity. Areas of interest were relocated based on the unique floc structure using stitched and 400× magnification FISH images and the NanoSIMS integrated CCD camera (Fig. S1 in the Supplementary material). Specific 150 × 150 to 200 × 200 μm areas were then rastered using secondary electron images to locate the FISH positive cells precisely (Fig. S2 in the Supplementary material).

Measurements were performed with a Cs$^+$ primary beam, a spot size of approximately 100 nm, impact energy of 16 keV, and a beam current of approximately 2 pA. The instrument was operated in multicollector mode, allowing the simultaneous detection of five ion species, namely ^{12}C$_2^-$, ^{12}C^{13}C$^-$, ^{12}C^{14}N$^-$, ^{12}C^{15}N$^-$ and ^{32}S, from the same analysis region. Secondary ion images were obtained by

rastering the primary ion beam across areas measuring 20×20 or $30 \times 30\,\mu m$ (Fig. S2 in the Supplementary material), at a resolution of 256×256 pixels, with dwell times of 15–30 ms per pixel.

Image processing was carried out using OpenMIMS (Copyright 2010 NRIMS, National Resource for Imaging Mass Spectrometry) an ImageJ plugin (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2015). Deadtime corrections were performed on images before data extraction. Regions of interest (ROI) were defined using $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratio HSI images. Each ROI corresponded to a tetrad because no differences in enrichment were observed in individual cells from the same tetrad. When the orientation of the tetrads did not allow the selection of the whole tetrad, ROIs where defined as single cells. The number of secondary ion images obtained and the ROIs defined by treatment are available in Table S1 in the Supplementary material.

The ^{13}C and ^{15}N abundance of selected ROIs was calculated as previously described by Li et al. [24] using the following formula: ^{13}C atom $\% = (^{13}C^-)/(^{12}C^- + ^{13}C^-) \times 100\%$.

DNA extraction and isotopic enrichment determination

Biomass DNA was extracted using a FastDNA™ SPIN Kit for soil (MP Biomedicals, Santa Ana, CA) following the standard protocol, except for increasing the bead beating duration by four. For the determination of ^{15}N enrichment, $5\,\mu L$ of the DNA samples were combusted in a EuroVector (EuroEA) elemental analyser, using standard settings without dilution. The gases were cleaned and separated using a GC column and then analysed on a CF-IRMS (Nu Instruments Horizon). Values were corrected to internal standards using glycine (Sigma Aldrich, purity >99%), L-glutamic acid (Sigma Aldrich, purity >99%), and EDTA (Ajax Finechem Univar, minimum purity 99.4%). These were calibrated to international standards ($\delta^{15}N$ values were calibrated using IAEA N1, N2, and N3, and USGS 32, 34, 35, 40, and 41).

Bacterial 16S rRNA gene sequencing and bioinformatics processing

Amplicon libraries and sequencing were performed by the Australian Centre for Ecogenomics (Brisbane, Australia) using primers targeting the V3-4 region of the 16S rRNA gene and the Miseq (Illumina) platform. Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 [6]. The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 [25] then dereplicated and formatted for use in the UPARSE workflow [17]. Taxonomy was assigned with QIIME [9], using the MiDAS database v. 2.1 [31]. The results were analysed with R [47] using the ampvis package v 1.9.1 [1].

Results and discussion

In situ identification and quantification of Defluviicoccus cluster II GAOs

The activated sludge sample was dominated by Gram-negative cocci arranged in tetrads and clumps distributed homogeneously throughout the sample as part of the flocs and as individual clusters (Figs. S3 and S4 in the Supplementary material). Positive FISH signals were obtained with the ALF968 probe, covering the *Alphaproteobacteria* (data not shown), and the DF2mix targeting the cluster II members of the genus *Defluviicoccus* (Fig. S3 in the Supplementary material). A positive signal with the DF2mix was also observed in filaments with a *Nostocoida limicola* morphotype (Fig. S4A in the Supplementary material), that gave a positive FISH signal with the Noli-644 probe (data not shown) targeting *Candidatus* 'Alysiosphaera europaea'. A similar event was observed by McIlroy et al.

Table 3
The 10 most abundant phyla in the control sample.

Phylum	Abundance (%)
Proteobacteria	71.0
Bacteroidetes	7.6
Acidobacteria	3.6
Planctomycetes	2.8
Verrucomicrobia	2.4
Chloroflexi	1.9
Actinobacteria	1.8
Saccharibacteria	1.8
Parcubacteria	1.6
Armatimonadetes	1.1

[28] in activated sludge samples from a WWW treatment plant, in which the inclusion of an unlabelled competitor probe eliminated the above background level fluorescence. However, in this study, a modified version of McIlroy et al. [28] competitor probe was included so that it targeted all *Alysiosphaera* spp. in the database and not just their specific clones (5′ TTTAGACGCCATGTCAAGGG 3′). Addition of this competitor successfully eliminated the false positive fluorescence (Fig. S4B in the Supplementary material).

A relative proportion of the DF2 population among the total bacterial population was extrapolated by the qFISH method [15] using the DF2mix and EUB338 mix probes (Table 2), indicating that $62 \pm 13\%$ of the bio-volume corresponded to DF2 cells. The high abundance of DF2 GAO is not rare for plants treating high COD industrial waste [27] and has previously been reported specifically for WWW treatment plants [28]. The lack of nutrient supplementation in the presence of high COD loads appears as a common trend in previous reports, suggesting that these conditions support the growth of DF2. Furthermore, the ability of high COD waste to support the growth of *Defluviicoccus*-related organisms has been investigated for their high PHA production potential useful in bioplastics manufacture [5,42].

16S rRNA gene microbial community profile

16S rRNA gene phylogenetic profiling of the control sample at time 0 was performed to confirm the high abundance of DF2 obtained by the qFISH method and acquire details of the community composition. This data was generated using the MiDAS database [31], a database specifically prepared for activated sludge.

The sample was dominated by OTUs belonging to the *Proteobacteria*, *Bacteroidetes* and *Acidobacteria* (Table 3). There are no previous reports of 16S rRNA gene community profiling for WWW; however, McIlroy et al. [28] estimated the bio-volumes of three Australian WWW treatment plants that revealed similar results with high abundances of *Proteobacteria* (>50% of the bio-volume) and less than 5% abundance of *Chloroflexi* and *Bacteroidetes*. The *Acidobacteria* were not quantified by McIlroy et al. [28], probably due to the limited number of probes available for this phylum.

In agreement with the qFISH bio-volume estimation, of the 16S rRNA gene sequences obtained, 56.9% corresponded to an OTU (KY271748) that fell among members of cluster II *Defluviicoccus* with 99% similarity to clones obtained from activated sludge. High abundance and diversity of bacteria corresponding to the *Bacteroidetes* phylum were observed (Tables 3 and 4), and their abundance in activated sludge has been reported previously [22]. Interestingly, the *Bacteroidetes* phylum was mainly represented by an OTU that fell within a *PHOS-HE51* family [11]. Although previously reported in other environmental conditions [4,45], there is no physiological information available for this family of microorganisms or previous reports of their abundance in such high numbers as observed in this study. We have performed 16S rRNA gene microbial community profiles of other WWW activated sludge samples

Table 4
The 10 most abundant OTUs in the control sample. Each OTU have both a broad group name (phylum) and a specific name (genus). If no genus name could be assigned, the best classification is provided.

OTUs	Abundance (%)
Proteobacteria; *Defluviicoccus* (Cluster II)	56.9
Proteobacteria; *f__Comamonadaceae*	4.4
Bacteroidetes; *f__PHOS-HE51*	2.9
Acidobacteria; *Blastocatella*	2.3
Bacteroidetes; *Candidatus* Epiflobacter	1.6
Proteobacteria; *Candidatus* Alysiosphaera	1.5
Proteobacteria; *f__Comamonadaceae*	1.3
Verrucomicrobia; *f__Verrucomicrobiaceae*	1.2
Parcubacteria; *c__Parcubacteria*	1.1
Saccharibacteria; *c__Saccharibacteria*	1.0

(data not shown) and have observed a high presence of this family of microorganisms. Consequently, their abundance suggests they may play an important role in the treatment of WWW and they should be investigated further.

16S rRNA gene data confirmed the FISH observations showing the presence of *Candidatus* 'Alysiosphaera europaea' (Table 4), which is easily observed with microscopic observations due to its unique morphology. However, the 16S rRNA gene data indicated it was only present in low numbers. 16S rRNA gene profiles were generated for all COD:N ratio incubations but a significant shift in populations was not observed within the 8 h period using this technique.

Evolution of storage polymers and DNA synthesis

Changes in the carbon consumption, storage polymer concentrations and DNA synthesis were investigated throughout the 8 h cycle. Supplemented acetate was rapidly depleted in 0.5 h (Fig. 1). Glycogen was consumed (0.5 h) and then restored in all treatments that included the addition of acetate (Fig. 1). No changes in the glycogen concentration were observed for treatments without the addition of an external carbon substrate.

Differences were observed in the PHA concentration (Fig. 1). A rapid increase in the acetate control (no supplemented nitrogen) was observed with the maximum concentration (0.45 mmol-C g TS^{-1}) being reached at 0.2 h (Fig. 1). On the other hand, treatments including nitrogen reached their maximum PHA concentration at 0.5 h (0.55 mmol-C g TS^{-1}) (Fig. 1). This is consistent with Valentino et al. [48] where faster PHA production was observed in a mixed microbial culture under nutrient starvation conditions, yet the overall biomass growth and production of PHA was enhanced by the addition of nutrients.

These data show that the activated sludge simultaneously consumed intracellular glycogen and depleted supplemented acetate. This metabolism is consistent with the known metabolism of *Defluviicoccus vanus*-related GAO [38,50]. In the presence of an external substrate, GAO used their stored glycogen as an energy source for substrate uptake, which was later restored by the consumption of PHA.

The rapid consumption of acetate indicates that the biomass was exposed to a long famine period. It is most likely that these long starvation periods will be beneficial for microbial communities that have storage compounds like the GAO. Moreover, alternating feast/famine stages, together with nutrient limitations and high COD loads have been described as the combination of factors required to apply a selective pressure that benefits organisms with internal carbon reservoirs [5]. These results suggest that full-scale WWW treatment plants performing 8 h or longer cycles [28] are exposing the sludge to long famine periods, possibly enhancing the growth of the GAO community. Therefore, changes in opera-
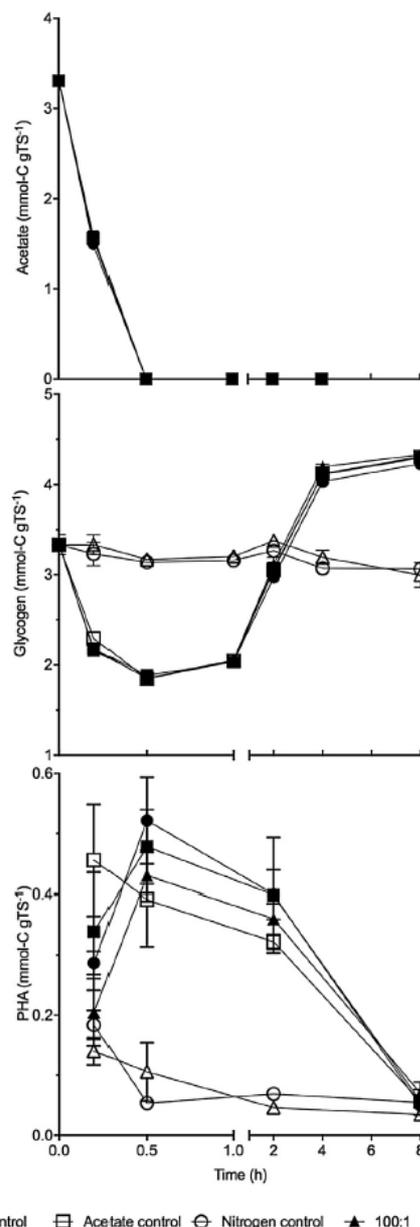
Fig. 1. Time course of acetate, glycogen and polyhydroxyalkanoates (PHAs) during the 8 h incubation period. Data points represent means of two biological replicates and show the standard deviation (SD) for each incubation condition. Symbols for acetate overlap.

Legend: △ Control   ▢ Acetate control   ⦶ Nitrogen control   ▲ 100:1   ■ 60:1   ● 20:1

tion conditions aiming to reduce the complete cycle time may be a feasible strategy to aid the reduction of the GAO community.

The growth of the microbial community was estimated by analysing the ^{15}N enrichment of extracted DNA using CF-IRMS analyses. The CF-IRMS instrument has high sensitivity and low sample volume requirements, allowing the rapid determination of stable isotope ratios in DNA samples, revealing the assimilation of labelled substrates in the DNA of the whole microbial community.

Higher δ^{15}N ratios after 8 h incubation were observed for treatments with ratios of 20:1 (Fig. 2), suggesting an increase in growth in the microbial community under these conditions. δ^{15}N ratio values higher than the control were observed in the nitrogen control DNA after 8 h, despite no addition of carbon substrates. This suggests that DNA production in the population was occurring using
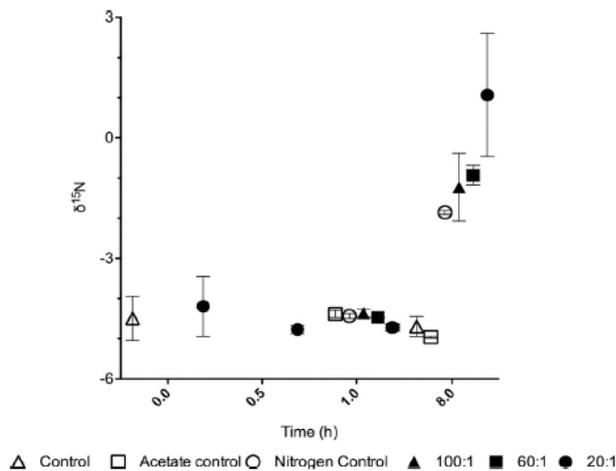
Fig. 2. δ^{15}N ratio values from extracted DNA at different time points. Data points represent the means of two biological replicates and show the SD for each incubation condition.

internal carbon storage, which correlates with the PHA consumption observed for the nitrogen control (Fig. 1). The data indicates that no growth occurred during the first hour of incubation, since no difference in the δ^{15}N ratio compared to the control was observed. Furthermore, the PHA and glycogen data supports this observation with the main glycogen production and PHA consumption occurring after the first hour of incubation (Fig. 1). COD:N ratios of 20:1 had a greater impact on the growth of the community with higher δ^{15}N ratio DNA values (Fig. 2).

These results are consistent with other previously reported enriched GAO and PHA accumulating microbial communities, where nitrogen supplementation promotes growth of the organism [2,5,12,43]. As DF2 is the dominant and, probably, the most metabolically active population, they will assimilate nutrients and carbon quickly, leading to their proliferation. For this reason, it is essential to determine which COD:N ratio would guarantee an homogenous distribution of substrates and growth throughout the microbial community, enhancing the competitiveness of the non-GAO bacteria.

FISH-NanoSIMS approach for activated sludge samples

The complex structure of activated sludge represents a challenge for the study of specific bacterial populations in environmental samples using NanoSIMS. Activated sludge microorganisms are not freely suspended in bulk liquid, but are organized into flocs composed of aggregates of bacteria and other organisms embedded in extracellular polymeric substances (EPS) [44]. Furthermore, NanoSIMS image acquisition can be time consuming, emphasizing the importance of knowing the exact location of the target cells prior to NanoSIMS analysis. Therefore, fluorescent image mapping of samples is essential for efficient NanoSIMS application on activated sludge samples. With the application of FISH probes targeting the DF2 cells and confocal microscopy, multiple areas of interest were identified and recorded within each sample. Additionally, each sample was mapped completely by recording sequential images of each area followed by image stitching.

The unique morphology and distribution of flocs throughout each sample allowed an easy recall of the target cells using the camera integrated in the NanoSIMS instrument and the subsequent stitched images (Fig. S1 in the Supplementary material).

Target cells were carefully selected from fluorescent images considering factors such as location, flatness of area and EPS abundance. Several target cells appeared to be covered by EPS, which interfered in the isotope enrichment acquisition. Therefore, a longer pre-sputter stage to shave the superficial layer of these cells was performed in order to obtain a reliable isotope enrichment measurement. Additionally, depth profiles in exposed target cells were performed to ensure that no relevant changes in isotopic enrichments occurred any deeper as the cells were destroyed. It is understood that spatial distribution of the cells within the floc would have an influence on the accessibility to nutrients and carbon sources creating microenvironments [44]. This study focused on DF2 cells that could be easily visually characterized and not obscured by other cells (Fig. S2 in the Supplementary material); however, the behaviour of DF2 cells within the floc can vary. It is therefore essential to complement this approach with another type of analysis in order to obtain a broad community perspective and support the trends observed in the single-cell NanoSIMS analysis.

Isotope analysis of Defluviicoccus *cluster II cells*

DF2 cells were analysed for their ^{13}C and ^{15}N enrichment using NanoSIMS after the 8 h incubation period. Areas of interest were predefined using DF2-positive FISH confocal microscopy images (Fig. 3A). For each area of interest, secondary ion images of ^{12}C$_2^-$, ^{12}C^{13}C$^-$, ^{12}C^{14}N$^-$, ^{12}C^{15}N$^-$ and ^{32}S were recorded simultaneously, from which quantitative isotope ratios were obtained. The bottom right-hand panel in Fig. 3A shows a ^{13}C/^{12}C ratio image, with the colour scale indicating the degree of ^{13}C enrichment, and blue in this case representing natural abundance. This approach allowed direct visualization of the uptake of the label by the DF2 cells.

NanoSIMS data showed a marked increase in the ^{13}C abundance of DF2 cells in the acetate control (5.2 at%) and 100:1 (6.3 at%) treatments (Fig. 3B). The results for treatments 60:1 and 20:1 showed that DF2 cells were assimilating comparatively less carbon, which was therefore assimilated by other microorganisms in the biomass. Under these conditions, more homogenous ^{13}C enrichment was observed throughout the microbial community with a clear lower enrichment in DF2 cells (Fig. 4). This information combined with previous reports of GAO excessive abundance in industrial activated sludge [27] support the view that high COD feeds with low nutrient levels enhance the carbon assimilation of DF2.

The ^{15}N abundance of DF2 cells corresponding to samples 60:1 and 20:1 was significantly higher than cells from other samples (Fig. 3B). However, unexpectedly there was no difference in the ^{15}N abundance of DF2 cells between samples 60:1 and 20:1 (Fig. 3B). Therefore, it can be speculated that a nitrogen uptake limit was reached in DF2 cells subjected to a COD:N ratio of 60:1. While higher nitrogen ratios did not appear to have an effect on DF2 cells, δ^{15}N-DNA data suggested it would favour the overall growth of the bacterial community (Fig. 2). Low ^{15}N abundances were observed in DF2 cells belonging to the nitrogen control (Fig. 3B), suggesting that nitrogen uptake in DF2 cells was limited by the availability of external carbon substrates.

COD:N ratios of 60:1 or higher appeared to have an important impact in the non-DF2 microbial population by increasing their competitiveness against the dominant DF2 community. These results help to understand the underlying effect of nutrient supplementation on enriched GAO communities. While low nitrogen addition favoured carbon uptake in the main metabolically active group of bacteria (GAOs), an increase of urea enhanced both carbon and nitrogen uptake by the non-GAO community, thus allowing them to compete. It is most likely that this event will result in a shift of the microbial population after multiple cycles of nitrogen dosing, as previously reported under changing C:P ratios [5,21].
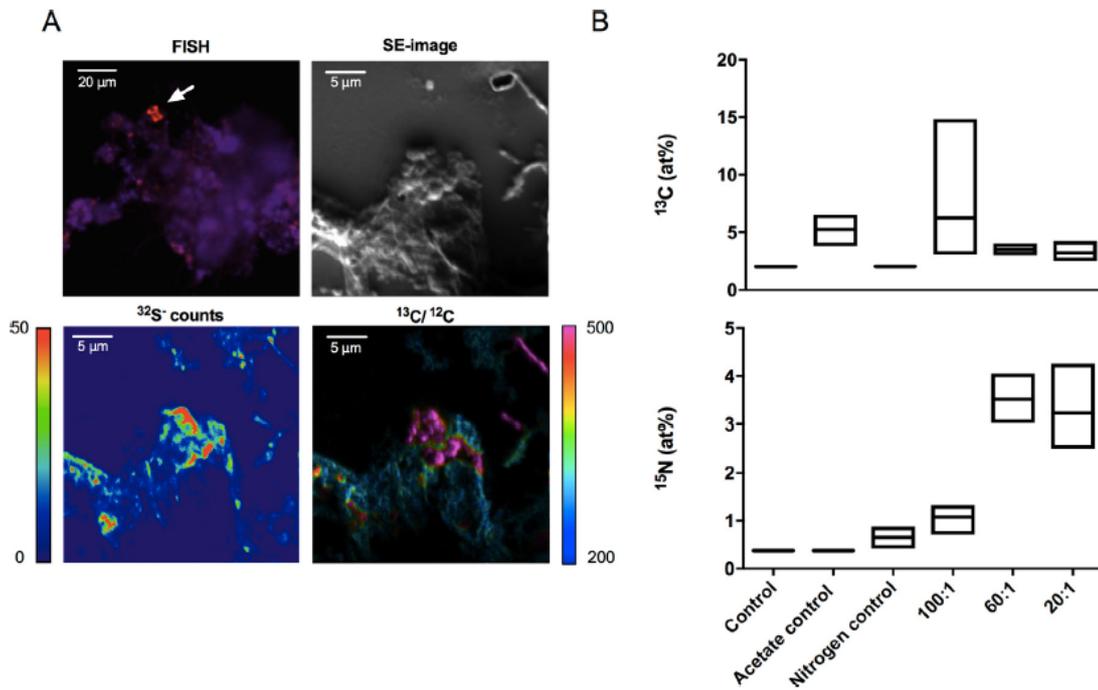
Fig. 3. (A) Representative FISH and NanoSIMS images showing DF2 cells in the same field of view after the 8 h incubation. Cells were located using FISH with the DF2mix (DF988 and DF1020). The ^{13}C/^{12}C ratio image demonstrates high ^{13}C uptake by DF2 cells, and the colour scale indicates the degree of ^{13}C enrichment, with blue representing natural abundance. The ^{32}S$^-$ ion count image shows the biomass arrangement. The arrow indicates the location of DF2 cells determined by FISH. (B) ^{13}C and ^{15}N uptake of *Defluviicoccus* cluster II (DF2) cells quantified by NanoSIMS after 8 h incubation. The isotope content is presented as the isotope fraction ^{13}C/(^{12}C + ^{13}C) given in atom %. The box plots summarize the mean, minimum, and maximum values.
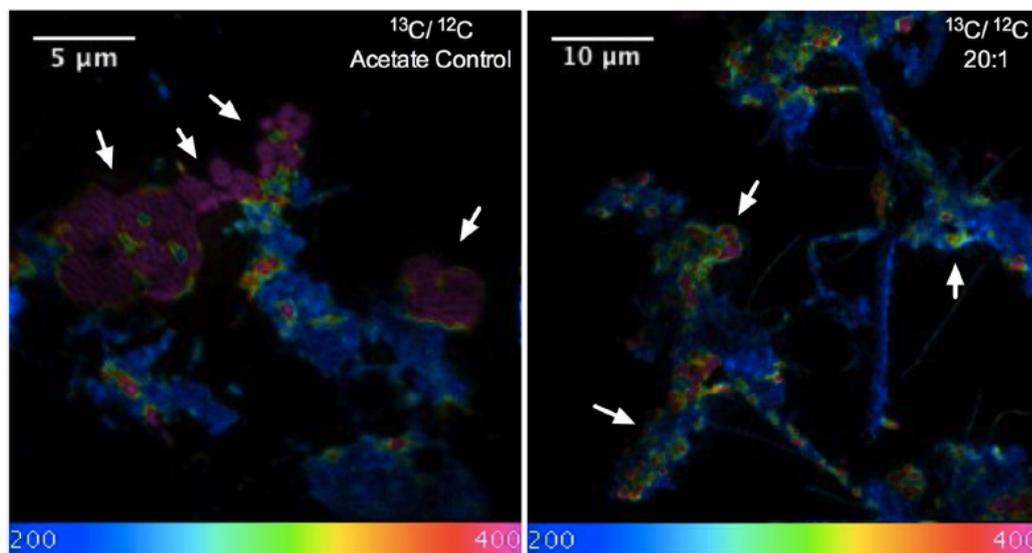
Fig. 4. NanoSIMS ^{13}C/^{12}C ratio images from the acetate control and 20:1 treatment after an 8 h incubation. The colour scale indicates the degree of ^{13}C enrichment, with blue representing natural abundance. The arrow indicates the location of DF2 cells determined by FISH. High ^{13}C uptake by DF2 cells was regularly observed in the acetate control samples. In 20:1 samples, a more homogenous ^{13}C enrichment is observed throughout the microbial community with lower ^{13}C enrichment in DF2 cells.

Conclusion

In this study, the effect of nitrogen addition on the carbon uptake of DF2 cells was investigated, and valuable results were obtained that support nitrogen dosage as a control strategy for problems associated with the high proliferation of DF2 GAO in rich COD waste industrial treatment plants. COD:N ratios of 60:1 and 20:1 reduced carbon assimilation by DF2 and maximized their nitrogen uptake, which should help control the growth of these organisms. Whilst a 20:1 ratio may theoretically increase biomass production in industrial plants, the quantity of urea required is often excessive and not economically viable. The results indicated that dosing at a more economically viable 60:1 ratio was also beneficial in reducing the favourable conditions for GAO.

In addition, it was demonstrated that FISH-NanoSIMS is a suitable technique for the study of bacterial ecophysiology in activated sludge samples.

Data availability

The raw 16S rRNA gene sequence data is available in the EBI database under project number PRJEB18425.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2017.07.006.

References

[1] Albertsen, M., Karst, S.r.M., Ziegler, A.S., Kirkegaard, R.H., Nielsen, P.H. (2015) Back to basics? The influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities. PLoS One 10, e0132783.

[2] Albuquerque, M.G.E., Eiroa, M., Torres, C., Nunes, B.R., Reis, M.A.M. (2007) Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. J. Biotechnol. 130, 411–421.

[3] Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.

[4] Baldwin, S.A., Khoshnoodi, M., Rezadehbashi, M., Taupp, M., Hallam, S., Mattes, A., Sanei, H. (2015) The microbial community of a passive biochemical reactor treating arsenic, zinc, and sulfate-rich seepage. Front. Bioeng. Biotechnol. 3, 27.

[5] Bengtsson, S., Werker, A., Welander, T. (2008) Production of polyhydroxyalkanoates by glycogen accumulating organisms treating a paper mill wastewater. Water Sci. Technol. 58, 323–330.

[6] Bolger, A.M., Lohse, M., Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics, btu170.

[7] Brito, A.G., Peixoto, J., Oliveira, J.M., Oliveira, J.A., Costa, C., Nogueira, R., Rodrigues, A. 2007 Brewery and winery wastewater treatment: some focal points of design and operation. In: Utilization of By-products and Treatment of Waste in the Food Industry, Springer, pp. , 109–131.

[8] Burow, L.C., Mabbett, A.N., Borras, L., Blackall, L.L. (2009) Anaerobic central metabolic pathways active during polyhydroxyalkanoate production in uncultured cluster 1 Defluviicoccus enriched in activated sludge communities. FEMS Microbiol. Lett. 298, 79–84.

[9] Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I. (2010) QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336.

[10] Chew, Y., Holmes, A., Cliff, J. (2014) Visualization of metabolic properties of bacterial cells using nanoscale secondary ion mass spectrometry (NanoSIMS). In: Paulsen, I.T., Holmes, A.J. (Eds.), Environ. Microbiol., Humana Press, pp. 133–146.

[11] Dabert, P., Sialve, B., Delgenès, J.-P., Moletta, R., Godon, J.-J. (2001) Characterisation of the microbial 16S rDNA diversity of an aerobic phosphorus-removal ecosystem and monitoring of its transition to nitrate respiration. Appl. Microbiol. Biotechnol. 55, 500–509.

[12] Dai, Y., Yuan, Z., Jack, K., Keller, J. (2007) Production of targeted poly(3-hydroxyalkanoates) copolymers by glycogen accumulating organisms using acetate as sole carbon source. J. Biotechnol. 129, 489–497.

[13] Dai, Y., Yuan, Z., Wang, X., Oehmen, A., Keller, J. (2007) Anaerobic metabolism of Defluviicoccus vanus related glycogen accumulating organisms (GAOs) with acetate and propionate as carbon sources. Water Res. 41, 1885–1896.

[14] Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., Wagner, M. (1999) The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22, 434–444.

[15] Daims, H., Lücker, S., Wagner, M. (2006) Daime, a novel image analysis program for microbial ecology and biofilm research. Environ. Microbiol. 8, 200–213.

[16] Dawson, K.S., Scheller, S., Dillon, J.G., Orphan, V.J. (2016) Stable isotope phenotyping via cluster analysis of NanoSIMS data as a method for characterizing distinct microbial ecophysiologies and sulfur-cycling in the environment. Front. Microbiol. 7, 774.

[17] Edgar, R.C. (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10, 996–998.

[18] Grbin, P., Eales, K. 2017 Developing a fundamental understanding of the microbiological treatment of winery wastewater, University of Adelaide, Adelaide, Australia.

[19] Kiss, B., Bakos, V., Liu, W.-T., Jobbágy, A. (2011) Full-scale use of glycogen-accumulating organisms for excess biological carbon removal. Water Environ. Res. 83, 855–864.

[20] Koch, H., Galushko, A., Albertsen, M., Schintlmeister, A., Gruber-Dorninger, C., Lücker, S., Pelletier, E., Le Paslier, D., Spieck, E., Richter, A., Nielsen, P.H., Wagner, M., Daims, H. (2014) Growth of nitrite-oxidizing bacteria by aerobic hydrogen oxidation. Science 345, 1052–1054.

[21] Kong, Y.H., Beer, M., Rees, G.N., Seviour, R.J. (2002) Functional analysis of microbial communities in aerobic–anaerobic sequencing batch reactors fed with different phosphorus/carbon (P/C) ratios. Microbiology 148, 2299–2307.

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

[23] Lanham, A.B., Ricardo, A.R., Coma, M., Fradinho, J., Carvalheira, M., Oehmen, A., Carvalho, G., Reis, M.A.M. (2012) Optimisation of glycogen quantification in mixed microbial cultures. Bioresour. Technol. 118, 518–525.

[24] Li, T., Wu, T.D., Mazéas, L., Toffin, L., Guerquin-Kern, J.L., Leblon, G., Bouchez, T. (2008) Simultaneous analysis of microbial identity and function using NanoSIMS. Environ. Microbiol. 10, 580–588.

[25] Magoč, T., Salzberg, S.L. (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27, 2957–2963.

[26] Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Janssen, P.H., Wanner, J. (2005) Defluvicoccus vanus gen. nov., sp. nov., a novel Gram-negative coccus/coccobacillus in the 'Alphaproteobacteria' from activated sludge. Int. J. Syst. Evol. Microbiol. 55, 2105–2111.

[27] McIlroy, S., Seviour, R.J. (2009) Elucidating further phylogenetic diversity among the Defluviicoccus-related glycogen-accumulating organisms in activated sludge. Environ. Microbiol. Rep. 1, 563–568.

[28] McIlroy, S., Speirs, L., Tucci, J., Seviour, R.J. (2011) In situ profiling of microbial communities in full-scale aerobic sequencing batch reactors treating winery waste in Australia. Environ. Sci. Technol. 45, 8794–8803.

[29] McIlroy, S.J., Albertsen, M., Andresen, E.K., Saunders, A.M., Kristiansen, R., Stokholm-Bjerregaard, M., Nielsen, K.L., Nielsen, P.H. (2013) 'Candidatus Competibacter'-lineage genomes retrieved from metagenomes reveal functional metabolic diversity. ISME J. 8, 613–624.

[30] McIlroy, S.J., Nittami, T., Seviour, E.M., Seviour, R.J. (2010) Filamentous members of cluster III Defluviicoccus have the in situ phenotype expected of a glycogen-accumulating organism in activated sludge. FEMS Microbiol. Ecol. 74, 248–256.

[31] McIlroy, S.J., Saunders, A.M., Albertsen, M., Nierychlo, M., McIlroy, B., Hansen, A.A., Karst, S.M., Nielsen, J.L., Nielsen, P.H. (2015) MiDAS: the field guide to the microbes of activated sludge. Database, bav062.

[32] Metcalf, E., Eddy, H. 2003 Wastewater Engineering: Treatment and Reuse, Fourth ed., McGraw-Hill, New York.

[33] Meyer, R.L., Saunders, A.M., Blackall, L.L. (2006) Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing. Microbiology 152, 419–429.

[34] Mosse, K.P.M., Patti, A.F., Christen, E.W., Cavagnaro, T.R. (2011) Review: winery wastewater quality and treatment options in Australia. Aust. J. Grape Wine Res. 17, 111–122.

[35] Musat, N., Foster, R., Vagner, T., Adam, B., Kuypers, M.M. (2012) Detecting metabolic activities in single cells, with emphasis on nanoSIMS. FEMS Microbiol. Rev. 36, 486–511.

[36] Musat, N., Halm, H., Winterholler, B., Hoppe, P., Peduzzi, S., Hillion, F., Horreard, F., Amann, R., Jørgensen, B.B., Kuypers, M.M. (2008) A single-cell view on the ecophysiology of anaerobic phototrophic bacteria. Proc. Natl. Acad. Sci. U.S.A. 105, 17861–17866.

[37] Neef, A., Witzenberger, R., Kämpfer, P. (1999) Detection of sphingomonads and in situ identification in activated sludge using 16S rRNA-targeted oligonucleotide probes. J. Ind. Microbiol. Biotechnol. 23, 261–267.

[38] Nobu, M.K., Tamaki, H., Kubota, K., Liu, W.T. (2014) Metagenomic characterization of 'Candidatus Defluviicoccus tetraformis strain TFO71', a tetrad-forming organism, predominant in an anaerobic–aerobic membrane bioreactor with deteriorated biological phosphorus removal. Environ. Microbiol. 16, 2739–2751.

[39] Oehmen, A., Keller-Lehmann, B., Zeng, R.J., Yuan, Z., Keller, J. (2005) Optimisation of poly-β-hydroxyalkanoate analysis using gas chromatography for enhanced biological phosphorus removal systems. J. Chromatogr. A 1070, 131–136.

[40] Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L., Reis, M.A. (2007) Advances in enhanced biological phosphorus removal: from micro to macro scale. Water Res. 41, 2271–2300.

[41] Oehmen, A., Zeng, R.J., Saunders, A.M., Blackall, L.L., Keller, J., Yuan, Z. (2006) Anaerobic and aerobic metabolism of glycogen-accumulating organisms selected with propionate as the sole carbon source. Microbiology 152, 2767–2778.

[42] Pisco, A.R., Bengtsson, S., Werker, A., Reis, M.A., Lemos, P.C. (2009) Community structure evolution and enrichment of glycogen-accumulating organisms

producing polyhydroxyalkanoates from fermented molasses. Appl. Environ. Microbiol. 75, 4676–4686.

[43] Serafim, L.S., Lemos, P.C., Oliveira, R., Reis, M.A.M. (2004) Optimization of poly-hydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions. Biotechnol. Bioeng. 87, 145–160.

[44] Seviour, R.J., Nielsen, P.H. 2010 Microbial Ecology of Activated Sludge, 1st ed., IWA Publishing, London.

[45] Shabarova, T., Villiger, J., Morenkov, O., Niggemann, J., Dittmar, T., Pernthaler, J. (2014) Bacterial community structure and dissolved organic matter in repeat-edly flooded subsurface karst water pools. FEMS Microbiol. Ecol. 89, 111–126.

[46] Snaidr, J., Beimfohr, C., Levantesi, C., Rossetti, S., van der Waarde, J., Geurkink, B., Eikelboom, D., Lemaitre, M., Tandoi, V. (2002) Phylogenetic analysis and *in situ* identification of "*Nostocoida limicola*"-like filamentous bacteria in activated sludge from industrial wastewater treatment plants. Water Sci. Technol. 46, 99–104.

[47] Team R.C, 2015 R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, 2013. Document freely available on the internet at: http://www.r-project.org.

[48] Valentino, F., Karabegovic, L., Majone, M., Morgan-Sagastume, F., Werker, A. (2015) Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels. Water Res. 77, 49–63.

[49] Wallner, G., Amann, R., Beisker, W. (1993) Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry 14, 136–143.

[50] Wang, Z., Guo, F., Mao, Y., Xia, Y., Zhang, T. (2014) Metabolic characteristics of a glycogen-accumulating organism in *Defluviicoccus* cluster II revealed by comparative genomics. Microb. Ecol. 68, 716–728.

Supplementary material

Managing the excessive proliferation of glycogen accumulating organisms in industrial activated sludge by nitrogen supplementation: A FISH-NanoSIMS approach.

Cristobal A Onetto[†], Kathryn L Eales[†], Paul Guagliardo[‡], Matt R Kilburn[‡], Joanna M Gambetta[†], Paul R Grbin[†*]

[†] Department of Wine & Food Science, University of Adelaide.

[‡] Centre for Microscopy, Characterisation and Analysis, University of Western Australia.
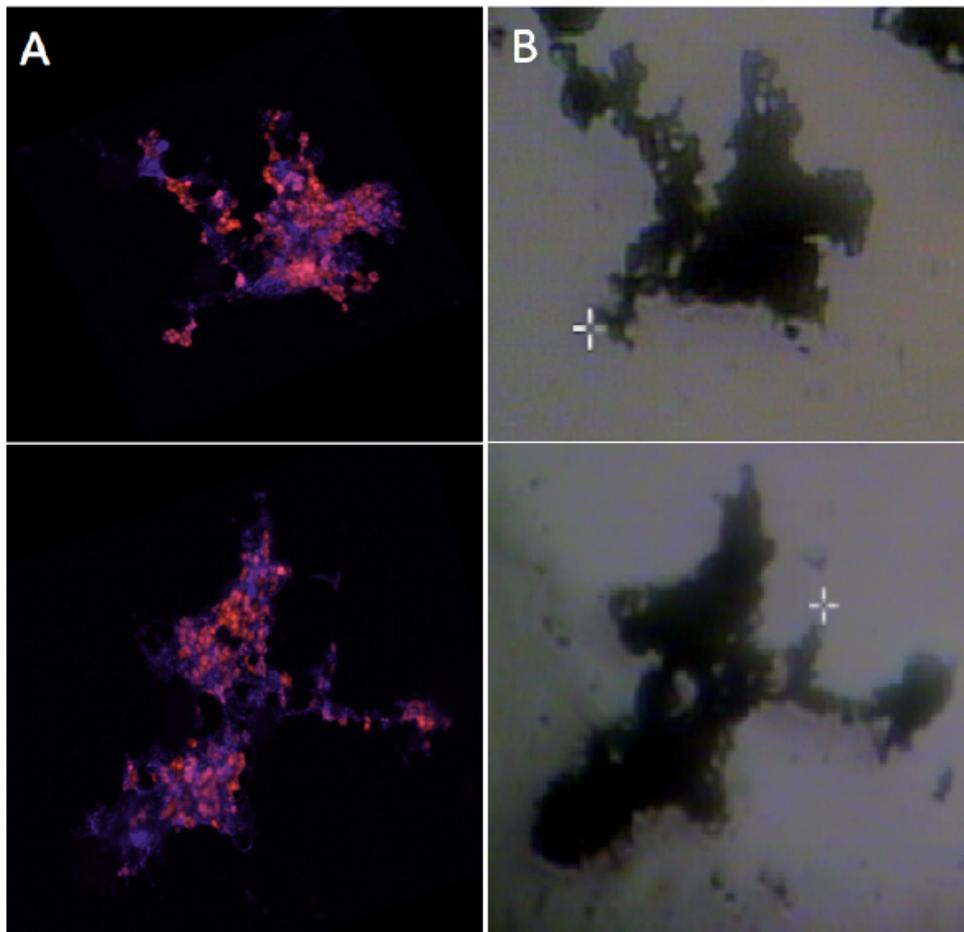
Figure S1. Relocation method of positive DF2 cells based on unique floc structure. (A) 400X magnification FISH micrographs of *Defluviicoccus* cluster II cells on a silicon chip. FISH probes applied included DF2mix (Cy3-label, red) and EUBmix (Cy5-label, blue). *Defluviicoccus* cluster II cells appear magenta. (B) Corresponding floc images obtained with the NanoSIMS integrated CCD camera, flocs were relocated using the stitched FISH images as reference.
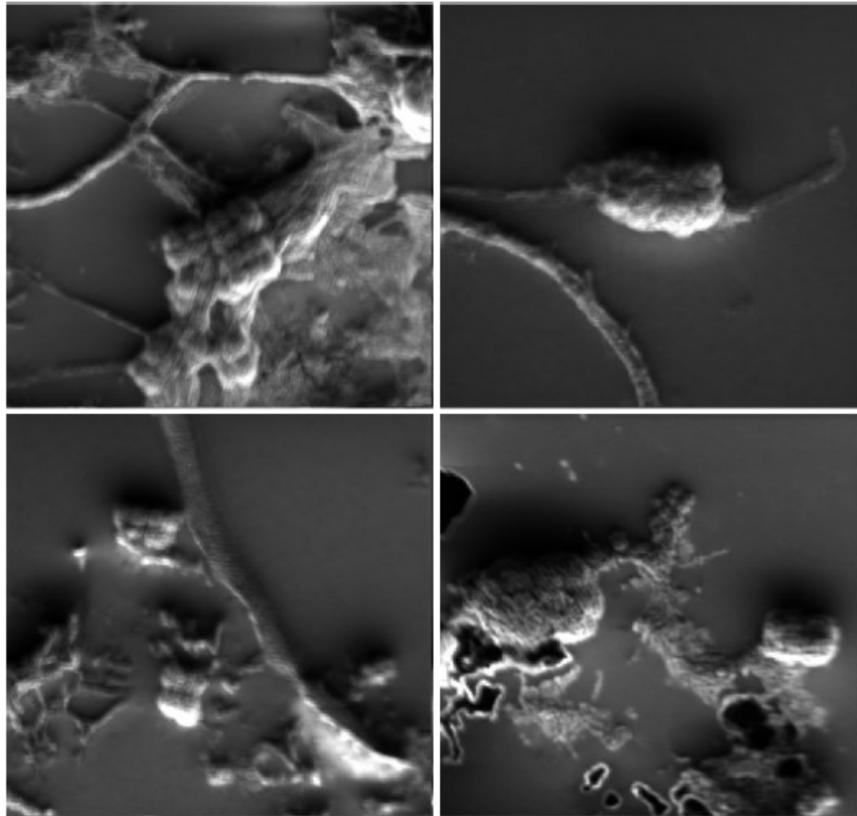
Figure S2. NanoSIMS secondary electron images of FISH positive *Defluviicoccus* cluster II tetrads relocated using stitched FISH images and unique floc structure.

Table S1. Number of areas and regions of interest (ROIs) analysed by treatment using NanoSIMS.

Treatment	Areas	ROIs
Control	7	21
Acetate Control	6	51
Nitrogen Control	5	36
100:1	9	14
60:1	4	15
20:1	3	24
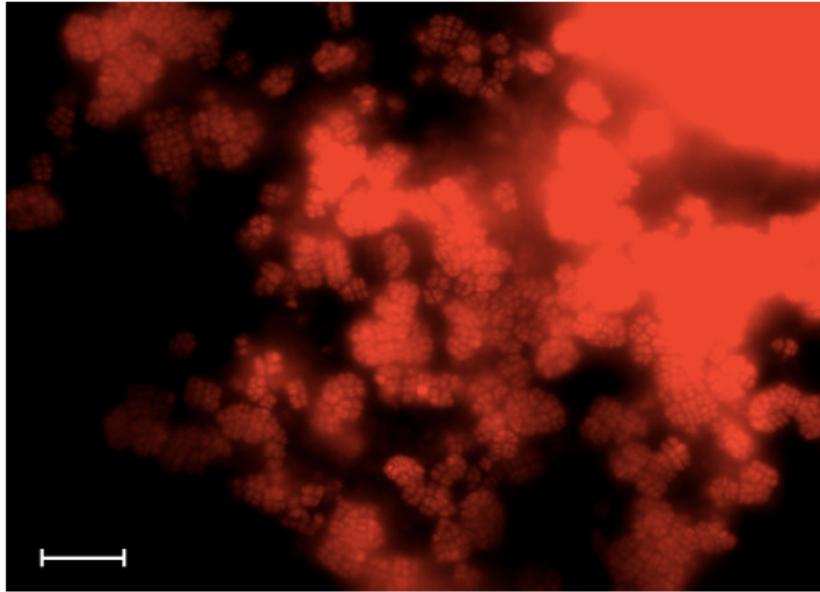
Figure S3. FISH micrograph of *Defluviicoccus* Cluster II in the SBR activated sludge sample. FISH probe applied included DF2mix (Cy3-label, red). Scale bar represents 10 μm.
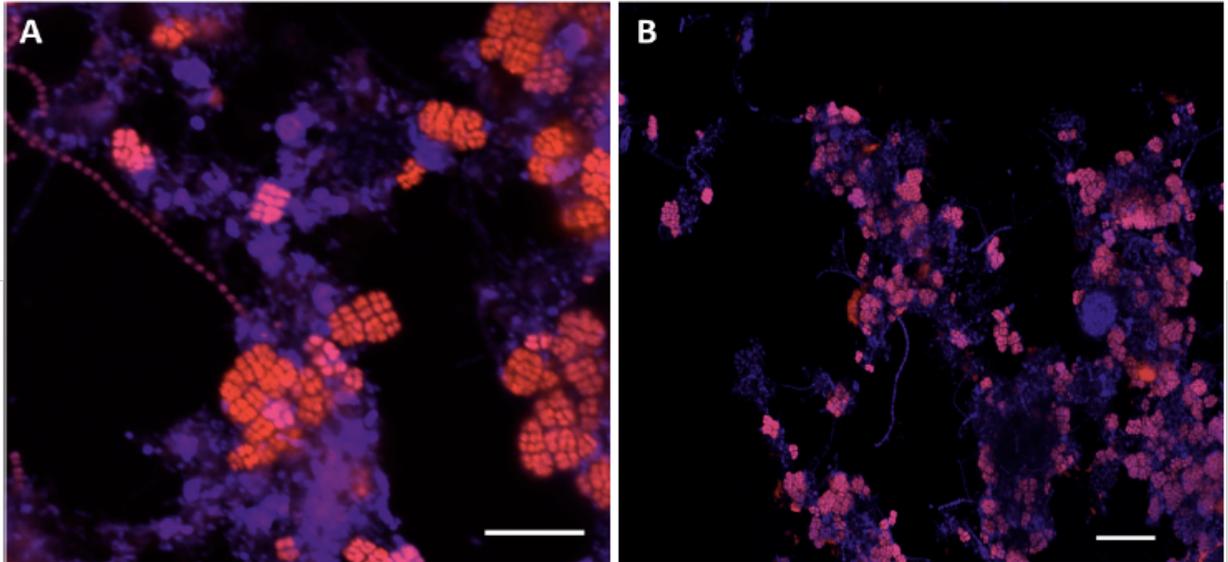
Figure S4. (A) Composite FISH micrograph of *Defluviicoccus* Cluster II in the SBR activated sludge sample. The FISH probes applied included DF2mix (Cy3-label, red) and EUBmix (Cy5-label, blue). Scale bar represents 10 μm. (B) Composite FISH micrograph of *Defluviicoccus* Cluster II in the SBR activated sludge sample. The FISH probes applied included DF2mix (Cy3-label, red), Competitor probe and EUBmix (Cy5-label, blue). Scale bar represents 20 μm.

Chapter 4

Genomic insights into the metabolism of "*Candidatus* Defluviicoccus seviourii", a member of *Defluviicoccus* cluster III abundant in industrial activated sludge

Contextual statement

Filamentous bacteria belonging to DF3 proliferate in the WWW survey presented in Chapter 2. This group of bacteria has been associated to bulking issues and reported as abundant in several industrial and domestic wastewater systems. The aim of this chapter is to extract and analyse the genome of DF3, in order to extrapolate what physiological traits allow them to compete in the activated sludge environment.

Statement of Authorship

Title of Paper	Genomic insights into the metabolism of '*Candidatus* Defluviicoccus seviourii', a member of *Defluviicoccus* cluster III abundant in industrial activated sludge.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Cristobal Onetto
Contribution to the Paper	Designed experiment, prepared samples for sequencing, performed bioinformatics, *in situ* and data analyses. Interpreted the data and drafted the manuscript.
Overall percentage (%)	85
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 22/02/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Kathryn Eales
Contribution to the Paper	Revised the manuscript and helped with data interpretation.
Signature	Date 21/02/18

Name of Co-Author	Paul Grbin
Contribution to the Paper	Revised the manuscript.
Signature	Date 22/02/2018

Genomic insights into the metabolism of "*Candidatus Defluviicoccus seviourii*", a member of *Defluviicoccus* cluster III abundant in industrial activated sludge.

Cristobal A Onetto[†], Paul R Grbin[†], Kathryn L Eales[†*]

[†] Department of Wine & Food Science, University of Adelaide.

*The Corresponding Author: kathryn.eales@adelaide.edu.au

ABSTRACT

Filamentous glycogen accumulating organisms (GAOs) belonging to the *Defluviicoccus* cluster III (DF3) are known to proliferate and cause bulking issues in industrial and domestic wastewater treatment plants. Yet, limited research has focused on understanding the physiological traits that allow them to compete in various wastewater environments.

In this study, a near complete genome of an abundant filamentous DF3 named "*Candidatus* Defluviicoccus seviourii" was obtained from a full-scale sequencing batch reactor (SBR) treating industrial wastewater. Annotation of the "*Ca.* D. seviourii" genome revealed interesting metabolic features that help to understand the competitiveness and abundance of this microorganism in wastewater treatment plants. The genetic potential to cycle trehalose through glycogen, nitrogen fixation, hydrogenase activity and urea uptake appear as adaptive strategies of "*Ca.* D. seviourii" to industrial activated sludge. The presence of the ethylmalonyl-CoA pathway as an alternative to the glyoxylate shunt, not previously reported in other GAO genomes, unveils new questions about the relevance of this pathway to the classical GAO metabolism.

INTRODUCTION

The excessive proliferation of specific filamentous bacteria can cause operational problems in wastewater treatment plants, commonly referred to as bulking (Jenkins et al., 2003; Seviour and Nielsen, 2010). Several filamentous morphotypes have been identified (Eikelboom, 1977; Martins et al., 2004) and their phylogeny resolved with the help of 16S rRNA gene sequencing and fluorescence *in situ* hybridization (FISH), revealing phylogenetic differences between morphologically similar bacteria (Thomsen et al., 2002; Kragelund et al., 2006; Nielsen et al., 2009). Although the majority of these filamentous bacteria are unculturable, techniques like FISH with the combination of microautoradiography (MAR) (Nielsen et al., 2009) and most recently genome extraction from metagenomes (McIlroy et al., 2016) have allowed important features of their ecophysiology to be elucidated.

Nostocoida limicola, a commonly observed filamentous bacterial morphotype, was originally classified into three morphotypes, *N. limicola* I, II and III based on their cell diameter (Eikelboom and Van Buijsen, 1983). Of these, *N. limicola* II morphotype filaments, regularly proliferate in industrial and domestic treatment plants (Eikelboom et al., 1998; Kragelund et al., 2006), and are proven to be phylogenetically diverse, with observed filaments belonging to the Actinobacteria (Blackall et al., 2000), Alphaproteobacteria (Levantesi et al., 2004) and Chloroflexi (Schade et al., 2002).

A previous study revealed that the alphaproteobacterial Candidatus *Monilibacter* spp., a *N. limicola* II morphotype filamentous bacteria dominant in industrial and domestic treatment plants (Levantesi et al., 2004; Kragelund et al., 2006) is actually a member of the *Defluviicoccus* cluster III (DF3) glycogen accumulating organisms (GAOs) (Nittami et al., 2009). GAOs and specifically the *Defluviicoccus*- related GAOs are an important core component of the bacterial community of both industrial and domestic treatment plants (McIlroy and Seviour, 2009; Onetto et al., 2017b; Stokholm-Bjerregaard et al., 2017). Much research has focused on these organisms, primarily due to their metabolic characteristic which are detrimental to the performance of enhanced biological phosphorus removal (EBPR) treatment plants (Oehmen et al., 2007). The classical GAO phenotype in EBPR treatment plants has been defined as the following; during the anaerobic phase GAOs take up volatile fatty acids (VFAs) and store them as polyhydroxyalkanoates (PHAs) using energy obtained from the hydrolysis of glycogen. PHAs stores are later used in the aerobic phase for replenishment of the glycogen pool and growth (Oehmen et al., 2007). This phenotype has been demonstrated for the gammaproteobacterial Competibacter-lineage (Crocetti et al., 2002; McIlroy et al., 2013) and the alphaproteobacterial *Defluviicoccus* (Wong and Liu, 2007).

Despite their reported dominance and associated bulking events in both industrial and domestic treatment plants (McIlroy and Seviour, 2009; Nittami et al., 2009), little attention has been paid to the filamentous DF3 GAOs. In contrast to the non-filamentous tetrad-forming *Defluviicoccus* of cluster I and II, with multiple *in situ* ecophysiology reports (Wong et al., 2004; Meyer et al., 2006; Burow et al., 2007; Wong and Liu, 2007; Schroeder et al., 2008) and genomes available (Nobu et al., 2014; Wang et al., 2014), only one study has focused on their ecophysiology, confirming the GAO phenotype (McIlroy et al., 2010). The observed proliferation in various waste streams suggest they possess metabolic traits that

allow them to adapt and compete in the activated sludge environment. This requires further investigation.

Through a metagenomic approach, the genome of a filamentous DF3 dominant in industrial activated sludge was investigated. The central metabolic pathways were characterised and valuable insights into their competition strategies were provided. This is the first study on the genomic characteristics of a member of the DF3 and serves as a basis for future physiology studies on this relevant microorganism for both industrial and domestic activated sludge.

MATERIALS AND METHODS

Sample collection

Activated sludge samples were collected for a period of 12 months from a full-scale SBR (5 ML capacity) treating winery wastewater (WWW) in Barossa, South Australia.

DNA extraction and sequencing

Biomass DNA was extracted using a FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA). Amplicon libraries and sequencing were performed by the Australian Centre for Ecogenomics (Brisbane, Australia). For the *in situ* estimation of DF3 and microbial diversity, 16S rRNA gene sequencing was performed using primers targeting the V3-4 region and the Miseq (Illumina) platform. For metagenome assembly, paired end libraries were prepared using the NexteraXT kit (Illumina Inc.). The prepared libraries were sequenced using the NextSeq 500 and the v2 reagent kit (2 x 150bp, Illumina Inc.).

16S rRNA bioinformatics processing and fluorescence in situ hybridization

Bioinformatic analysis of 16S rRNA gene sequences was performed as previously described (Albertsen et al., 2015). Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32. (Bolger et al., 2014). The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 (Magoč and Salzberg, 2011) then dereplicated and formatted for use in the UPARSE workflow (Edgar, 2013). Taxonomy was assigned with QIIME (Caporaso et al., 2010), using the MiDAS database v. 2.1 (McIlroy et al., 2015). The results were analysed with R (Team, 2015) using the ampvis package v 1.9.1 (Albertsen et al., 2015). Phylogenetic trees were performed using ARB (Ludwig et al., 2004).

SBR samples were also assessed by FISH (Daims et al., 2005) using formaldehyde fixed cells and probes EUB338mix (Amann et al., 1990; Daims et al., 1999) and DF198 (Nittami et al., 2009) to identify DF3 filaments.

Histochemical staining

Intracellular PHA and polyphosphate granules were examined using Nile blue A (Ostle and Holt, 1982) and 4', 6-diamidino-2-phenylindole (DAPI) (Kawaharasaki et al., 1999), as previously described by (Ahn et al., 2007), with modifications in the Nile blue A destaining

procedure (overnight destaining) prior to FISH. Gram staining was performed as previously described by Forster et al. (2002).

Metagenome assembly and genome extraction

Metagenome assembly was performed as previously described (Albertsen et al., 2016). Paired-end reads were imported to CLC Genomics Workbench v. 10.0 (CLCbio, Qiagen) and trimmed for quality and Illumina sequencing adaptors. The trimmed metagenome reads were *de novo* assembled using CLC with a kmer size of 63 and a minimum scaffold length of 1 kpb. Reads from each time point were then mapped independently to scaffolds from the metagenome assembly using CLC's map to reference algorithm. Open reading frames from the metagenome assembly were predicted using Prodigal (Hyatt et al., 2010) and searched with HHMER3 (http://hmmer.org) against a set of 107 HMMs essential single-copy genes (Dupont et al., 2012). Identified proteins were taxonomically classified using BLASTP (Altschul et al., 1990). MEGAN v. 6.6 (Huson et al., 2011) was used to extract class level taxonomic assignments.

Genome bins extraction from the metagenome was performed as previously described by (Albertsen et al., 2016) using mmgenome (Karst et al., 2016) and the differential coverage principle (Albertsen et al., 2013). Extracted paired-end reads from the genome bins were re-assembled using SPAdes (Bankevich et al., 2012). Contigs with low coverage (< 100 X) were removed from the assembly. Final estimation of genome completeness was performed using CheckM (Parks et al., 2015).

Genome annotation and metabolic pathways analysis

The assembled genome was uploaded to the Microscope platform (Vallenet et al., 2013). Pathways and genes of interest were validated manually using the integrated KEGG (Kanehisa et al., 2014) and MicroCyc (Caspi et al., 2008) databases.

Data availability

The genome annotation and genome sequence will be submitted to the European Nucleotide Archive (ENA) database prior to submission of the manuscript.

RESULTS AND DISCUSSION

In situ identification and quantification of DF3 in activated sludge samples

16S rRNA gene analyses of eight time points revealed the common occurrence of an OTU (OTU_9) classified as DF3 (**Figure 1**). Operational data obtained from the treatment plant showed a correlation between the abundance of this OTU and bulking events (data not shown). To confirm the identity of this organism *in situ*, FISH was performed on fixed samples from dates that showed a high abundance of OTU_9 using the DF198 probe, specifically designed to target this group of microorganisms (Nittami et al., 2009). A positive

FISH signal was observed on abundant filaments with a *"Nostocoida limicola* II*"* morphotype **(Figure 2)**, confirming the identity and abundance of this filament as a member of the DF3. Histochemical staining of FISH positive filaments showed the presence of intracellular PHAs stores (Supplementary material **Figure 2**) and absence of polyphosphate granules (data not shown) as previously reported by McIlroy et al. (2010).

High abundances of DF3 have previously been reported as dominant in industrial and domestic wastewater treatment plants with EBPR and non-EBPR configurations (McIlroy and Seviour, 2009; Nittami et al., 2009; McIlroy et al., 2010). Besides the observed abundance of DF3, an OTU classified as *Defluviicoccus* cluster II (OTU_1) was also observed in the activated sludge samples (**Figure 1**). Bacteria belonging to the Betaproteobacteria, Flavobacteria, Sphingobacteria and Alphaproteobacteria were also abundant within the samples. Previous microbial community surveys of WWW treatment plants showed abundance of bacteria belonging to these classes (McIlroy et al., 2011; Onetto et al., 2017a; Onetto et al., 2017b), specifically *Defluviicoccus* spp. appear as core components of the microbial community of activated sludge treating WWW.

Genome extraction

In order to elucidate the metabolic characteristics of the dominant GAO observed in the activated sludge samples, the genome of this organism was extracted using the differential coverage principle (Albertsen et al., 2013). Three time points from a wastewater treatment plants showing high abundance of DF3 were sequenced. Differential coverage plots were performed on the three dates, however, one set (showing better binning potential) was selected for final genome extraction (**Figure 3**). The genome statistics after binning cleaning and reassembly are described in **Table 1**, showing a genome size of 3.2 Mbp, an estimated completeness of 98% predicted using the Rhodospirillalles lineage specific marker set and a GC content of 64%, similar to the characteristics of the two available *Defluviicoccus* genomes belonging to cluster I (Nobu et al., 2014) and cluster II (Wang et al., 2014).

Phylogenetic analysis was performed on the 16S rRNA gene of the extracted genome (**Figure 4**) indicating it was closely related to clones A40 and B29 (99% similarity Blastn) (Nittami et al., 2009), which are classified as members of DF3.

The genome extracted in this study has been designated as *"Candidatus* Defluviicoccus seviourii"*, with the species name referring to Robert Seviour, an Australian microbiologist who has made a considerable contribution to resolving the phylogeny of *N. limicola*, understanding the diversity of GAOs and activated sludge microbiology in general.

Genomic potential for GAO metabolism

The proposed metabolism that describes most GAOs is that under anaerobic conditions carbon is assimilated and converted to PHAs with energy obtained from the hydrolysis of glycogen. To balance the excess of reducing equivalents produced from this hydrolysis, pyruvate would be converted to propionyl-CoA through the reductive branch of the TCA cycle, using a fumarate reductase complex, and the methylmalonyl-CoA pathway, also contributing to the proton motive force (PMF) required for the uptake of VFAs (Oehmen et

al., 2007). Under aerobic conditions, PHAs are used for growth and replenishment of the glycogen pool. It is of great importance to consider that this model metabolism is not necessarily what defines GAOs. Recent genomic and *in situ* studies have suggested alternative strategies to balance the reducing equivalents produced from the hydrolysis of glycogen, such as fermentation of sugars (McIlroy et al., 2013) or hydrogenase activity (Albertsen et al., 2016). Even the absence of PHAs storage has been recently described in *Micropruina* spp. GAOs, which store glycogen under anaerobic conditions through a fermentative metabolism (McIlroy et al., unpublished**)**

The "*Ca*. D. seviourii" genome contains genes associated to the classical GAO metabolism, this includes glycogen synthesis (glgABC) and hydrolysis (glgXP) (Preiss, 2006), a fumarate reductase complex, a succinate to propionyl-CoA pathway including a possible sodium-export methylmalonyl-CoA decarboxylase (Supplementary material **Table 1**) (**Figure 5**) and a F_1F_0 ATPase also suggested to be involved in the PMF generation for VFA uptake (Saunders et al., 2007). Genes for PHA metabolism are also present in the "*Ca*. D. seviourii" genome with multiple PHA synthases annotated.

Interestingly, two trehalose pathways (TreYZ and TreS) (Ruhal et al., 2013) were annotated in the "*Ca*. D. seviourii" genome, highlighting this organism has the genetic potential to cycle trehalose and glycogen. A similar metabolism was reported in the *Candidatus* Contendobacter odensis GAO genome (McIlroy et al., 2013), however, this was not described in previous *Defluviicoccus* reports. The ability to store trehalose is associated with multiple stress related functions such as resistance to osmotic stress, extreme temperatures (Ruhal et al., 2013) and desiccation (Elbein et al., 2003). DF3 ability to proliferate in a broad range of wastewater environments may be partially explained by their capacity to cycle trehalose and glucose, which would aid in their resistance to adverse environmental conditions.

McIlroy et al. (2010) showed the absence of polyphosphate granules in filaments that hybridized with the DF1013 and DF1004 probes. The "*Ca*. D. seviourii" genome contains genes associated to polyP metabolism (Supplementary material **Table 1**) however it lacks a low affinity phosphate transporter (Pit). The absence of this transporter has been observed in other organisms with GAO metabolism (McIlroy et al., 2013; Nobu et al., 2014; Albertsen et al., 2016) displaying a negative DAPI staining for polyP and has been postulated to be essential for the PAO phenotype (McIlroy et al., 2013). Thus, "*Ca*. D. seviourii" capacity to cycle polyP might be limited by the absence of the low affinity Pit transporter, given that DF3 are observed proliferating in non-EBPR systems it is logical that they do not rely on polyP cycling.

Substrate uptake

Previous *in situ* ecophysiology studies have reported the ability of DF3 to uptake propionate, acetate and glutamate, implying that DF3 is a highly specialised feeder in activated sludge communities (Kragelund et al., 2006; McIlroy et al., 2010). Discrepancies have been reported specifically in regard to DF3 ability to uptake acetate anaerobically. The *C*. Monilibacter batavus member of DF3 investigated by Kragelund et al. (2006) showed no acetate uptake under anaerobic conditions. However, McIlroy et al. (2010) reported acetate uptake under

anaerobic in DF3 filaments present in three wastewater treatment plants. The "*Ca*. D. seviourii" genome contains the genes actP, acs, ackA and pta (Supplementary material **Table 1),** involved in the uptake and activation of acetate and propionate in symport with a cation. Furthermore, a Na/Glutamate symporter was also annotated in the genome, along with two ABC type amino acid transport systems (**Figure 5**), the aapJQMP (Walshaw and Poole, 1996) which transports a broad range of L-amino acids and a LIV branched-chain amino acid transport system (Hosie and Poole, 2001). This would provide "*Ca*. D. seviourii" the genetic potential to uptake a wide range of amino acids.

Interestingly, a mannose specific PTS transport system was also observed in the genome along with genes for the activation of mannose (Supplementary material **Table 1**). The ability to uptake these substrates *in situ* was not tested on filaments binding to the DF1013 and DF1004 probes (McIlroy et al., 2010), however, an overview of this study suggests DF3 has a more diverse substrate utilisation pattern than previously reported and could be examined in future ecophysiology studies.

Central carbon metabolism

Annotation of the "*Ca*. D. seviourii" genome showed the presence of genes involved in central metabolic pathways previously described in *Defluviicoccus* studies (Nobu et al., 2014; Wang et al., 2014) including the TCA cycle, glycolysis (EMP pathway) and gluconeogenesis (Supplementary material **Table 1**). Interestingly, the "*Ca*. D. seviourii" genome lacks both genes Icl (Isocitrate lyase) and Mas (Malate synthase) associated with the glyoxylate shunt (Kornberg and Krebs, 1957). This pathway has been shown to be active in other *Defluviicoccus* related GAOs (Burow et al., 2009) and the genes were present in both *Defluviicoccus* genomes (Nobu et al., 2014; Wang et al., 2014). The glyoxylate shunt is considered an important pathway for microorganisms growing on acetyl-CoA generating carbon sources such as acetate and PHAs, allowing the bypass of the decarboxilating enzymes of the TCA cycle (Anthony, 2011). The ability of DF3 to cycle PHA along with strong acetate uptake has been demonstrated *in situ* (McIlroy et al., 2010). Furthermore, the chemical composition of WWW is mainly composed of acetate (Grbin and Eales, 2017), and DF3 is shown to proliferate under these conditions (**Figure 1**). This evidence indicates that DF3 bacteria rely on acetyl-CoA generating compounds for growth in accordance with the classical GAO phenotype. For this reason, the genetic potential for alternative pathways was investigated. Unique genes associated to the anaplerotic ethylmalonyl-CoA pathway were observed in the genome of "*Ca*. D. seviourii" (Ccr, Epi, Mea and Mcd) (Anthony, 2011) (Supplementary material **Table 1**), indicating "*Ca*. D. seviourii" has the genetic potential to grow on acetate through the conversion of two molecules of acetyl-CoA to glyoxylate and propionyl-CoA (**Figure 5**). Glyoxylate would be later condensed to malate by Mcl-2 and enter the TCA cycle. Propionyl-CoA would be converted to succinyl-CoA through the methylmalonyl-CoA (**Figure 5**). Furthermore, the genes Ccr, Mea and Mcd are clustered in the same genetic locus (Supplementary material **Table 1**), commonly observed in bacteria that grow on acetate but not having the classic glyoxylate cycle (Erb et al., 2007).

The reasons why DF3 would use this pathway instead of the glyoxylate cycle as observed in other *Defluviicoccus* related GAOs is unknown and needs to be further investigated. Future

inhibition studies need to be performed on this cluster to confirm the activity of this pathway and elucidate the actual implications that this pathway has over the well described PHA-glycogen metabolism of the *Defluviicoccus* GAOs (Oehmen et al., 2007).

Nitrogen metabolism

Annotation of the "*Ca*. D. seviourii" genome revealed interesting insights into the nitrogen metabolism of DF3. Multiple observations indicate that "*Ca*. D. seviourii" is highly adapted to low nitrogen environments. Along with possessing the genetic potential to reduce nitrate to ammonia through an assimilatory metabolism including a high affinity ABC-type nitrate transporter (nrtABCD), it also contains the genetic potential to fix nitrogen (**Figure 5**). Trehalose storage is also associated to nitrogen fixing organisms, functioning as the preferred nitrogen-free compatible solute in nitrogen limited conditions (Galinski and Herzog, 1990). Nitrogen fixation is not an energy efficient process, requiring ATP and low potential reductant to function. A characteristic of the nitrogenase activity is the production of hydrogen during nitrogen reduction. In the absence of nitrogen, the activity of this enzyme is the reduction of protons to hydrogen, therefore incurring a waste of energy (Vignais and Billoud, 2007). The interrelationship of hydrogenase activity to nitrogen fixation has been postulated as an efficiency strategy to recycle the hydrogen produced. The "*Ca*. D. seviourii" genome encodes an uptake NiFe-hydrogenase Hyd-2 complex (HybABCDO) (Pinske et al., 2015), that could be essential for the adaptation of this organism to low nitrogen environments, allowing a more energy efficient nitrogen fixation process. The activity of this hydrogenase may also be associated to the reduction of other electron acceptors such as fumarate through the fumarate reductase complex (frdABCD) (Pinske et al., 2015), formate through a formate hydrogen lyase complex and/or nitrate (**Figure 5**).

A urea uptake system operon urtABCDE and a urease operon ureABCEFGD (Beckers et al., 2004) were also observed in the "*Ca*. D. seviourii" genome, allowing active uptake of urea along with its conversion to ammonia. Interestingly, the activity of this urea uptake and urease system has been demonstrated to work only under nitrogen starvation conditions (Beckers et al., 2004), such as the conditions observed in WWW (McIlroy et al., 2011; Onetto et al., 2017b) and other industrial wastewater streams (Ammary, 2004).

From an observation of the overall nitrogen related genomic features of "*Ca*. D. seviourii", speculations can be made in relation to DF3 adaptation to industrial activated sludge and specifically WWW. Besides being characterised by low nutrient levels, many industrial waste streams such as winery waste are highly seasonal (Mosse et al., 2011), exposing the activated sludge to feeding fluctuations. It is logical to expect that activated sludge communities with mechanisms to overcome the adverse environmental conditions (e.g. trehalose-glycogen cycling and nitrogen fixation) will be able to compete and proliferate, as observed for "*Ca*. D. seviourii".

CONCLUSIONS

In this study, metagenomics techniques were used to extract the first draft genome of an abundant filamentous GAO belonging to the *Defluviicoccus* cluster III. Through the analyses

of its genetic potential, relevant features related to its adaptation and competition strategies in activated sludge were extrapolated. In contrast to the other closely related *Defluviicoccus* clusters (I, II, IV) described so far, which exhibit a tetrad morphology, DF3 filamentous morphology links its abundance to severe bulking issues in wastewater treatment plants. Their abundance in EBPR and non-EBPR configuration treatment plants treating both domestic and industrial wastewater, along with their impact in floc settleability and competition with polyphosphate accumulating organisms, makes them highly relevant for the wastewater treatment field. The genomic insight of "*Ca*. D. seviourii" described in this study are an important contribution to further understand the physiology of this organisms in order to elaborate control strategies for their proliferation.

REFERENCES

Ahn, J., Schroeder, S., Beer, M., McIlroy, S., Bayly, R.C., May, J.W. *et al.* (2007) Ecology of the microbial community removing phosphate from wastewater under continuously aerobic conditions in a sequencing batch reactor. Appl Environ Microbiol 73: 2257-2270.

Albertsen, M., Karst, S.r.M., Ziegler, A.S., Kirkegaard, R.H., and Nielsen, P.H. (2015) Back to basics ? The influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities. PLoS ONE 10: e0132783.

Albertsen, M., McIlroy, S.J., Stokholm-Bjerregaard, M., Karst, S.M., and Nielsen, P.H. (2016) "*Candidatus* Propionivibrio aalborgensis": A novel glycogen accumulating organism abundant in full-scale enhanced biological phosphorus removal plants. Front Microbiol 7: 1033.

Albertsen, M., Hugenholtz, P., Skarshewski, A., Nielsen, K.L., Tyson, G.W., and Nielsen, P.H. (2013) Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. Nat biotechnol 31: 533.

Altschul, S., Gish, W., Miller, W., Myers, E., and Lipman, D. (1990) Basic local alignment search tool. J Mol Biol 215: 403-410.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 56: 1919-1925.

Ammary, B.Y. (2004) Nutrients requirements in biological industrial wastewater treatment. Afr J biotechnol 3: 236-238.

Anthony, C. (2011) How half a century of research was required to understand bacterial growth on C1 and C2 compounds; the story of the serine cycle and the ethylmalonyl-CoA pathway. Sci prog 94: 109-137.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S. *et al.* (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19: 455-477.

Beckers, G., Bendt, A.K., Krämer, R., and Burkovski, A. (2004) Molecular identification of the urea uptake system and transcriptional analysis of urea transporter- and urease-encoding genes in corynebacterium glutamicum. J Bacteriol 186: 7645-7652.

Blackall, L.L., Seviour, E.M., Bradford, D., Rossetti, S., Tandoi, V., and Seviour, R.J. (2000) '*Candidatus* Nostocoida limicola', a filamentous bacterium from activated sludge. Int J Syst Evol Microbiol 50: 703-709.

Bolger, A.M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics: btu170.

Burow, L.C., Mabbett, A.N., Borras, L., and Blackall, L.L. (2009) Anaerobic central metabolic pathways active during polyhydroxyalkanoate production in uncultured cluster 1 Defluviicoccus enriched in activated sludge communities. FEMS Microbiol Lett 298: 79-84.

Burow, L.C., Kong, Y., Nielsen, J.L., Blackall, L.L., and Nielsen, P.H. (2007) Abundance and ecophysiology of *Defluviicoccus* spp., glycogen-accumulating organisms in full-scale wastewater treatment processes. Microbiol 153: 178-185.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K. *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7: 335-336.

Caspi, R., Foerster, H., Fulcher, C.A., Kaipa, P., Krummenacker, M., Latendresse, M. *et al.* (2008) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Res 36: D623-D631.

Crocetti, G.R., Banfield, J.F., Keller, J., Bond, P.L., and Blackall, L.L. (2002) Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. Microbiol 148: 3353-3364.

Daims, H., Stoecker, K., and Wagner, M. (2005) Fluorescence *in situ* hybridization for the detection of prokaryotes. Mol Microb Ecol 213: 239.

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., and Wagner, M. (1999) The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: development and evaluation of a more comprehensive probe set. Syst Appl Microbiol 22: 434-444.

Dupont, C.L., Rusch, D.B., Yooseph, S., Lombardo, M.-J., Alexander Richter, R., Valas, R. *et al.* (2012) Genomic insights to SAR86, an abundant and uncultivated marine bacterial lineage. ISME J 6: 1186-1199.

Edgar, R.C. (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 10: 996-998.

Eikelboom, D. (1977) Identification of filamentous organisms in bulking activated sludge. Prog Water Tech 8: 153-161.

Eikelboom, D.H., and Van Buijsen, H. (1983) Microscopic sludge investigation manual. 2nd ed. In: TNO research institute for environmental hygiene. Delft. The Netherlands.

Eikelboom, D.H., Andreadakis, A., and Andreasen, K. (1998) Survey of filamentous populations in nutrient removal plants in four European countries. Water Sci Technol 37: 281-289.

Elbein, A.D., Pan, Y.T., Pastuszak, I., and Carroll, D. (2003) New insights on trehalose: a multifunctional molecule. Glycobiology 13: 17-27.

Erb, T.J., Berg, I.A., Brecht, V., Müller, M., Fuchs, G., and Alber, B.E. (2007) Synthesis of C5-dicarboxylic acids from C2-units involving crotonyl-CoA carboxylase/reductase: the ethylmalonyl-CoA pathway. Proc Natl Acad Sci 104: 10631-10636.

Forster, S., Snape, J.R., Lappin-Scott, H.M., and Porter, J. (2002) Simultaneous fluorescent gram staining and activity assessment of activated sludge bacteria. Appl Environ Microbiol 68: 4772-4779.

Galinski, E.A., and Herzog, R.M. (1990) The role of trehalose as a substitute for nitrogen-containing compatible solutes (*Ectothiorhodospira halochloris*). Arch Microbiol 153: 607-613.

Grbin, P., and Eales, K. (2017) Developing a fundamental understanding of the microbiological treatment of winery wastewater. In. Adelaide, Australia: University of Adelaide.

Hosie, A.H.F., and Poole, P.S. (2001) Bacterial ABC transporters of amino acids. Res Microbiol 152: 259-270.

Huson, D.H., Mitra, S., Ruscheweyh, H.-J., Weber, N., and Schuster, S.C. (2011) Integrative analysis of environmental sequences using MEGAN4. Genome Res 21: 1552-1560.

Hyatt, D., Chen, G.-L., LoCascio, P.F., Land, M.L., Larimer, F.W., and Hauser, L.J. (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinf 11: 119.

Jenkins, D., Richard, M.G., and Daigger, G.T. (2003) Manual on the causes and control of activated sludge bulking, foaming and other solids separation problems, 3rd ed. London, UK: IWA Publishing.

Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M. (2014) Data, information, knowledge and principle: back to metabolism in KEGG. Nucleic Acids Res 42: 199-205.

Karst, S.M., Kirkegaard, R.H., and Albertsen, M. (2016) mmgenome: a toolbox for reproducible genome extraction from metagenomes. bioRxiv. 059121.

Kawaharasaki, M., Tanaka, H., Kanagawa, T., and Nakamura, K. (1999) *In situ* identification of polyphosphate-accumulating bacteria in activated sludge by dual staining with rRNA-targeted oligonucleotide probes and 4′, 6-diamidino-2-phenylindol (DAPI) at a polyphosphate-probing concentration. Water Res 33: 257-265.

Kornberg, H., and Krebs, e.H. (1957) Synthesis of cell constituents from C2-units by a modified tricarboxylic acid cycle. Nature 179: 988-991.

Kragelund, C., Kong, Y., van der Waarde, J., Thelen, K., Eikelboom, D., Tandoi, V. *et al.* (2006) Ecophysiology of different filamentous Alphaproteobacteria in industrial wastewater treatment plants. Microbiol 152: 3003-3012.

Levantesi, C., Beimfohr, C., Geurkink, B., Rossetti, S., Thelen, K., Krooneman, J. *et al.* (2004) Filamentous Alphaproteobacteria associated with bulking in industrial wastewater treatment plants. Syst Appl Microbiol 27: 716-727.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar *et al.* (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32: 1363-1371.

Magoč, T., and Salzberg, S.L. (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27: 2597-2963.

Martins, A.M.P., Pagilla, K., Heijnen, J.J., and van Loosdrecht, M.C.M. (2004) Filamentous bulking sludge—a critical review. Water Res 38: 793-817.

McIlroy, S., and Seviour, R.J. (2009) Elucidating further phylogenetic diversity among the *Defluviicoccus*-related glycogen-accumulating organisms in activated sludge. Environ Microbiol Rep 1: 563-568.

McIlroy, S., Speirs, L., Tucci, J., and Seviour, R.J. (2011) *In situ* profiling of microbial communities in full-scale aerobic sequencing batch reactors treating winery waste in Australia. Environ Sci Technol 45: 8794-8803.

McIlroy, S.J., Nittami, T., Seviour, E.M., and Seviour, R.J. (2010) Filamentous members of cluster III *Defluviicoccus* have the *in situ* phenotype expected of a glycogen-accumulating organism in activated sludge. FEMS Microbiol Ecol 74: 248-256.

McIlroy, S.J., Albertsen, M., Andresen, E.K., Saunders, A.M., Kristiansen, R., Stokholm-Bjerregaard, M. *et al.* (2013) '*Candidatus* Competibacter'-lineage genomes retrieved from metagenomes reveal functional metabolic diversity. ISME J 8: 613.

McIlroy, S.J., Karst, S.M., Nierychlo, M., Dueholm, M.S., Albertsen, M., Kirkegaard, R.H. *et al.* (2016) Genomic and *in situ* investigations of the novel uncultured Chloroflexi associated with 0092 morphotype filamentous bulking in activated sludge. ISME J 10: 2223-2234.

McIlroy, S.J., Saunders, A.M., Albertsen, M., Nierychlo, M., McIlroy, B., Hansen, A.A. et al. (2015) MiDAS: the field guide to the microbes of activated sludge. Database 2015: bav062.

Meyer, R.L., Saunders, A.M., and Blackall, L.L. (2006) Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing. Microbiol 152: 419-429.

Mosse, K.P.M., Patti, A.F., Christen, E.W., and Cavagnaro, T.R. (2011) Review: winery wastewater quality and treatment options in Australia. Aust J Grape Wine Res 17: 111-122.

Nielsen, P.H., Kragelund, C., Seviour, R.J., and Nielsen, J.L. (2009) Identity and ecophysiology of filamentous bacteria in activated sludge. FEMS Microbiol Rev 33: 969-998.

Nittami, T., McIlroy, S., Seviour, E.M., Schroeder, S., and Seviour, R.J. (2009) *Candidatus* Monilibacter spp., common bulking filaments in activated sludge, are members of Cluster III *Defluviicoccus*. Syst Appl Microbiol 32: 480-489.

Nobu, M.K., Tamaki, H., Kubota, K., and Liu, W.T. (2014) Metagenomic characterization of 'Candidatus* Defluviicoccus tetraformis strain TFO71', a tetrad-forming organism, predominant in an anaerobic–aerobic membrane bioreactor with deteriorated biological phosphorus removal. Environ Microbiol 16: 2739-2751.

Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L., and Reis, M.A. (2007) Advances in enhanced biological phosphorus removal: from micro to macro scale. Water Res 41: 2271-2300.

Onetto, C.A., Eales, K.L., and Grbin, P.R. (2017a) Remediation of *Thiothrix* spp. associated bulking problems by raw wastewater feeding: a full-scale experience. Syst Appl Microbiol 40: 396-399.

Onetto, C.A., Eales, K.L., Guagliardo, P., Kilburn, M.R., Gambetta, J.M., and Grbin, P.R. (2017b) Managing the excessive proliferation of glycogen accumulating organisms in industrial activated sludge by nitrogen supplementation: a FISH-NanoSIMS approach. Syst Appl Microbiol 40: 500-507.

Ostle, A.G., and Holt, J.G. (1982) Nile blue A as a fluorescent stain for poly-beta-hydroxybutyrate. Appl Environ Microbiol 44: 238-241.

Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., and Tyson, G.W. (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res 25: 1043-1055.

Pinske, C., Jaroschinsky, M., Linek, S., Kelly, C.L., Sargent, F., and Sawers, R.G. (2015) Physiology and bioenergetics of [NiFe]-Hydrogenase 2-Catalyzed H_2-consuming and H_2-producing reactions in *Escherichia coli*. J Bacteriol 197: 296-306.

Preiss, J. (2006) Bacterial glycogen inclusions: enzymology and regulation of synthesis. In Inclusions in Prokaryotes. Shively, J.M. (ed). Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 71-108.

Ruhal, R., Kataria, R., and Choudhury, B. (2013) Trends in bacterial trehalose metabolism and significant nodes of metabolic pathway in the direction of trehalose accumulation. Microb Biotechnol 6: 493-502.

Saunders, A.M., Mabbett, A.N., McEwan, A.G., and Blackall, L.L. (2007) Proton motive force generation from stored polymers for the uptake of acetate under anaerobic conditions. FEMS Microbiol Lett 274: 245-251.

Schade, M., Beimfohr, C., and Lemmer, H. (2002) Phylogenetic and physiological characterization of a "*Nostocoida limicola*"-like organism isolated from activated sludge. Water Sci Technol 46: 91-97.

Schroeder, S., Ahn, J., and Seviour, R.J. (2008) Ecophysiology of polyphosphate-accumulating organisms and glycogen-accumulating organisms in a continuously aerated enhanced biological phosphorus removal process. J Appl Microbiol 105: 1412-1420.

Seviour, R.J., and Nielsen, P.H. (2010) Microbial ecology of activated sludge. London, UK: IWA Publishing.

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

Team, R.C. (2015) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2013. Document freely available on the internet at: http://www r-project org.

Thomsen, T.R., Kjellerup, B.V., Nielsen, J.L., Hugenholtz, P., and Nielsen, P.H. (2002) *In situ* studies of the phylogeny and physiology of filamentous bacteria with attached growth. Environ Microbiol 4: 383-391.

Vallenet, D., Belda, E., Calteau, A., Cruveiller, S., Engelen, S., Lajus, A. *et al.* (2013) MicroScope—an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. Nucleic Acids Res 41: 636-647.

Vignais, P.M., and Billoud, B. (2007) Occurrence, classification, and biological function of hydrogenases: an overview. Chem Rev 107: 4206-4272.

Walshaw, D.L., and Poole, P.S. (1996) The general l-amino acid permease of *Rhizobium leguminosarum* is an ABC uptake system that also influences efflux of solutes. Mol Microbiol 21: 1239-1252.

Wang, Z., Guo, F., Mao, Y., Xia, Y., and Zhang, T. (2014) Metabolic characteristics of a glycogen-accumulating organism in *Defluviicoccus* cluster II revealed by comparative genomics. Microb Ecol 68: 716-728.

Wong, M., Tan, F.M., Ng, W.J., and Liu, W.-T. (2004) Identification and occurrence of tetrad-forming Alphaproteobacteria in anaerobic–aerobic activated sludge processes. Microbiol 150: 3741-3748.

Wong, M.T., and Liu, W.T. (2007) Ecophysiology of *Defluviicoccus*-related tetrad-forming organisms in an anaerobic–aerobic activated sludge process. Environ Microbiol 9: 1485-1496.

TABLES

Table 1. Genome characteristics of "*Candidatus* Defluviicoccus seviourii".

Parameter	"*Ca*. D. seviourii"
Size (Mbp)	3.2
Contigs	54
Scaffolds	47
Completeness (%)	98
Contamination (%)	0
Strain heterogeinity (%)	0
GC content (%)	64.83
N50	139823
CDS	3252
Protein coding density (%)	90.3
rRNA copies	1

CDS = Coding DNA sequence.

FIGURES
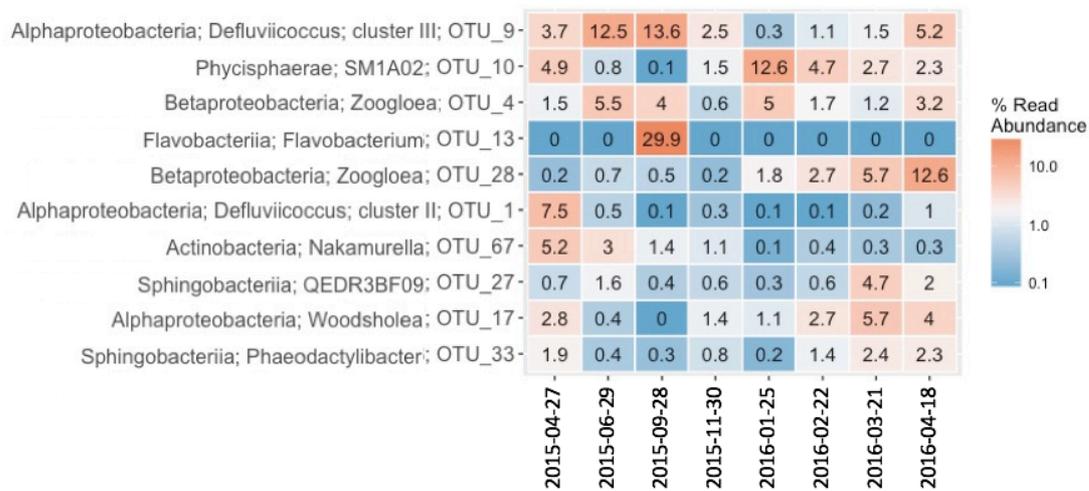
Figure 1. Heatmap representing the percentage read abundance of the 10 most abundant OTUs in SBR samples of a treatment plant over a 12-month period. Class and genus taxonomic classification for each OTU are provided.
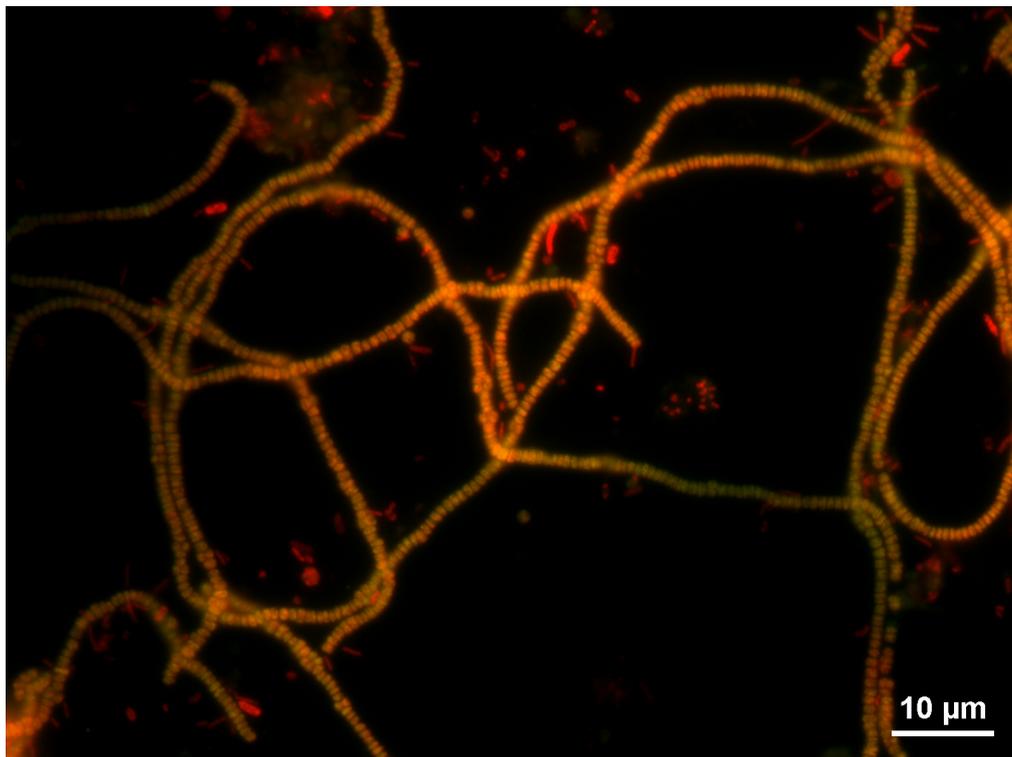
Figure 2. Composite FISH micrograph from a SBR sample (2015-09-28). FISH probes applied include DF198 (Fluos-label, green) and EUBmix (Cy3-label, red). *Defluviicoccus* cluster III filaments appear yellow/orange.
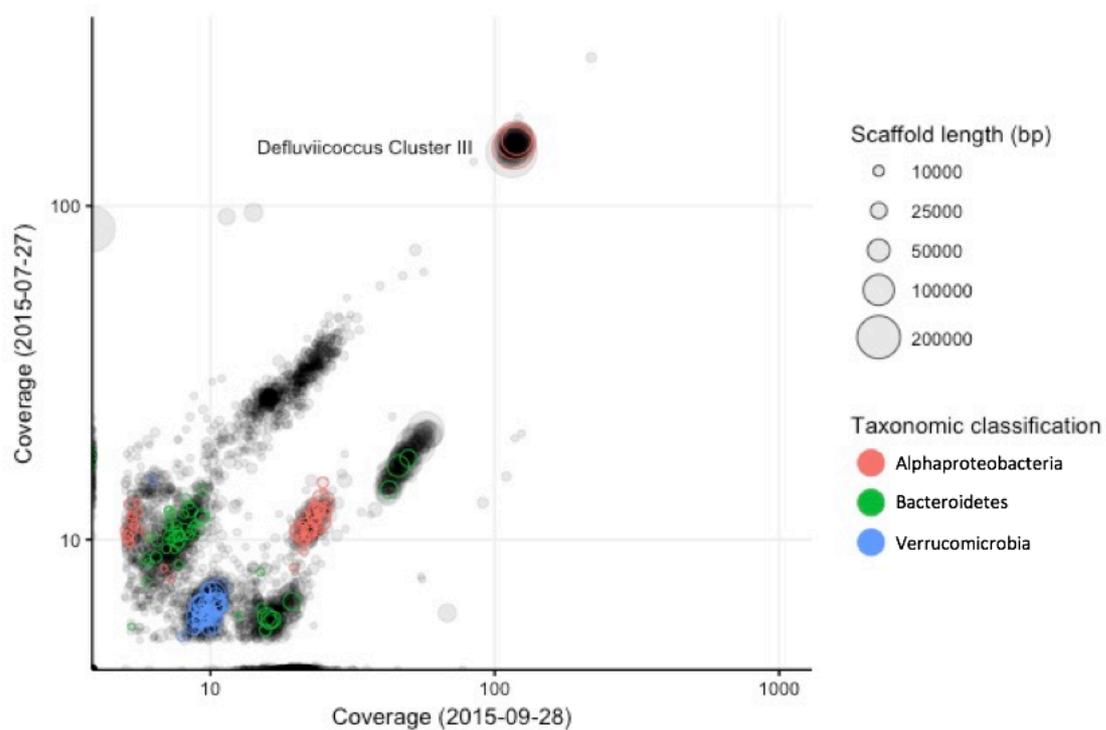
Figure 3. Differential coverage plot of two metagenomes obtained from a full-scale SBR. Each circle represents a metagenomic scaffold with size proportional to length. Colours indicate taxonomic classification of scaffolds using single essential copy genes.
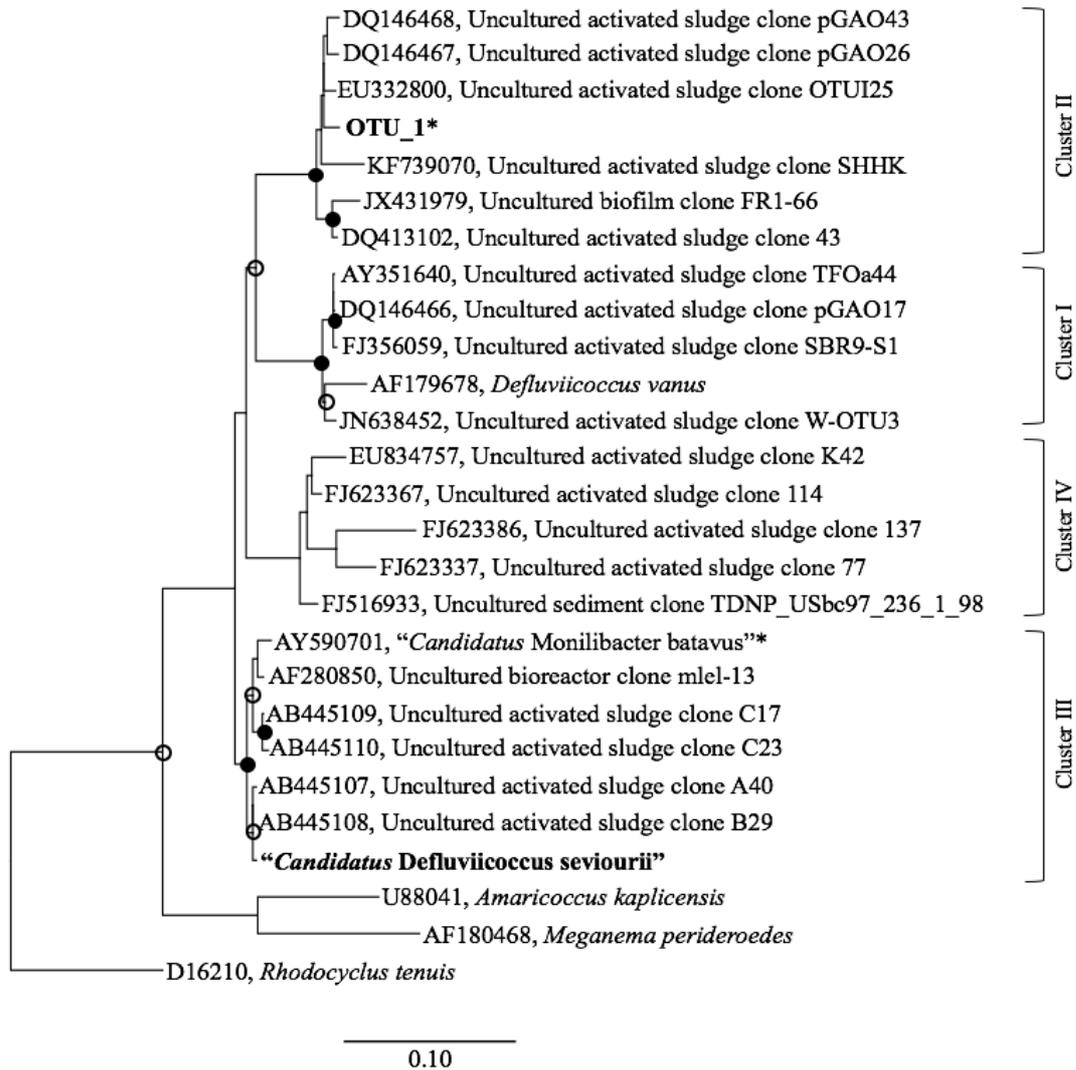
Figure 4. PhyML maximum likelihood 16S rRNA phylogenetic tree of *Defluviicoccus*- related sequences (> 1250 bp). Sequences in bold correspond to this study. Bootstraps values were calculated as percentages of 100 analyses. Black circles represent a branch support >70% and empty circles > 50%. Partial sequences (*) were added using the 'quick add' function in ARB. Scale bar represents substitutions per nucleotide base.
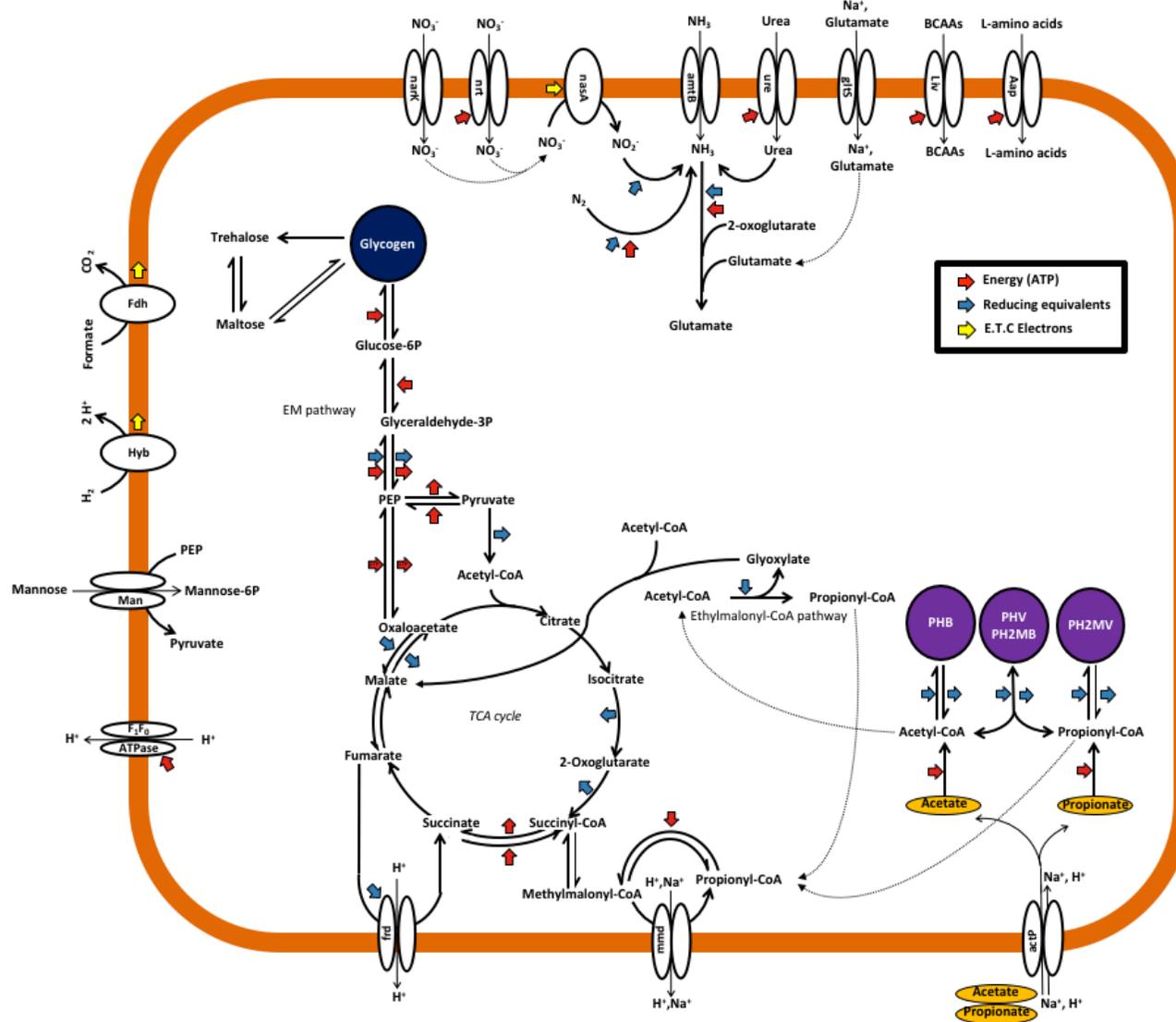
Figure 5. Metabolic model for "*Ca.* D. seviourii". Only relevant carbon, nitrogen and energy related pathways are represented. Aerobic and anaerobic pathways are represented in the same figure. For details on all the genes corresponding to each pathway please see supplementary material. NarK, Nitrate/nitrite transporter; nrt, ABC-type nitrate transporter; nasA, Nitrate reductase; amtB, Ammonia channel; ure, ABC-type urea transporter; gltS, Sodium/glutamate symporter; Liv, Branched-chain amino acid ABC-type transporter; Aap, General L-amino acid ABC-type transporter; actP, Acetate transporter; mmd, Methylmalonyl-CoA decarboxylase; frd, Fumarate reductase; Man, PTS mannose/fructose transporter; Hyb, Hydrogenase 2; Fdh, Formate dehydrogenase; EM, Embden-Meyerhof-Parnas; PEP, Phosphoenolpyruvate; TCA, Tricarboxylic acid.

Supplementary Material

Genomic insights into the metabolism of '*Candidatus Defluviicoccus seviourii*', a member of *Defluviicoccus* cluster III abundant in industrial activated sludge.

Cristobal A Onetto[†], Paul R Grbin[†], Kathryn L Eales[†*]

[†] Department of Wine & Food Science, University of Adelaide.

*The Corresponding Author: kathryn.eales@adelaide.edu.au

Table 1. List of annotated genes for the "*Ca*. D. seviourii" genome.

Gene	Protein	EC no.	"*Ca*. D. seviourii"
TCA cycle			
gltA	Citrate synthase	2.3.3.1	DF3_v1_180016
acnA	Aconitate hydratase	4.2.1.3	DF3_v1_90004
icd	Isocitrate dehydrogenase (NADP(+))	1.1.1.42	DF3_v1_70155
lpd	Dihydrolipoyl dehydrogenase	1.8.1.4	DF3_v1_50114
			DF3_v1_160069
			DF3_v1_160074
sucA	Oxoglutarate dehydrogenase	1.2.4.2	DF3_v1_160067
sucB	Dihydrolipoyllysine-residue succinyltransferase	2.3.1.61	DF3_v1_160068
sucC	Succinate--CoA ligase (ADP-forming), beta subunit	6.2.1.5	DF3_v1_160065
sucD	Succinate--CoA ligase (ADP-forming), alpha subunit	6.2.1.5	DF3_v1_160066
sdhA	Succinate dehydrogenase/fumarate reductase, flavoprotein subunit	1.3.5.1	DF3_v1_160057
sdhB	Succinate dehydrogenase/fumarate reductase, iron sulphur subunit	1.3.5.1	DF3_v1_160058
sdhC	Succinate dehydrogenase/fumarate reductase, cytochrome B subunit	1.3.5.1	DF3_v1_160056
sdhD	Succinate dehydrogenase, hydrophobic membrane anchor protein	1.3.5.1	DF3_v1_160055
frdA	Succinate dehydrogenase/fumarate reductase, flavoprotein subunit	1.3.99.1	DF3_v1_70088
frdB	Succinate dehydrogenase/fumarate reductase, iron sulphur subunit	1.3.99.1	DF3_v1_70089
frdC	Succinate dehydrogenase/fumarate reductase, cytochrome B subunit	1.3.99.1	DF3_v1_70090
frdD	Succinate dehydrogenase, hydrophobic membrane anchor protein	1.3.99.1	DF3_v1_70091
fumA	Fumarate hydratase	4.2.1.2	DF3_v1_320019
mdh	Malate dehydrogenase	1.1.1.37	DF3_v1_160064
Pyruvate metabolism			

pckG	Phosphoenolpyruvate carboxykinase [GTP]	4.1.1.32	DF3_v1_30084
pdhC	Dihydrolipoyllysine-residue acetyltransferase	2.3.1.12	DF3_v1_50115
pdhB	Pyruvate dehydrogenase E1 component subunit beta	1.2.4.1	DF3_v1_50116
pdhA	Pyruvate dehydrogenase E1 component subunit alpha	1.2.4.1	DF3_v1_50117

Glycolisis/gluconeogenesis

glk	Glucokinase	2.7.1.2	DF3_v1_220048
pgi	Glucose-6-phosphate isomerase	5.3.1.9	DF3_v1_40111
pfkA	6-phosphofructokinase	2.7.1.11	DF3_v1_100017
pfp	Diphosphate---fructose-6-phosphate 1-phosphotransferase	2.7.1.90	DF3_v1_10079
fba	Fructose-bisphosphate aldolase	4.1.2.13	DF3_v1_250031
			DF3_v1_10155
tpiA	Triose-phosphate isomerase	5.3.1.1	DF3_v1_50125
gapB	Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	DF3_v1_10272
pgk	Phosphoglycerate kinase	2.7.2.3	DF3_v1_60133
gpm	Phosphoglycerate mutase	5.4.2.12	DF3_v1_130008
eno	Phosphopyruvate hydratase	4.2.1.11	DF3_v1_50119
ttuE	Pyruvate kinase	2.7.1.40	DF3_v1_20038
pps	Pyruvate, phosphate dikinase	2.7.9.1	DF3_v1_160016

Entner-Duodoroff pathway

gdh	Glucose 1-dehydrogenase	1.1.1.47	DF3_v1_240046
			DF3_v1_80059

Pentose phosphate pathway (non-oxidative)

rpe	Ribulose-phosphate isomerase	5.1.3.1	DF3_v1_10156
rpiA	Ribose-5-phosphate isomerase	5.3.1.6	DF3_v1_130102
tkt	Transketolase	2.2.1.1	DF3_v1_10154
			DF3_v1_90046
prsA	Ribose-phosphate diphosphokinase	2.7.6.1	DF3_v1_140065

Glycogen metabolism

pgm	Phosphoglucomutase	5.4.2.2	DF3_v1_10173
glgC	Glucose-1-phosphate adenylyltransferase	2.7.7.27	DF3_v1_20023
glgA	Glycogen synthase	2.4.1.21	DF3_v1_50042
glgB	1,4-alpha-glucan branching enzyme	2.4.1.18	DF3_v1_10237
			DF3_v1_50037
			DF3_v1_90065
glgP	Glycogen phosphorylase	2.4.1.1	DF3_v1_50041
glgX	Glycogen debranching enzyme	3.2.1.-	DF3_v1_90066
			DF3_v1_180039
malZ	Alpha-glucosidase	3.2.1.20	DF3_v1_140091
malQ	4-alpha-glucanotransferase	2.4.1.25	DF3_v1_90067

Trehalose related

celA	UTP--glucose-1-phosphate uridylyltransferase	2.7.7.9	DF3_v1_10168
treX	Isoamylase	3.2.1.68	Glycogen metabolism
treY	Malto-oligosyltrehalose synthase	5.4.99.15	DF3_v1_180037
treZ	Malto-oligosyltrehalose trehalohydrolase	3.2.1.141	DF3_v1_180038
treS	Trehalose synthase	5.4.99.16	DF3_v1_180040 Fused genes
pep2	Maltokinase	2.7.1.175	DF3_v1_180040 Fused genes
glgE	Alpha-1,4-glucan:maltose-1-phosphate maltosyltransferase	2.4.99.16	DF3_v1_90069
glgB	1,4-alpha-glucan branching enzyme	2.4.1.18	Glycogen metabolism

PHA metabolism

phaA	Acetyl-CoA acetyltransferase	2.3.1.9	DF3_v1_30022
phaB	Acetoacetyl-CoA reductase	1.1.1.36	DF3_v1_60142
phaC	PHA synthase	2.3.1.-	DF3_v1_30019 DF3_v1_150036
phaE	PHA synthase type III	2.3.1.-	DF3_v1_30018
phaZ	Poly(3-hydroxyalkanoate) depolymerase	3.1.1.-	DF3_v1_10082 DF3_v1_120083
phaR	Polyhydroxyalkanoate synthesis repressor	-	DF3_v1_50085

Methylmalonyl-CoA

pathway

pccA	Propionyl-CoA carboxylase alpha subunit	6.4.1.3	DF3_v1_140016
pccB	Propionyl-CoA carboxylase beta subunit	6.4.1.3	DF3_v1_140017
epi	Methylmalonyl-CoA/ethylmalonyl-CoA epimerase	5.1.99.1	DF3_v1_260020
mcm	Methylmalonyl-CoA mutase	5.4.99.2	DF3_v1_50002

Succinate conversion to propionate

mcm	Methylmalonyl-CoA mutase	5.4.99.2	Methylmalony-Coa pathway
scpB	Methylmalonyl-CoA decarboxylase	4.1.1.41	DF3_v1_50030
mmdA	Methylmalonyl-CoA decarboxylase alpha subunit	4.1.1.41	(DF3_v1_50056)
mmdB	Methylmalonyl-CoA decarboxylase beta subunit	4.1.1.41	(DF3_v1_50053)
mmdC	Methylmalonyl-CoA decarboxylase gamma subunit	4.1.1.41	(DF3_v1_50054)

Ethylmalonyl-CoA pathway

phaA	Acetyl-CoA acetyltransferase	2.3.1.9	PHA metabolism
phaB	Acetoacetyl-CoA reductase	1.1.1.36	PHA metabolism
crt	3-hydroxybutyryl-CoA dehydratase	4.2.1.55	(DF3_v1_50084)
ccr	Crotonyl-CoA reductase	1.3.1.85	DF3_v1_60074
epi	Methylmalonyl-CoA/ethylmalonyl-CoA epimerase	5.1.99.1	Methylmalony-Coa pathway
ecm	(2R)-ethylmalonyl-CoA Mutase	5.4.99.63	DF3_v1_60073
mcd	(2S)-methylsuccinyl-CoA dehydrogenase	1.3.8.12	DF3_v1_60075
mch	2-methylfumaryl-CoA hydratase	4.2.1.148	DF3_v1_180054

mcl-1	B- methylmalyl-CoA/(3S)-malyl-CoA lyase	4.1.3.24, 4.1.3.25	DF3_v1_180055
mcl-2	(3S)-malyl-CoA thioesterase	3.1.2.30	DF3_v1_20061
pccA	Propionyl-CoA carboxylase alpha subunit	6.4.1.3	Methylmalony-Coa pathway
pccB	Propionyl-CoA carboxylase beta subunit	6.4.1.3	Methylmalony-Coa pathway
mcm	Methylmalonyl-CoA mutase	5.4.99.2	Methylmalony-Coa pathway

Short chain fatty acid uptake metabolism

actP	Acetate transporter		DF3_v1_130002
			DF3_v1_140009
			DF3_v1_150030
acs	acetyl-CoA synthetase	6.2.1.1	DF3_v1_50059
			DF3_v1_130012
ach	Acetyl-CoA hydrolase/transferase	3.1.2.1	DF3_v1_120080
pta	Phosphate acetyl/butyryltransferase	2.3.1.8	DF3_v1_60102
			DF3_v1_280020
ackA	Acetate kinase	2.7.2.1	DF3_v1_60101
			DF3_v1_280019

Nitrogen metabolism

assimilatory nitrate reduction

nasA	fragment of nitrate reductase, large subunit (part 2)	1.7.99.4	DF3_v1_100034
nasA	fragment of nitrate reductase, large subunit (part 1)	1.7.99.4	DF3_v1_100039
nirA	Ferredoxin--nitrite reductase	1.7.7.1	DF3_v1_100041
nrtD	Nitrate ABC transporter, ATP-binding protein		DF3_v1_100044
nrtB	Nitrate ABC transporter, permease protein		DF3_v1_100045
nrtC	Nitrate ABC transporter, ATP-binding protein		DF3_v1_100046
nrtA	Nitrate ABC transporter, susbtrate-binding protein		DF3_v1_100048
narK	Nitrate/nitrite transporter		DF3_v1_160076

nitrogen fixation

nifA	Nif-specific regulatory protein		DF3_v1_20133
nifB	Iron-molibdenum cofactor biosynthesis protein		DF3_v1_20134
fdxN	Ferredoxin, nitrogenase associated		DF3_v1_20135
nifT	Nitrogen fixation protein		DF3_v1_20136
draG	Dinitrogenase reductase activating glycohydrolase	3.2.2.24	DF3_v1_20137
			DF3_v1_140086
draT	NAD-dinitrogen-reductase ADP-D-ribosyltransferase	2.4.2.37	DF3_v1_20138
nifH	Nitrogenase iron protein	1.18.6.1	DF3_v1_20139
nifD	Nitrogenase molybdenum-iron protein alpha chain	1.18.6.1	DF3_v1_20140
nifK	Nitrogenase molybdenum-iron protein beta chain	1.18.6.1	DF3_v1_20141
nifE	Nitrogenase iron-molybdenum cofactor biosynthesis protein	1.18.6.1	DF3_v1_20142
nifN	Nitrogenase iron-molybdenum cofactor biosynthesis protein	1.18.6.1	DF3_v1_20143
nifX	Dinitrogenase iron-molybdenum cofactor biosynthesis (modular protein)	-	DF3_v1_20144
			DF3_v1_290013
nifW	Nitrogenase-stabilizing/protective protein	-	DF3_v1_20149

glnK	Nitrogen assimilation regulatory protein	-	DF3_v1_30186
fixI	Nitrogen fixation protein	-	DF3_v1_40018
fixG	Nitrogen fixation protein	-	DF3_v1_40020
ntrB	Nitrogen regulation protein	2.7.13.3	DF3_v1_10005
ntrY	Nitrogen regulation protein	2.7.13.3	DF3_v1_10007
ntrX	Nitrogen assimilation regulatory protein		DF3_v1_10008

urea uptake

ureG	Urease accessory protein		DF3_v1_230018
ureF	Urease accessory protein		DF3_v1_230019
ureE	Urease accessory protein		DF3_v1_230020
ureC	Urease alpha subunit	3.5.1.5	DF3_v1_230021
ureB	Urease beta subunit	3.5.1.5	DF3_v1_230022
ureA	Urease gamma subunit	3.5.1.5	DF3_v1_230023
ureD	Urease accessory protein		DF3_v1_230024
urtE	Urea ABC transporter ATP-binding protein		DF3_v1_230025
urtD	Urea ABC transporter ATP-binding protein		DF3_v1_230026
urtC	Urea ABC transporter permease subunit		DF3_v1_230027
urtB	Urea ABC transporter permease subunit		DF3_v1_230028
urtA	Urea ABC transporter permease subunit		DF3_v1_230029

other

amtB	Ammonia channel		DF3_v1_80071
			DF3_v1_110022
glnT	Glutamine synthetase	6.3.1.2	DF3_v1_80073

			DF3_v1_230035
gdhA	Glutamate dehydrogenase (NADP(+))	1.4.1.4	DF3_v1_90025
gltD	Glutamate synthase, beta subunit	1.4.1.13	DF3_v1_30087
gltB	Glutamate synthase, alpha subunit	1.4.1.13	DF3_v1_30088
			DF3_v1_80079
ptsN	PTS system, nitrogen regulatory IIA component	2.7.1.-	DF3_v1_60007

Phosphorus metabolism

ppk1	Polyphosphate kinase 1	2.7.4.1	DF3_v1_10201
			DF3_v1_260022
ppx	Exopolyphosphatase ppx	3.6.1.11	DF3_v1_10203
adk	Adenylate kinase	2.7.4.3	DF3_v1_270027
pap	Polyphosphate AMP phosphotransferase		DF3_v1_100106
hppa	K(+)-insensitive pyrophosphate-energized proton pump	3.6.1.1	DF3_v1_130093
pstC	Phosphate ABC transporter, trans-membrane component		DF3_v1_280009
			DF3_v1_340018
pstA	Phosphate ABC transporter, trans-membrane component		DF3_v1_280010
			DF3_v1_340017
pstB	Phosphate ABC transporter, ATP-binding component		DF3_v1_280011
			DF3_v1_340016
pstS	Phosphate ABC transporter, periplasmic-binding component		DF3_v1_340019
phoB	Two-component system, sensor kinase		DF3_v1_340014
phoU	Phosphate concentration transducer of pho regulon		DF3_v1_340015
sixA	Phosphohistidine phosphatase		DF3_v1_20180
atpC	ATP synthase F1, epsilon subunit	3.6.3.14	DF3_v1_20181

atpD	ATP synthase F1, beta subunit	3.6.3.14	DF3_v1_20182
atpG	ATP synthase F1, gamma subunit	3.6.3.14	DF3_v1_20183
			DF3_v1_30118
atpA	ATP synthase F1, alpha subunit	3.6.3.14	DF3_v1_20184
atpH	ATP synthase F1 delta subunit	3.6.3.14	DF3_v1_20185
atpI	ATP synthase F0, subunit I	3.6.3.15	DF3_v1_30115
atpB	ATP synthase F0, subunit A	3.6.3.14	DF3_v1_30116
atpE	ATP synthase F0, subunit C	3.6.3.14	DF3_v1_30117
atpF	ATP synthase F0, subunit B	3.6.3.14	DF3_v1_30119

Hydrogenase

hybO	Hydrogenase 2, small subunit		DF3_v1_110083
hybA	Hydrogenase 2 4Fe-4S ferredoxin-type component		DF3_v1_110084
hybB	Hydrogenase 2 cytochrome b type component		DF3_v1_110085
hybC	Hydrogenase 2, large subunit		DF3_v1_110086
hybD	Maturation element for hydrogenase 2		DF3_v1_110087
hypE	Carbamoyl phosphate phosphatase, hydrogenase 3 maturation protein		DF3_v1_50031
hypC	Hydrogenase 2 accessory protein		DF3_v1_50033
hypF	Hydrogenase maturation factor		DF3_v1_50034
hypB	Hydrogenase nickel incorporation protein		DF3_v1_50035

Amino acids transport systems

aapP	Amino-acid transporter, ATP-binding subunit		DF3_v1_180007
aapM	Amino-acid transporter, membrane component		DF3_v1_180008
aapQ	Amino-acid transporter, membrane component		DF3_v1_180009
aapJ	Amino-acid transporter, periplasmic-binding component		DF3_v1_180010
livK	Branched-chain amino acid transport system substrate-binding protein		DF3_v1_130104
			DF3_v1_40152
livF	Branched-chain amino acid transport system ATP-binding protein		DF3_v1_130106
			DF3_v1_20152
			DF3_v1_20113
			DF3_v1_40155
			DF3_v1_230025
			DF3_v1_240034
livG	Branched-chain amino acid transport system ATP-binding protein		DF3_v1_130107
			DF3_v1_40154
			DF3_v1_20153
			DF3_v1_240039
livM	Branched-chain amino acid transport system permease protein		DF3_v1_130108
			DF3_v1_40153
livH	Branched-chain amino acid transport system permease protein		DF3_v1_130109
gltS	Sodium/glutamate symporter		DF3_v1_230012

Mannose uptake/activation

manX	PTS system, mannose-specific IIA component	2.7.1.191	DF3_v1_30059
pstH	Phosphocarrier protein HPr	2.7.11.-	DF3_v1_30060

ptsl	Phosnoenolpyruvate-protein phosphotransferase	2.7.3.9	DF3_v1_30061
mak	D-fructose(D-mannose)kinase	2.7.1.4	DF3_v1_330010
exoC	Phosphomannomutase	5.4.2.8	DF3_v1_10170
cpsB	Mannose-1-phosphate guanylyltransferase (GDP)	2.7.7.22	DF3_v1_30007
	Mannose-1-phosphate guanylyltransferase	2.7.7.13	DF3_v1_30065
gmd	GDP-mannose 4,6-dehydratase	4.2.1.47	DF3_v1_200044
fcl	GDP-L-fucose synthase	1.1.1.271	DF3_v1_200043
algD	GDP-mannose 6-dehydrogenase	1.1.1.132	DF3_v1_160084

Other

Methanol metabolism

mdh	Methanol dehydrogenase	1.1.2.7, 1.1.	DF3_v1_140053
frmA	Alcohol dehydrogenase class III/glutathione-dependent formaldehyde dehydrogenase	1.1.1.1, 1.1.1.284	DF3_v1_20119
frmC	S-formylglutathione hydrolase	3.1.2.12	DF3_v1_20120
fdol	Cytochrome b subunit of formate dehydrogenase	1.2.1.2	DF3_v1_390004
fdhB	Formate dehydrogenase iron-sulfur subunit	1.2.1.2	DF3_v1_390006
fdhA	Formate dehydrogenase	1.2.1.2	DF3_v1_390007

( ) = There is evidence for this assignment but there is a higher evidence for a different gene assignment.
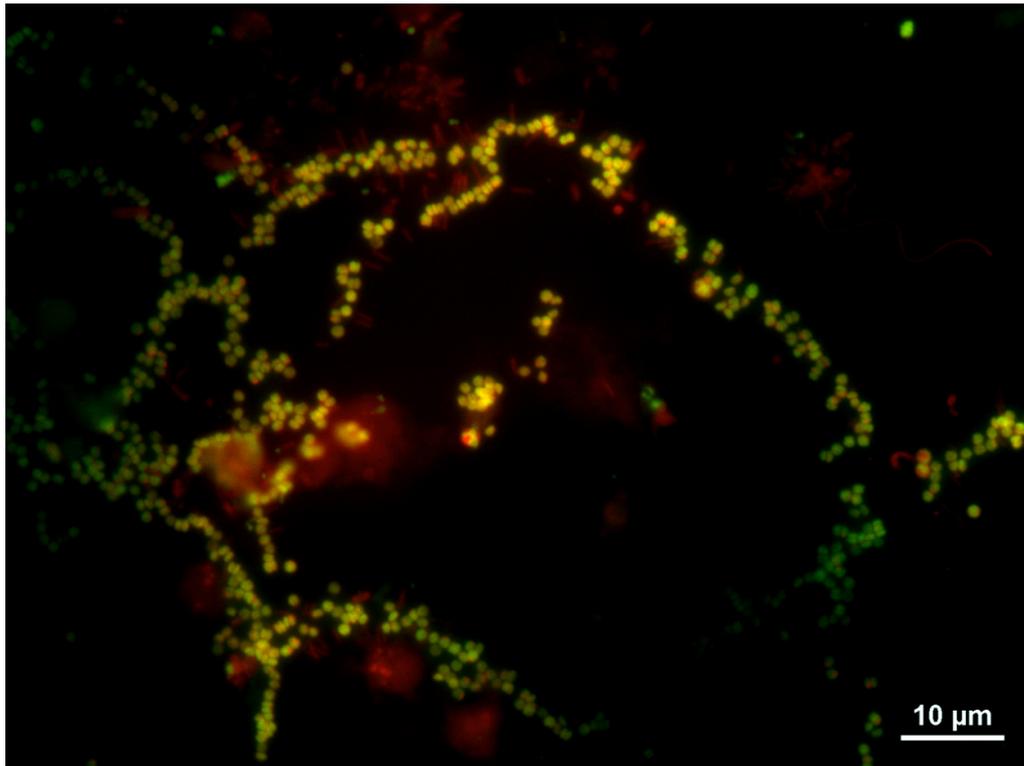
Figure 1. Composite FISH micrograph from a SBR sample after Nile Blue A staining protocol. FISH probes applied include DF198 (Fluos-label, green) and EUBmix (Cy3-label, red). *Defluviicoccus* cluster III cells appear yellow/orange. Cells lost their filamentous arrangement after destaining procedures.
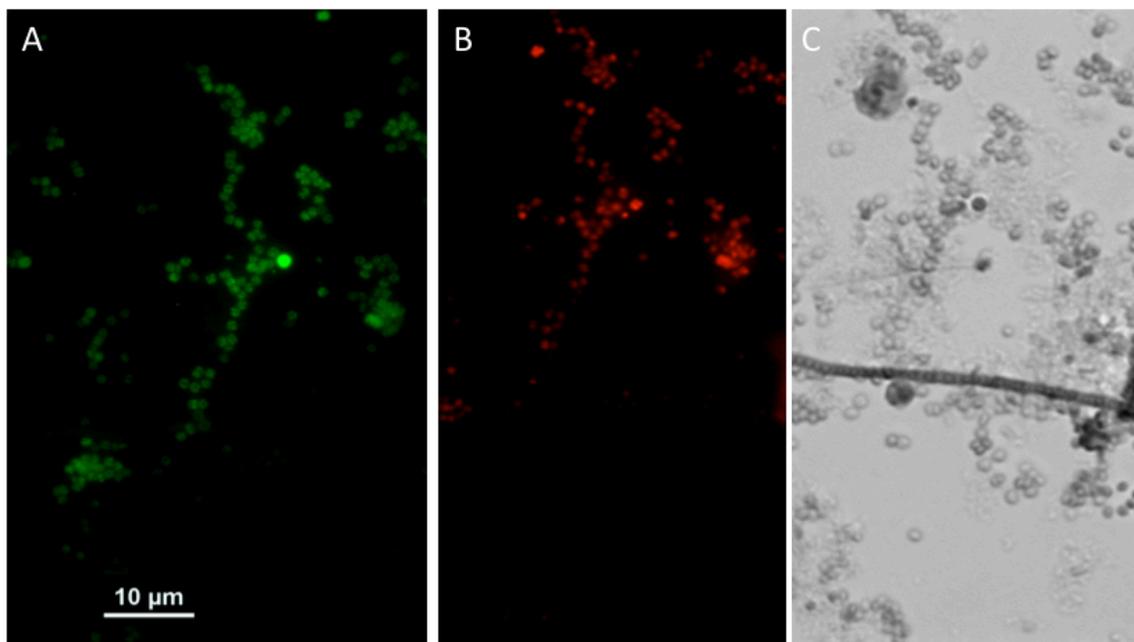
Figure 2. Nile blue A staining micrographs. (A) Positive FISH *Defluviicoccus* cluster III cells using probe DF198 (Fluos-label, green). (B) Corresponding Nile blue A staining and (C) phase – contrast images.

Chapter 5

Genomic and *in situ* analyses reveal the *Micropruina* spp. as abundant fermentative glycogen accumulating organism in enhanced biological phosphorus removal systems

Contextual statement

The microbial survey of WWW presented in Chapter 2 revealed the presence of bacteria belonging to the genus *Micropruina*. *Micropruina* spp. are putative GAOs and the most dominant GAO in Danish EBPR treatment plants. The aim of this study was to understand their physiology using state-of-the-art techniques for both *in situ* and pure culture studies, gaining insights into the role of this genus in activated sludge.

Statement of Authorship

Title of Paper	Genomic and *in situ* analyses reveal the *Micropruina* spp. as abundant fermentative glycogen accumulating organism in enhanced biological phosphorus removal systems.

Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Co-Author

Name of Co-Author (PhD Candidate)	Cristobal Onetto
Contribution to the Paper	Planned experiments, performed pure culture incubations, adapted genomic DNA extraction method for Nanopore sequencing, prepared DNA for Illumina sequencing, performed single cell RAMAN analyses, performed autoradiographic procedures including FISH and image acquisition for *in situ* analyses (MAR). Revised the manuscript.
Overall percentage (%)	30
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am a co-author of this paper.
Signature	Date 22-02-2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i.   the candidate's stated contribution to the publication is accurate (as detailed above);
ii.   permission is granted for the candidate in include the publication in the thesis; and
iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Principal Author	Simon McIlroy
Contribution to the Paper	Planned experiments, designed FISH probes, annotated the genome and drafted the manuscript.
Signature	Date 19-02-2018.

Name of Co-Author	Bianca McIlroy
Contribution to the Paper	Performed pure culture incubations, designed FISH probes and revised the manuscript.
Signature	Date 21/2/2018

Name of Co-Author	Florian-Alexander Herbst
Contribution to the Paper	Designed experiments, performed NMR analyses, pure culture incubations and revised the manuscript.
Signature		Date	14.02.2018

Name of Co-Author	Morten Dueholm
Contribution to the Paper	Sequenced genomic DNA, assembled genome and revised manuscript.
Signature		Date	13/2 - 18

Name of Co-Author	Rasmus Kirkegaard
Contribution to the Paper	Sequenced genomic DNA, assembled genome and revised manuscript.
Signature		Date	13/2 - 2018

Name of Co-Author	Eustace Fernando
Contribution to the Paper	Performed RAMAN single cell analyses and revised the manuscript.
Signature		Date	13 02 2015

Name of Co-Author	Soren Karst
Contribution to the Paper	Sequenced genomic DNA, assembled genome and revised manuscript.
Signature		Date	13 2 - 2018

Name of Co-Author	Marta Nierychlo
Contribution to the Paper	Performed radiotracer incubations, cell fixation (MAR) and revised the manuscript.
Signature		Date	13/2 - 20

Name of Co-Author	Jannie Kristensen
Contribution to the Paper	Prepared genomic DNA for Illumina sequencing and revised the manuscript.
Signature	Date 13-02-18

Name of Co-Author	Kathryn Eales
Contribution to the Paper	Revised the manuscript.
Signature	Date 21/02/18

Name of Co-Author	Paul Grbin
Contribution to the Paper	Revised the manuscript.
Signature	Date 22/2/2018

Name of Co-Author	Reinhard Wimmer
Contribution to the Paper	Helped in NMR analyses and revised the manuscript
Signature	Date 17.2.2018

Name of Co-Author	Per H. Nielsen
Contribution to the Paper	Planned experiments and revised the manuscript.
Signature	Date 13.2.2018

Submitted to Frontiers in Microbiology

Genomic and *in situ* analyses reveal the *Micropruina* spp. as abundant fermentative glycogen accumulating organisms in enhanced biological phosphorus removal systems

Simon J. McIlroy[1,§], Cristobal A. Onetto[2], Bianca McIlroy[1], Florian-Alexander Herbst[1], Morten Simonsen Dueholm[1], Rasmus H. Kirkegaard[1], Eustace Fernando[1], Søren M. Karst[1], Marta Nierychlo[1], Jannie M. Kristensen, Kathryn L. Eales[2], Paul R. Grbin[2], Reinhard Wimmer[1] and Per Halkjær Nielsen[1*]

[1] Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, Aalborg, Denmark

[2] School of Agriculture, Food, and Wine, The University of Adelaide, Adelaide, SA, Australia

*Correspondence:
Per H. Nielsen
Email: phn@bio.aau.dk.

Running Title: Characterisation of the *Micropruina* GAO

ABSTRACT

Enhanced biological phosphorus removal (EBPR) configurations, for the treatment of wastewater, involve the cycling of the biomass through carbon-rich (feast) and carbon-deficient (famine) conditions promoting the activity of polyphosphate accumulating organisms (PAO). However, several alternate metabolic strategies, without polyphosphate storage, are possessed by other organisms, which can compete with the PAO for carbon at the potential expense of EBPR efficiency. The most studied are the glycogen accumulating organisms (GAO), which utilise aerobically stored glycogen to energise anaerobic substrate uptake and storage. In a recent 16S rRNA amplicon survey of full-scale EBPR plants in Denmark, *Micropruina* spp. were identified as the most abundant of the previously proposed GAO, yet little is known about their ecophysiology. In the current study, genomic and metabolomic studies were made of *Micropruina glycogenica* str. Lg2^T and compared to the *in situ* physiology of the genus in EBPR plants using state-of-the-art single cell techniques. These studies included the design of probes for fluorescence *in situ* hybridisation for the genus and the assembly of a closed genome for the type strain. The *Micropruina* spp. were observed to take up carbon, including sugars and amino acids, under anaerobic conditions, which were partly fermented to lactic acid, acetate, propionate and ethanol, and partly stored as glycogen for potential aerobic use. Anaerobic uptake of carbon and glycogen storage, in the absence of detectable polyhydroxyalkanoates or polyphosphate reserves, was confirmed for the abundant *in situ* members of the genus. This physiology is markedly different from the classical GAO model. Fermenters are generally viewed as essential for good EBPR, where they provide fermentation products for the classical PAO. However, the amount of carbon they store has potentially important implications for phosphorus removal - as they compete for substrates with the *Tetrasphaera* PAO and stored carbon is not made available to the "*Candidatus* Accumulibacter" PAO under anaerobic conditions. This study shows that the current models of the competition between PAO and GAO are too simplistic and that we need to revise the view of the GAO phenotype, the likely diversity of organisms potentially competing with the PAO, and the role of fermentation in EBPR.

Keywords: Activated sludge; EPBR; Fermentation; *Micropruina*; GAO; PAO

INTRODUCTION

Enhanced biological phosphorus removal (EBPR) activated sludge systems have been widely implemented for the removal of nutrients from wastewaters. Phosphorus (P) removal is achieved in these systems by cycling of the biomass through carbon-rich (feast) anaerobic and carbon-deficient (famine) conditions to encourage the activity of the so-called polyphosphate accumulating organisms (PAO) (see Oehmen et al., 2007 for review).

Classical models for the PAO phenotype stipulate that aerobically stored polyphosphate provides energy for anaerobic uptake and storage of volatile fatty acids (VFAs) as polyhydroxyalkanoates (PHAs). Hydrolysis of aerobically stored glycogen, and activity of the tricarboxylic acid (TCA) cycle provides required reducing power and additional energy. Stored PHAs are utilised under subsequent aerobic conditions supporting growth and replenishing glycogen and polyphosphate stores, with wastage of aerobic biomass giving net P removal (Comeau et al., 1986; Wentzel et al., 1986: Mino et al., 1987). Such a phenotype has been demonstrated in the widely studied "*Ca.* Accumulibacter" genus within the Betaproteobacteria (Hesselmann et al., 1999; He and McMahon, 2011).

Although EBPR systems are considered as an economical strategy for wastewater treatment, they are subject to periods of inefficiency and failure. Deterioration of EBPR has been attributed to a variety of conditions; such as high rainfall, nutrient limitation and high nitrate loading to the anaerobic zone (Oehmen et al., 2007). Another proposed reason is microbial competition, where the proliferation of organisms competing for anaerobic carbon supply, without excess polyphosphate storage, is at the theoretical expense of P removal efficiency (Satoh et al., 1994). Bacteria with the glycogen accumulating organism (GAO) phenotype have received considerable attention as potential competitors of the PAO (Oehmen et al., 2007). The GAO phenotype is similar to the classical PAO phenotype, except that polyphosphate is not stored in excess under aerobic conditions. As polyphosphate is not stored for anaerobic use, there is an increased reliance on aerobically stored glycogen as an energy source for anaerobic carbon uptake (Liu et al., 1994; Mino et al., 1995). The phenotype has been demonstrated for members of the alphaproteobacterial *Defluviicoccus* (Wong et al., 2004), and the gammaproteobacterial Competibacteraceae family (Crocetti et al., 2002; Kong et al., 2002; McIlroy et al., 2015a) and at least partially for several other taxa (see Stokholm-Bjerregaard et al., 2017).

Research efforts have overwhelmingly focused on the competing classical PAO-GAO phenotypes in EBPR with acetate as carbon source. It is generally believed that VFAs are made available to the PAO and GAO through the hydrolysis and fermentation of more complex substrates, such as carbohydrates and proteinaceous material, by other heterotrophic organisms (Henze et al., 2000; Kong et al., 2008). However, more

diverse strategies for organisms with a direct influence on EBPR have long been suggested (Carucci et al., 1999). Several anaerobic carbon storage compounds are reported for full-scale activated sludge, including triacylglycerols (TAGs), gamma-aminobutyric acid (GABA) as well as intracellular pools of non-polymerised fermentation by-products, long chain fatty acids, amino acids and trehalose (Satoh et al., 1998; Santos et al., 1999; Kristiansen et al., 2013; McIlroy et al., 2013; Nguyen et al., 2015; Marques et al., 2017). Some organisms exhibiting the classical GAO and PAO phenotypes reportedly utilise sugars and amino acids directly for PHA production (Liu et al., 1996; Burow et al., 2007; Oyserman et al., 2015) and some may also ferment glycogen stores or glucose to lactate as an additional anaerobic energy source (McIlroy et al., 2014). Most notably, the *Tetrasphaera* spp. and *Microlunatus phosphovorus*[T], both within the phylum Actinobacteria, cycle polyphosphate without PHA storage with dynamic feast-famine conditions. Instead, these organisms exhibit a fermentative metabolism where polyphosphate supplements anaerobic energy demands (Nakamura et al., 1995; Kong et al., 2005; Kristiansen et al., 2013). Importantly, surveys of full-scale systems revealed that the *Tetrasphaera* spp. are in much higher abundance than the "*Ca.* Accumulibacter" PAO, questioning the long-held belief that the latter classical-PAO are the most important for EBPR (Nguyen et al., 2011; Mielczarek et al., 2013a; Saunders et al., 2016) and reinforcing the need to consider diverse physiologies for organisms important for EBPR.

In addition to the fermentative actinobacterial PAO, it has been shown that in dynamic feast-famine systems unidentified organisms can store glucose directly as glycogen anaerobically, energised by fermentation, without cycling polyphosphate (Carucci et al., 1999). The role of these 'fermentative GAO' in EBPR is of interest, given their potential as competitors of the abundant fermentative *Tetrasphaera* PAO. A likely candidate for this phenotype is the activated sludge isolate *Micropruina glycogenica*[T] – a member of the family Propionibacteriaceae within the Actinobacteria - shown to accumulate large amounts of unidentified intracellular carbohydrate (up to 8.4% dry cell weight), in the absence of detected polyphosphate storage, under both aerobic and anaerobic conditions (Shintani et al., 2000). Members of the *Micropruina* genus were reportedly abundant in a non-EBPR lab-scale system fed with acetate and glucose (Kong et al., 2001). *In situ* analyses of the genus revealed that both acetate and glucose were taken up anaerobically with some PHA storage. A relatively high biomass polymerised-glucose content was attributed to storage by the *Micropruina* spp. present. The organism has received little attention in EBPR research since. However, in our recent comprehensive survey of full-scale EBPR plants in Denmark, the genus was observed to be the most abundant of the putative GAO proposed thus far (Stokholm-Bjerregaard et al., 2017). In the current study, several approaches were applied to the characterisation of members of the *Micropruina* genus, in both pure culture and *in situ*, to give a comprehensive view of their ecology in EBPR systems and their subsequent importance therein. The results show that the classical view of PAO-GAO interactions in full-scale EBPR systems needs to be revised.

MATERIALS AND METHODS

Pure culture studies

Micropruina glycogenica str. Lg2^T (DSM15918) was obtained from DSMZ and maintained on R2A agar (Reasoner and Geldreich, 1985) at 30°C. Anaerobic cultivation was performed in quadruplicate by autoclaving 40 ml R2A broth, without soluble starch, in serum flasks closed with rubber plugs perforated by syringe needles. Afterwards, 0.2 μm filters were attached to the needles and nitrogen was bubbled through the hot media until it reached room temperature. A *Micropruina* colony was suspended in 200 μl R2A broth and distributed as inoculum to the nitrogen-saturated serum flasks. These were incubated for seven days at 30°C.

Nuclear magnetic resonance (NMR) spectroscopy

Following incubation, cells were removed by centrifugation (10,000 x g, 10 min, 4°C). For NMR analysis two approaches were chosen: with and without a concentration step. Samples without concentration were prepared by adding 60 μl of buffer (for 50 ml: 10 ml D_2O, 0.04 g sodium 2,2,3,3-tetradeutero 3-trimethylsilyl propionate (TSP-d_4), 1.005 g Na_2HPO_4 x 7 H_2O, 0.5 ml NaN_3 (4%), adjusted to 50 ml and pH 7.0 with H_2O and NaOH/HCl) directly to 540 μl of the cultures supernatant or the R2A control. For samples with a concentration step, 15 ml of the cultures supernatant or R2A were freeze-dried and suspended in 600 μl NMR sample solution (D_2O containing 2 mM sodium azide and ~ 0.83 mM trimethylsilylpropanoic acid (TSP)). All samples were adjusted to a pH of 7.0 before NMR analysis.

All NMR spectra were recorded at 298.1 K on a BRUKER AVIII-600 MHz NMR spectrometer equipped with a 5 mm cryogenic inverse triple-resonance probe. A blank containing only water and buffer or the NMR sample solution was analyzed by a PULCON experiment and the exact TSP concentration was determined (Wider and Dreier, 2006). For each sample, a 1D-NOESY experiment with a relaxation delay of 1 s, a mixing time of 10 ms, and an acquisition time of 4 s was recorded. During the relaxation delay, the water resonance was presaturated with a continuous wave irradiation at $\gamma B_1/2\pi$ = 48 Hz. A pulsed field gradient of 1.2 ms length and 30 G/cm strength was applied between the presaturation period and the first 90° pulse. Another pulsed field gradient of 1.2 ms length and 7.2 G/cm was applied during the mixing period. Metabolite identification and quantification were performed using the ChenomX NMR Suite v. 8.1 using the known concentration of TSP as an internal standard.

Genome sequencing

Genomic DNA was extracted from *M. glycogenica* Lg2^T using a DNeasy Blood & Tissue kit (Qiagen, Copenhagen, Denmark) according to manufacturer's instruction with changes in the pre-treatment protocol for Gram-positive Bacteria (2 h of lysis buffer treatment and 1 h of proteinase K treatment). A library for Illumina paired-end sequencing was prepared from 50 ng of total DNA using the Nextera DNA library

preparation kit (Illumina, CA, USA) according to manufacturer's instructions (Part # 15027987 v01), but with an AMPure XP bead to sample ratio of 0.5:1 instead of 1.5:1 during the library clean-up step. The library was sequenced on the Illumina MiSeq platform (v3 chemistry, 2×300 bp). Paired-end reads were quality filtered (minimum length of 240 bp, allowing no ambiguous nucleotides) and trimmed for sequencing adaptors using the CLC genomics workbench (v. 9.5.2). In addition, long-read nanopore sequencing was performed. Genomic DNA was sequenced using the MinION (Oxford Nanopore Technologies, UK) following the manufacturer's protocol (1D gDNA sequencing SQK-LSK108). The library was sequenced on a FLO-MIN106 flowcell on a MinION MK1b sequencer using Minknow software (v. 1.4.2). The reads were basecalled using Metrichor (v. 2.45.3). Fastq read files were extracted using Poretools (v.0.6.0, (Loman and Quinlan, 2014)). The nanopore sequencing reads were assembled into a closed, single contig, draft genome using CANU (v. 1.4, (Koren et al., 2016)). The draft genome was subsequently polished with the Illumina paired-end reads using Pilon (v. 1.18, (Walker et al., 2014)) and by manual read mapping in CLC genomics workbench to remove SNPs and ensure a high-quality assembly.

FISH probe design and optimisation

Phylogenetic analyses and probe design were performed with the MiDAS database v. 2.1 (McIlroy et al., 2015b) (derived from the SILVA taxonomy (Quast et al., 2013)) within the ARB software package (Ludwig et al., 2004). Potential probes were assessed *in silico* with the mathFISH software for hybridisation efficiencies of target, and potentially weak, non-target matches (Yilmaz et al., 2011). Unlabeled competitor probes were designed for single base mismatched non-target sequences (Manz et al., 1992). The Ribosomal Database Project (RDP) PROBE MATCH function (Cole et al., 2014) was used to screen for the existence of non-target indel sequences (McIlroy et al., 2011). Probe validation and optimisation were based on generated formamide dissociation curves (Daims et al., 2005) where fluorescent intensities of at least 50 cells were measured with ImageJ software (National Institutes of Health, Maryland, USA). Calculated fluorescence intensity average values were compared for hybridisation buffers with formamide concentrations over a range of 0-70% (v/v) with 5% increments (data not shown). *Micropruina glycogenica* Lg2[T] was used to optimize the MGL-67 and MGL-1223 probes designed to target the genus. Other isolates were used to assess the potential for non-specific binding of the designed probes (see **Table 1**).

Fluorescence *in situ* hybridization (FISH)

FISH was performed essentially as detailed by Daims *et al.,* (2005). Activated sludge biomass samples from full-scale EBPR wastewater treatment plants (WWTPs) were taken from the aerobic tank and transported on ice to the laboratory as part of the broader MiDAS project (Mielczarek et al., 2013b). Activated sludge biomass and axenic cultures were fixed for FISH with 50 % ethanol (v/v) and stored at -20°C. The 5′ end of oligonucleotide FISH probes were labelled with 5(6)-carboxyfluorescein-N-

hydroxysuccinimide ester (FLUOS) or with the sulfoindocyanine dyes (Cy3 and Cy5) (Thermo Fisher Scientific GmbH, Ulm, Germany). The NON-EUB nonsense probe was used as a negative hybridisation control (Wallner et al., 1993). Quantitative FISH (qFISH) values were calculated as a percentage area of the total biovolume, hybridising the EUBmix probes (Amann et al., 1990; Daims et al., 1999), that also hybridised with the specific probe. qFISH analyses were based on 30 fields of view taken at 630 x magnification using the daime image analysis software (DOME, Vienna, Austria) (Daims et al., 2006). Increased permeabilisation of cells for FISH was achieved with the enzymatic pre-treatment method described by Kragelund and colleagues (2007). The protocol includes lysozyme treatment (Sigma-Aldrich, Brøndby, Denmark) (10 mg mL^{-1} in 0.05M EDTA, 0.1M Tris-HCl, pH 8) for 30 min at 37°C, achromopeptidase treatment (Sigma-Aldrich) (60U mL^{-1} in 0.01M NaCl, 0.01M Tris-HCl, pH 8) for 30 min at 37°C and acid treatment with 0.1M HCl for 10 mins with a final dehydration step with 96% [v/v] ethanol for 1 min. Microscopy was performed with either an Axioskop epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) or a white light laser confocal microscope (Leica TCS SP8 X).

Microautoradiography-FISH

Samples were taken from the aerobic tank of the WWTPs in Odense North-West and Ejby Mølle, Denmark. Both plants are configured for EBPR with stable performance (see Mielczarek *et al.*, (2013a) for further operational details). Samples were stored at 4°C and microautoradiography (MAR) analyses performed within 24 h of sampling. The MAR protocol was essentially as detailed by Nierychlo et al., (2015). Activated sludge was aerated for 40 min at room temperature prior to MAR incubation, to reduce any residual substrates, oxygen and NO$_x$ present. Sludge was then diluted with filtered sludge water from the same plant to yield a biomass concentration of 1 mgSS mL^{-1} for a final volume of 2 ml in 11 ml serum bottles. Radiolabelled substrates were added to yield a total radioactivity of 10 μCi mg^{-1} SS. The following were used: [^{14}C]-pyruvic acid (Perkin-Elmer) Waltham MA, USA, [^{3}H]-acetic acid, [^{3}H]-galactose, [^{3}H]-oleic acid (Amersham Biosciences, UK), [^{3}H]-glucose (Perkin-Elmer, Waltham MA, USA), [^{14}C]-propionic acid, [^{3}H]-NAG, [^{3}H]-fructose, [^{14}C]-butyric acid, [^{3}H]-glycerol, [^{3}H]-ethanol and [^{3}H]-amino acid mix (American Radiolabeled Chemicals Inc., Saint Louis MO, USA). The corresponding cold substrate was added to yield a total concentration of 2 mM. Oxygen was removed by repeated evacuation of the headspace and subsequent injection of O$_2$-free N$_2$ to achieve anaerobic conditions, prior to substrate addition. Samples were incubated with each labelled substrate for 3 h at room temperature (approx. 21°C) on a rotary shaker at 250 rpm. A pasteurized biomass (heated to 70°C for 10 min) was prepared as a negative control to assess possible silver grain formation due to chemography. Incubations were terminated by the addition of cold PFA to a final concentration of 4% [w/v]. Samples were fixed for 3 h at 4°C and subsequently washed 3 times with sterile filtered tap water. Aliquots of 30 μl of the biomass were gently homogenized between glass coverslips. Following FISH (see earlier), coverslips were coated with Ilford K5D emulsion (Polysciences,

Inc., Warrington, PA, USA), exposed in the dark for periods of 10 days and developed with Kodak D-19 developer.

Raman microspectroscopy

Raman spectra from single-cells of *Micropruina glycogenica* Lg2^T were obtained using a Horiba LabRam HR 800 Evolution (Jobin Yvon – France) equipped with a Torus MPC 3000 (UK) 532 nm 341 mW solid-state semiconductor laser. The incident laser power density on the sample was attenuated down to 2.1 mW/μm^2 using a set of neutral density (ND) filters. The Raman system is equipped with an in-built Olympus (model BX-41) fluorescence microscope. A 50X, 0.75 numerical aperture dry objective (Olympus M Plan Achromat- Japan), with a working distance of 0.38 mm was used throughout the work. A diffraction grating of 600 mm/groove was used, and the Raman spectra collected spanned the wavenumber region of 200 cm^{-1} to 1800 cm^{-1}. The slit width of the Raman spectrometer and the confocal pinhole diameter were set to 100 μm and 150 μm, respectively. Raman spectrometer operation and subsequent processing of spectra were conducted using LabSpec version 6.4 software (Horiba Scientific, France). *Micropruina* cells from the pure cultures were directly mounted on optically polished CaF$_2$ Raman windows (Crystran, UK) and air-dried before analysis. Spectra presented are average measurements from 30 individual *Micropruina* cells. Prior to all measurements, the Raman spectrometer was calibrated based on the first-order Raman signal of silicon, occurring at 520.7 cm^{-1}. The CaF$_2$ Raman substrate also contains a single-sharp Raman marker at 321 cm^{-1}, which serves as an internal reference point in every spectrum. Glycogen (sourced from oyster – CAS # 9005-79-2) (Sigma Aldrich, UK) was used as reference compound. Glycogen produces characteristically strong Raman markers between wavenumbers 478 cm^{-1} - 484 cm^{-1} and 840 cm^{-1} – 860 cm^{-1}, attributed respectively to skeletal deformation and CC skeletal stretch (Lin-Vien et al., 1991). PHA produces characteristic Raman bands at 432 cm^{-1}, 840 cm^{-1} and 1726 cm^{-1}, attributed respectively to δ (CC) skeletal deformations and ν(C=O) stretching vibrations (Lin-Vien et al., 1991) (**Figure S1**). Polyphosphate produces marker Raman bands at 1170 cm^{-1} and 690 cm^{-1}, attributed respectively to –P-O-P- stretching vibrations and PO$_2^-$ stretching vibrations (Majed et al., 2009) (**Figure S1**).

FISH-Raman analysis of *Micropruina*

In order to measure storage compounds (glycogen, PHA and polyphosphate) *in situ* for the *Micropruina* spp., Raman microspectroscopy was combined with FISH. Activated sludge samples from the Ejby Mølle WWTP were incubated anaerobically with either glucose or butyrate (see MAR incubation details) and PFA fixed. FISH was performed on calcium fluoride slides using the MGL-67 and EUBmix probes. FISH-positive cells were located under fluorescence and, before Raman measurements, fluorescent labels were bleached by constantly illuminating the Raman laser on the area of interest for 5 min. Raman spectra were obtained from 30 single cells tagged with the *Micropruina* probe, as described above, and the averaged spectrum was calculated.

RESULTS

Pure culture studies

The ability for *M. glycogenica* to grow under anaerobic conditions was not explicitly stated in the original description of the organism (Shintani et al., 2000) but was confirmed in the current study; with visible growth in liquid culture after 7 days in R2A media (with no starch). Metabolite analyses with NMR suggested that, under anaerobic conditions, *M. glycogenica* primarily utilised available glucose and alanine as the primary carbon sources. Smaller amounts of trehalose (all present in the media was utilized), and the amino acids aspartate, glycine, histidine, leucine and threonine were also detectably taken up from the media. Large amounts of acetate and lactate accumulated in the media, along with smaller fractions of ethanol and propionate, indicating active fermentation (see **Figure S2**). All of these by-products are reportedly utilised by at least one known PAO and GAO *in situ* (Kong et al., 2004; 2006; Burow et al., 2007; Wong and Liu, 2007; Nguyen et al., 2012). Raman microspectroscopy identified the storage polymer glycogen, but not PHA or polyphosphate, in anaerobically grown *M. glycogenica* cells (**Figure S3**).

Genome annotation

Genome sequencing and assembly using Illumina short reads and Nanopore long reads for scaffolding gave a closed 3.8 Mbp circular chromosome for *M. glycogenica* Lg2^T (see **Table 2** for details). The genome contained genes for a complete TCA cycle, a cytochrome *c* oxidase and other electron transport chain complexes, supporting an ability for aerobic respiration. A respiratory nitrate reductase (narG: MPLG2_2671) along with a putative copper-containing nitrite reductase (nirK: MPLG2_2662) were also annotated, despite the organism reportedly being able to reduce nitrate to nitrite but no further (Shintani et al., 2000). The Lg2 genome also contained complete pathways for glycolysis, the pentose phosphate pathway and glycogen synthesis. The absence of an annotated PHA synthase indicates an inability to form PHAs, supporting the Raman analyses.

Several annotated genes support the fermentative physiology observed with pure culture investigations (see earlier). Alanine and glucose, the main carbon sources utilized in pure culture incubations, are likely converted to pyruvate via the Embden-Meyerhof-Parnas (EMP) glycolysis pathway and by annotated alanine dehydrogenases (MPLG2_3627; MPLG2_3728), respectively. Pyruvate can be converted to acetyl-CoA by a pyruvate: ferredoxin oxidoreductase (MPLG2_2718), pyruvate dehydrogenase (MPLG2_1287; MPLG2_2466) or a pyruvate formate lyase (MPLG2_2587), with formate released from activity of the latter potentially oxidised to CO_2 by an annotated formate dehydrogenase (MPLG2_0499-0500). Fermentation by-products from acetyl-CoA include acetate, mediated by a phosphate acetyltransferase (MPLG2_3027) and an acetate kinase (MPLG2_v2_2721) and ethanol, facilitated by a possible acetylaldehyde dehydrogenase (MPLG2_v2_0028) and an alcohol dehydrogenase (MPLG2_0623). Detected lactate was likely generated

from pyruvate, catalyzed by an annotated lactate dehydrogenase (MPLG2_0671). Key genes were missing from the acrylate pathway (Cardon and Barker, 1947) ruling it out as the source of observed propionate accumulation under anaerobic conditions. The small amount of propionate produced may come from the metabolism of some amino acids and/or the anaerobic activity of the TCA cycle in conjunction with the methylmalonyl-CoA pathway – similar to proposed pathways for the production of propionyl-CoA in the classical GAO phenotype (Mino et al., 1998).

Fluorescence *in situ* hybridisation
In this study, two FISH probes were designed to target the *Micropruina* genus (**Table 1**). Both have the same coverage of the genus with *in silico* analyses and can be applied together with different fluorochromes to increase confidence in their specificity (**Table 1; Figure 1**). These probes replace the MIC184 probe (Kong et al., 2001), which has inferior specificity (**Table 1**). Pre-treatment with lysozyme was required to give good FISH signal for the *Micropruina* cells. Neither achromopeptidase nor mild acid treatment, which have been applied to increase permeability for other Gram-positives (Carr et al., 2005), led to a noticeable increase in signal. Cells responding to the MGL-67 and MGL-1223 FISH probes were cocci, typically between 0.5 and 1.0 μm in diameter, forming tetrads and microcolonies (**Figure 2**), which is consistent with the description of *Micropruina glycogenica* Lg2^T (Shintani et al., 2000). Despite covering 90% of near full-length sequences assigned to the genus in the MiDAS database (**Table 1**), the probes of this study do not cover all members of the genus in full-scale systems, and their use may underestimate *Micropruina* abundance (see **Supplementary text**). However, they do cover the most abundant member of the genus (represented by OTU_91: **Figures 1, 3 & S4**).

***In situ* physiology**
MAR-FISH analyses of activated sludge from two WWTPs showed that FISH-positive *Micropruina* cells were positive for uptake of a broad range of tested substrates, including glucose, fructose, galactose, amino acids, pyruvate, glycerol and butyrate, under both aerobic and anaerobic conditions. NAG, ethanol, acetate, propionate and oleate were not assimilated (**Table S1; Figures S6 & S7**). In contrast, *M. glycogenica* Lg2^T is reportedly able to utilise both acetate and propionate under aerobic conditions (Shintani et al., 2000). This may indicate a metabolic difference between the pure culture and *in situ* strains; noting that activated sludge organisms are known to behave different in pure culture to when they are in mixed communities (e.g. Kindaichi et al., 2013; McIlroy et al., 2013). The uptake of sugars and amino acids is consistent with the fermentative metabolism observed for *M. glycogenica* Lg2^T, however, future efforts to obtain genomes from *in situ* members of the genus will more definitively support a role for these organisms in fermentation in EBPR systems. The ability for the *in situ Micropruina* spp. to utilise sugars and amino acids is shared by the *Tetrasphaera* PAO (Kong et al., 2005), indicating direct competition for anaerobic carbon for these organisms. Raman microspectroscopy combined with FISH was applied to show that cells contained glycogen, but not PHAs, after

anaerobic incubation with either glucose or butyrate (**Figure S3**). MAR-FISH confirmed these substrates were incorporated into the *Micropruina* cells *in situ* – noting that the change in internal glycogen during the incubation would need to be quantified to empirically determine if supplied substrates are used for growth or storage. Cells from the aeration tank did not contain detectable polyphosphate. These results are consistant with the Raman results for *M. glycogenica* Lg2^T (see earlier). Anaerobic utilisation of butyrate *in situ* is difficult to explain, noting that Raman-FISH analyses indicated that PHAs were not produced with butyrate uptake (data not shown). The pure culture did not utilise butyrate when supplemented into the R2A media (at 2 mM) – noting that a range of other carbon sources were available that may be utilised preferentially – and the Lg2^T genome lacks a butyrate kinase. It may be that *in situ* strains utilise butyrate for anabolic reactions, with stored glycogen as the primary source of carbon and energy. Further work is required to determine this.

DISCUSSION

In this study, quantitative FISH confirmed the relatively high abundance of members of the *Micropruina* genus *in situ* in full-scale EBPR systems in Denmark, which was previously suggested by amplicon sequencing of the same plants (**Figure 3**) (Stokholm-Bjerregaard et al., 2017). *Micropruina* have also been reported in Japanese full-scale EBPR systems at up to 8% of the biovolume (Wong et al., 2005) and amplicon sequencing surveys suggest that they are abundant, and therefore likely important, in WWTPs globally (Nierychlo, M., and Nielsen, P.H., unpublished).

The *Micropruina* genus did not possess the classical GAO metabolism, but likely exhibits a fermentative metabolism in EBPR systems. Under anaerobic conditions these organisms are proposed to ferment sugars and amino acids to provide the carbon source and energy required for glycogen storage and possibly growth. As the *Micropruina* spp. are facultative anaerobes, stored glycogen may be oxidised under aerobic conditions for growth. Such a metabolic model makes them ideally suited to the dynamic feast-famine conditions of EBPR. Members of the genus enriched in a previous lab-scale study, reportedly able to assimilate acetate as PHA (Kong et al., 2001), may represent atypical members of the genus as neither the abundant *in situ* members of the genus, nor the axenic culture of *M. glycogenica,* behaved in this way. Therefore, the abundant *Micropruina* did not behave according to the classical GAO model where VFAs are stored as PHA under anaerobic conditions. They do accumulate glycogen, but under anaerobic conditions where excess substrate is available, and are therefore considered to behave as 'fermentative GAO' (fGAO). Anaerobic glycogen accumulation has been reported for non-EBPR sludges previously (Liu et al., 1996; Carucci et al., 1997), that may have contained undescribed fermentative GAOs, as well as for the *Tetrasphaera* PAO isolates (Kristiansen et al., 2013).

Therefore, competition for resources likely occurs on two levels, where the fermentative PAO (fPAO) compete with the fGAO and other fermenters for sugars and amino acids, and the classical PAO and GAO compete for fermentation by-products such as VFAs (summarised in **Figure 4**). Interestingly, the ability of the fPAO and fGAO to grow under both aerobic and anaerobic conditions may provide them with an advantage over the organisms with the classical PAO and GAO phenotypes, which is at least consistent with their higher relative abundances in Danish WWTPs (Stokholm-Bjerregaard et al., 2017). In addition, while the *Micropruina* spp., and other unknown fGAO, may ferment substrates to supply VFAs, any carbon stored as glycogen and utilised aerobically is not made available anaerobically to the classical PAOs for subsequent P removal – which may be important in systems where VFAs are limiting. As such, organisms storing carbon under anaerobic conditions, in the absence of polyphosphate cycling, are potential competitors of the PAO.

The dynamic feast-famine EBPR environment is selective for organisms able to store carbon anaerobically. Fermenters storing carbon when it is in excess will be able to use it for growth under aerobic conditions, but also during any anaerobic starvation periods (van Loosdrecht et al., 1997). Supporting the anaerobic storage of carbon by fermenters, Kong and colleagues (2008) observed the continual accumulation of VFAs with anaerobic incubation of full-scale sludge. This occurred when the carbon storage capacity of the classical PAO and GAO were saturated and added exogenous carbon sources became exhausted, which likely indicates fermentation of storage products by unidentified bacteria. In full-scale BNR plants, *in situ* measures estimate that bacteria identified to be actively fermentative constitute up to 40% of the biovolume and appear to be largely refined to the Actinobacteria and Firmicutes (Kong et al., 2008; Nielsen et al., 2012). Previous *in situ* studies have identified the genera *Propionicimonas*, *Tetrasphaera* and "*Ca*. Promineofilum" (Chloroflexi) as abundant fermenters in full-scale systems (Kong et al., 2008; Nielsen et al., 2012; McIlroy et al., 2016). These genera, along with several others known to include fermentative species, are among the most abundant in comprehensive surveys of full-scale systems in Denmark - in even higher abundances than *Micropruina* (**Figure 3**). Future application of Raman-FISH, or equivalent methods, to quantify the storage polymer dynamics of abundant organisms, will provide important information regarding the flow of carbon through these systems and the potential impact of specific abundant organisms on P removal and the ecology of EBPR. Elucidating the physiology of these abundant members of the community will importantly contribute to the broader goal of an in-depth understanding of the ecology of EBPR systems. To this end, the current study provides a detailed insight into the ecology of the abundant *Micropruina* spp., with the designed FISH probes and genome providing the foundation for more detailed studies – including *in situ* storage polymer dynamics and gene expression studies.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

SJM, PHN, FAH and CAO planned the experiments. FAH, CAO and BM performed pure culture incubations and FAH carried out the NMR analyses with help from RW. CAO and JMK prepared the genomic DNA, MSD, RHK, SMK sequenced and assembled the genome, and SJM annotated the genome. FISH probe design, optimisation and quantitative FISH analyses were performed by SJM and BM. CAO, EF and MN performed the single cell analyses. The manuscript was drafted by SJM and revised by all authors.

REFERENCES

Amann, R. I., Binder, B. J., Olson, R. J., Chisolm, S. W., Devereux, R., and Stahl, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Env. Microbiol* 56, 1919–1925.

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

Cardon, B. P., and Barker, H. A. (1947). Amino acid fermentations by *Clostridium propionicum* and *Diplococcus glycinophilus*. *Arch. Biochem.* 12, 165–180.

Carr, E. L., Eales, K., Soddell, J., and Seviour, R. J. (2005). Improved permeabilization protocols for fluorescence *in situ* hybridization (FISH) of mycolic-acid-containing bacteria found in foams. *J Microbiol Methods* 61, 47–54. doi: 10.1016/j.mimet.2004.10.023

Carucci, A., Lindrea, K. C., Majone, M., and Ramadori, R. (1999). Different mechanisms for the anaerobic storage of organic substrates and their effect on enhanced biological phosphate removal (EBPR). *Water Sci Technol* 39 (6), 21–28. doi: https://doi.org/10.1016/S0273-1223(99)00119-5

Carucci, A., Majone, M., Ramadori, R., and Rossetti, S. (1997). Biological phosphorus removal with different organic substrates in an anaerobic/aerobic sequencing batch reactor. *Water Sci. Technol.* 35 (1), 161–168. doi: https://doi.org/10.1016/S0273-1223(96)00892-X

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., et al. (2014). Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* 42, D633-42. doi:10.1093/nar/gkt1244.

Comeau, Y., Hall, K. J., Hancock, R. E. W., and Oldham, W. K. (1986). Biochemical model for enhanced biological phosphorus removal. *Water Res.* 20, 1511–1521. doi: https://doi.org/10.1016/0043-1354(86)90115-6

Crocetti, G. R., Banfield, J. F., Keller, J., Bond, P. L., and Blackall, L. L. (2002). Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. *Microbiology* 148, 3353–3364. doi:10.1099/00221287-14811-3353

Daims, H., Brühl, A., Amann, R., Schleifer, K. H., and Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* 22, 434–444. doi:10.1016/S0723-2020(99)80053-8

Daims, H., Lücker, S., and Wagner, M. (2006). daime, a novel image analysis program for microbial ecology and biofilm research. *Env. Microbiol* 8, 200–213. doi:10.1111/j.1462-2920.2005.00880.x

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

He, S., and McMahon, K. D. (2011). Microbiology of "Candidatus Accumulibacter" in activated sludge. *Microb. Biotechnol.* 4, 603–619. doi: 10.1111/j.17517915.2011.00248.x

Henze, M., Gujer, W., Mino, T., and van Loosdrecht, M. C. M. (2000). *Activated Sludge Models ASM1, ASM2, ASM2d and ASM3*. Cornwall, UK: IWA Publishing.

Hesselmann, R. P., Werlen, C., Hahn, D., van der Meer, J. R., and Zehnder, A. J. (1999). Enrichment, phylogenetic analysis and detection of a bacterium that performs enhanced biological phosphate removal in activated sludge. *Syst Appl Microbiol* 22, 454–465. doi:10.1016/S0723-2020(99)80055-1

Kindaichi T, Nierychlo M, Kragelund C, Nielsen JL, Nielsen PH. (2013). High and stable substrate specificities of microorganisms in enhanced biological phosphorus removal plants. *Environ Microbiol.* 15, 1821-1831. doi:10.1111/1462-2920.12074.

Kong, Y. H., Beer, M., Seviour, R. J., Lindrea, K. C., and Rees, G. N. (2001). Structure and functional analysis of the microbial community in an aerobic: anaerobic sequencing batch reactor (SBR) with no phosphorus removal. *Syst Appl Microbiol* 24, 597–609. doi: https://doi.org/10.1078/0723-2020-00075

Kong, Y., Nielsen, J. L., and Nielsen, P. H. (2004). Microautoradiographic study of *Rhodocyclus*-related polyphosphate-accumulating bacteria in full-scale enhanced biological phosphorus removal plants. *Appl Env. Microbiol* 70, 5383–5390. doi: 10.1128/AEM.70.9.5383-5390.2004

Kong, Y., Nielsen, J. L., and Nielsen, P. H. (2005). Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. *Appl Env. Microbiol* 71, 4076–4085. doi:10.1128/AEM.71.7.4076-4085.2005

Kong, Y., Ong, S. L., Ng, W. J., and Liu, W.-T. (2002). Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic-aerobic activated sludge processes. *Env. Microbiol* 4, 753–757. doi: 10.1046/j.1462-2920.2002.00357.x

Kong, Y., Xia, Y., and Nielsen, P. H. (2008). Activity and identity of fermenting microorganisms in full-scale biological nutrient removing wastewater treatment plants. *Env. Microbiol* 10, 2008–2019. doi: 10.1111/j.1462-2920.2008.01617.x

Kong, Y., Xia, Y., Nielsen, J. L., and Nielsen, P. H. (2006). Ecophysiology of a group of uncultured Gammaproteobacterial glycogen-accumulating organisms in full-scale enhanced biological phosphorus removal wastewater treatment plants. *Env. Microbiol* 8, 479–489. doi: 10.1111/j.1462-2920.2005.00914.x

Koren, S., Walenz, B. P., Berlin, K., Miller, J. R., and Phillippy, A. M. (2016). Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *bioRxiv*. doi: https://doi.org/10.1101/071282

Kristiansen, R., Nguyen, H. T. T., Saunders, A. M., Nielsen, J. L., Wimmer, R., Le, V. Q., et al. (2013). A metabolic model for members of the genus *Tetrasphaera* involved in enhanced biological phosphorus removal. *ISME J.* 7, 543–54. doi:10.1038/ismej.2012.136.

Lin-Vien, D., Colthup, N. B., Fateley, W. G., and Grasselli, J. G. (1991). *The Handbook of Infrared and Raman Characteristic Frequencies of Organic Molecules*. London, England: Academic Press.

Liu, W.-T., Mino, T., Nakamura, K., and Matsuo, T. (1994). Role of glycogen in acetate uptake and polyhydroxyalkanoate synthesis in anaerobic-aerobic activated sludge with a minimized polyphosphate content. *J. Ferment. Bioeng.* 77, 535–540. doi:10.1016/0922-338X(94)90124-4

Liu, W. T., Mino, T., Nakamura, K., and Matsuo, T. (1996). Glycogen accumulating population and its anaerobic substrate uptake in anaerobic-aerobic activated sludge without biological phosphorus removal. *Water Res* 30, 75–82. doi: https://doi.org/10.1016/0043-1354(95)00121-Z

Loman, N. J., and Quinlan, A. R. (2014). Poretools: a toolkit for analyzing nanopore sequence data. *Bioinformatics* 30, 3399–401. doi:10.1093/bioinformatics/btu555.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, et al. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res.* 32, 1363–1371. doi:10.1093/nar/gkh293

Majed, N., Matthäus, C., Diem, M., and Gu, A. Z. (2009). Evaluation of intracellular polyphosphate dynamics in enhanced biological phosphorus removal process using raman microscopy. *Environ. Sci. Technol.* 43, 5436–5442. doi:10.1021/es900251n.

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Scheifer, K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst Appl Microbiol* 15, 593–600. doi: 10.1016/S0723-2020(11)80121-9

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

McIlroy, S. J., Albertsen, M., Andresen, E. K., Saunders, A. M., Kristiansen, R., Stokholm-Bjerregaard, M., et al. (2014). "*Candidatus* Competibacter"-lineage genomes retrieved from metagenomes reveal functional metabolic diversity. *ISME J.* 8, 613–624. doi:10.1038/ismej.2013.162

McIlroy, S. J., Karst, S. M., Nierychlo, M., Dueholm, M. S., Albertsen, M., Kirkegaard, R. H., et al. (2016). Genomic and *in situ* investigations of the novel uncultured Chloroflexi associated with 0092 morphotype filamentous bulking in activated sludge. *ISME J.* 10, 2223–2234. doi:10.1038/ismej.2016.14

McIlroy, S. J., Kristiansen, R., Albertsen, M., Karst, S. M., Rossetti, S., Nielsen, J. L., et al. (2013). Metabolic model for the filamentous "*Candidatus* Microthrix parvicella" based on genomic and metagenomic analyses. *ISME J.* 7, 1161–1172. doi: 10.1038/ismej.2013.6

McIlroy, S. J., Nittami, T., Kanai, E., Fukuda, J., Saunders, A. M., and Nielsen, P. H. (2015a). Re-appraisal of the phylogeny and fluorescence in situ hybridization probes for the analysis of the Competibacteraceae in wastewater treatment systems. *Environ. Microbiol. Rep.* 7, 166–74. doi:10.1111/1758-2229.12215.

McIlroy, S. J., Saunders, A. M., Albertsen, M., Nierychlo, M., McIlroy, B., Hansen, A. A., et al. (2015b). MiDAS: the field guide to the microbes of activated sludge. *Database* 2015, 1–8. doi:10.1093/database/bav062

McIlroy, S. J., Tillett, D., Petrovski, S., and Seviour, R. J. (2011). Non-target sites with single nucleotide insertions or deletions are frequently found in 16S rRNA sequences and can lead to false positives in fluorescence in situ hybridization (FISH). *Env. Microbiol* 13, 38–47. doi:10.1111/j.1462-2920.2010.02306.x

Mielczarek, A. T., Nguyen, H. T., Nielsen, J. L., and Nielsen, P. H. (2013a). Population dynamics of bacteria involved in enhanced biological phosphorus removal in Danish wastewater treatment plants. *Water Res.* 47, 1529–1544. doi: 10.1016/j.watres.2012.12.003

Mielczarek, A. T., Saunders, A. M., Larsen, P., Albertsen, M., Stevenson, M., Nielsen, J. L., et al. (2013b). The Microbial Database for Danish wastewater treatment plants with nutrient removal (MiDAS-DK) - a tool for understanding activated sludge population dynamics and community stability. *Water Sci. Technol.* 67 (11), 2519–2526. doi:10.2166/wst.2013.151.

Mino, T., Arun, V., Tsuzuki, Y., and Matsuo, T. (1987). "Effect of phosphorus accumulation on acetate metabolism in the biological phosphorus removal process" in *Biological phosphorus removal from wastewaters*, ed. R. Ramadori (Oxford: Pergamon Press), 27–38.

Mino, T., Liu, W.-T., Kurisu, F., and Matsuo, T. (1995). Modelling glycogen storage and denitrification capability of microorganisms in enhanced biological phosphate removal processes. *Water Sci Technol* 31 (2), 25–34. doi: https://doi.org/10.1016/0273-1223(95)00177-O

Mino, T., Van Loosdrecht, M. C. M., and Heijnen, J. J. (1998). Microbiology and biochemistry of the enhanced biological phosphate removal process. *Water Res.* 32, 3193–3207. doi: https://doi.org/10.1016/S0043-1354(98)00129-8

Nakamura, K., Ishikawa, S., and Kawaharasaki, M. (1995). Phosphate uptake and release activity in immobilized polyphosphate-accumulating bacterium *Microlunatus phosphovorus* strain NM-1. *J. Ferment. Bioeng.* 80, 377–382. doi: https://doi.org/10.1016/0922-338X(95)94207-8

Nguyen, H. T., Le, V. Q., Hansen, A. A., Nielsen, J. L., and Nielsen, P. H. (2011). High diversity and abundance of putative polyphosphate-accumulating *Tetrasphaera*-related bacteria in activated sludge systems. *FEMS Microbiol Ecol* 76, 256–267. doi: 10.1111/j.1574-6941.2011.01049.x

Nguyen, H. T. T., Kristiansen, R., Vestergaard, M., Wimmer, R., and Nielsen, P. H. (2015). Intracellular accumulation of glycine in polyphosphate-accumulating organisms in activated sludge, a novel storage mechanism under dynamic anaerobic-aerobic conditions. *Appl. Environ. Microbiol.* 81, 4809–18. doi:10.1128/AEM.01012-15. doi: 10.1128/AEM.01012-15

Nguyen, H. T., Nielsen, J. L., and Nielsen, P. H. (2012). "*Candidatus* Halomonas phosphatis", a novel polyphosphate-accumulating organism in full-scale enhanced biological phosphorus removal plants. *Environ. Microbiol.* 14, 2826–2837. doi: 10.1111/j.1462-2920.2012.02826.x

Nielsen, J. L., Nguyen, H., Meyer, R. L., and Nielsen, P. H. (2012). Identification of glucose-fermenting bacteria in a full-scale enhanced biological phosphorus removal

plant by stable isotope probing. *Microbiology* 158, 1818–1825. doi:10.1099/mic.0.058818-0.

Nierychlo, M., Nielsen, J. L., and Nielsen, P. H. (2015). "Studies of the ecophysiology of single cells in microbial communities by (quantitative) microautoradiography and fluorescence *in situ* hybridization (MAR-FISH)," in *Hydrocarbon and Lipid Microbiology Protocols, Springer Protocols Handbooks*, eds. T. J. McGenity, K. N. Timmis, and B. Nogales Fernández (Berlin-Heidelberg: Springer-Verlag). doi:10.1007/8623_2015_66.

Oehmen, A., Lemos, P. C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L. L., et al. (2007). Advances in enhanced biological phosphorus removal: from micro to macro scale. *Water Res.* 41, 2271–2300. doi:10.1016/j.watres.2007.02.030

Oyserman, B. O., Noguera, D. R., Del Rio, T. G., Tringe, S. G., and McMahon, K. D. (2015). Metatranscriptomic insights on gene expression and regulatory controls in *Candidatus* Accumulibacter phosphatis. *ISME J.* doi:10.1038/ismej.2015.155. doi: 10.1038/ismej.2015.155

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590-6. doi:10.1093/nar/gks1219. doi:10.1093/nar/gks1219

Reasoner, D. J., and Geldreich, E. E. (1985). A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.* 45, 1–7.

Santos, M. M., Lemos, P. C., Reis, M. A., and Santos, H. (1999). Glucose metabolism and kinetics of phosphorus removal by the fermentative bacterium *Microlunatus phosphovorus*. *Appl Env. Microbiol* 65, 3920–3928.

Satoh, H., Mino, T., and Matsuo, T. (1994). Deterioration of enhanced biological phosphorus removal by the domination of microorganisms without polyphosphate accumulation. *Water Sci Technol* 30(6), 203–211. doi: 0273-1223(94)00541-9

Satoh, H., Mino, T., and Matsuo, T. (1998). Anaerobic uptake of glutamate and aspartate by enhanced biological phosphorus removal activated sludge. *Water Sci Technol* 37(4-5), 579–582. doi: https://doi.org/10.1016/S0273-1223(98)00163-2

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

Shintani, T., Liu, W. T., Hanada, S., Kamagata, Y., Miyaoka, S., Suzuki, T., et al. (2000). *Micropruina glycogenica* gen. nov., sp. nov., a new Gram-positive glycogen-accumulating bacterium isolated from activated sludge. *Int J Syst Evol Microbiol* 50 Pt 1, 201–207. doi: 10.1099/00207713-50-1-201

Stokholm-Bjerregaard, M., McIlroy, S. J., Nierychlo, M., Karst, S. M., Albertsen, M., and Nielsen, P. H. (2017). A critical assessment of the microorganisms proposed to be important to enhanced biological phosphorus removal in full-scale wastewater treatment systems. *Front. Microbiol.* 8, 718. doi:10.3389/fmicb.2017.00718.

van Loosdrecht, M. C. M., Pot, M. A., and Heijnen, J. J. (1997). Importance of bacterial storage polymers in bioprocesses. *Water Sci. Technol.* 35 (1), 41-47. https://doi.org/10.1016/S0273-1223(96)00877-3

Walker, B. J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., et al. (2014). Pilon: An integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One* 9, e112963. doi:10.1371/journal.pone.0112963.

Wallner, G., Amann, R., and Beisker, W. (1993). Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* 14, 136–143. doi: 10.1002/cyto.990140205

Wentzel, M. C., Lotter, L. H., Loewenthal, R. E., and Marais, G. v. R. (1986). Metabolic behaviour of *Acinetobacter* spp. in enhanced biological phosphorus removal - a biochemical model. *Water SA* 12, 209–224.

Wider, G., and Dreier, L. (2006). Measuring protein concentrations by NMR spectroscopy. *J. Am. Chem. Soc.* 128, 2571-2576. doi:10.1021/JA055336T.

Wong, M.-T., and Liu, W.-T. (2007). Ecophysiology of *Defluviicoccus*-related tetrad-forming organisms in an anaerobic-aerobic activated sludge process. *Env. Microbiol* 9, 1485–1496. doi: 10.1111/j.1462-2920.2007.01267.x

Wong, M.-T., Tan, F. M., Ng, W. J., and Liu, W.-T. (2004). Identification and occurrence of tetrad-forming Alphaproteobacteria in anaerobic-aerobic activated sludge processes. *Microbiology* 150, 3741–3748. doi: 10.1099/mic.0. 27291-0

Wong, M. T., Mino, T., Seviour, R. J., Onuki, M., and Liu, W.-T. (2005). *In situ* identification and characterization of the microbial community structure of full-scale enhanced biological phosphorous removal plants in Japan. *Water Res.* 39, 2901–2914. doi:10.1016/j.watres.2005.05.015

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

TABLES

Table 1. FISH probes designed for the detection of members of the genus *Micropruina*

Probe	*E. coli* pos.	Target	Coverage[1]	Non-target hits[1]	Sequence 5'-3'	[FA] %[2]	Ref.
MGL-67	67-97	*Micropruina* spp.	8/9 (89%)	0	CAG AAG AGC AAG CTC TTC GTC ACC G	50	This study
MGL-67_C1[3]	67-97	Competitor probe for MGL-67	-	-	CAG AAG AGC AAG CTC TCC GTC ACC G	-	This study
MGL-1223	1223-1246	*Micropruina* spp.	8/9 (89%)	0	CCA GCC ATT GTA GCA TGT TTC AAG	40	This study
MGL-1223_C1[3]	1223-1246	Competitor probe for MGL-1223	-	-	CCT GCC ATT GTA GCA TGT TTC AAG	-	This study
MGL-1223_C2[3]	1223-1246	Competitor probe for MGL-1223	-	-	CCA GCC ATT GTA GCA TGT TTG CAG	-	This study
MIC184	645-661	*Micropruina* spp.	8/9 (89%)	22	CAT TCC TCA AGT CTG CC	20	Kong *et al.,* 2001

[1] Coverage and specificity values are based on the MiDAS taxonomy v. 1.20 (McIlroy *et al.,* 2015) noting that the only sequence not covered by the three probes (Accession No. JN038683) does not consistently cluster with other sequences in the genus with phylogenetic analysis (see **Figure 1**). [2] Recommended hybridisation buffer formamide concentration % [v/v]. [3] Pure cultures representing non-target sequences with a single mismatch were used to assess the need for competitor probes. MGL-67_C1 and MGL-1223_C1 were required to remove non-specific binding of the MGL-67 and MGL-1223 probes to *Propionicicella superfundia*[T] DSM22317 and *Aestuariimicrobium kwangyangense*[T] DSM21549, respectively. Several database sequences contain two terminal mismatches to the MGL-1223 probe, which are covered by the MGL-1223_C2 competitor. *Nonomuraea soli*[T] DSM45533 could not be used to assess the need for MGL-1223_C2 due to high autofluorescence. It is recommended that all listed competitor probes be applied with their respective probe.

Table 2. Genome properties of the closed *Micropruina glycogenica* Lg2^T genome

Property	
Size	3.84 Mbp
GC content	68.3%
Protein coding density	90.2%
CDS	3952
CDS assigned function*	15.4%
rRNA operons	1
Sequencing project accession no.	PRJEB23532

CDS = Coding DNA sequence; *MicroScope software prediction classes 1-3.

FIGURES
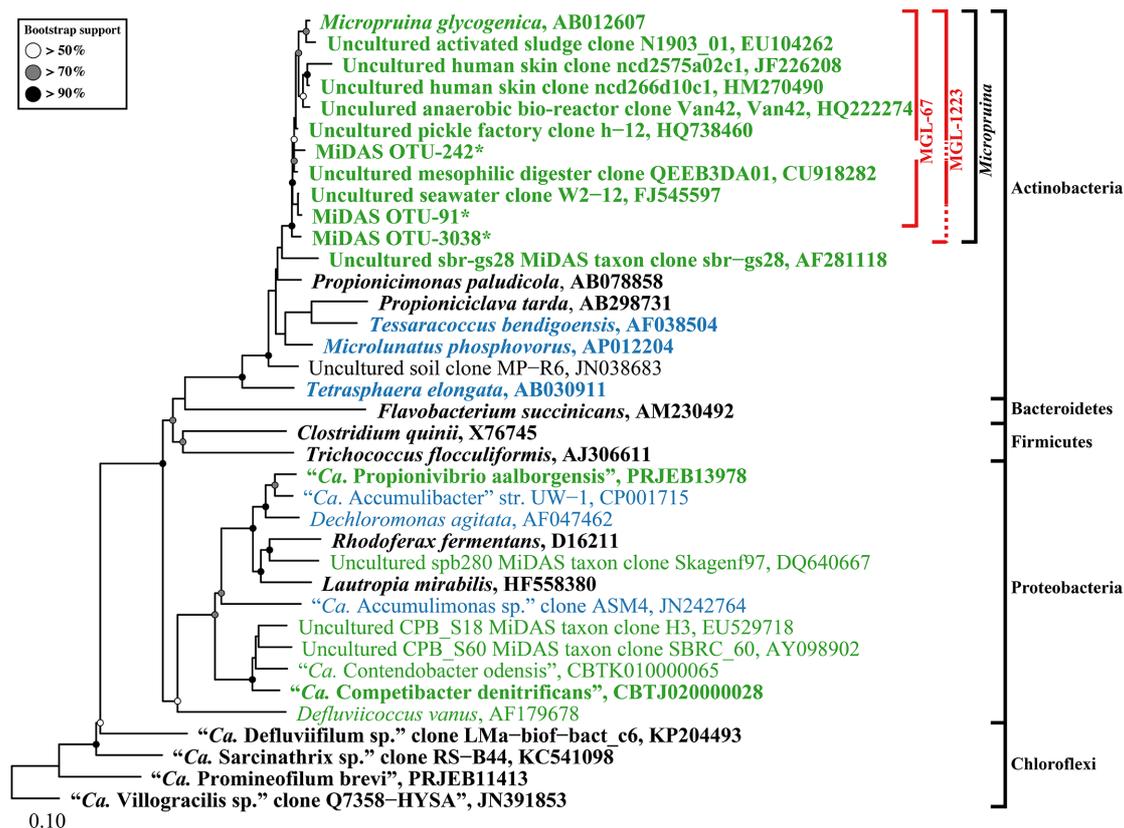
Figure 1. Maximum-likelihood (PhyML) 16S rRNA gene phylogenetic tree for PAOs, GAOs and abundant fermenters in EBPR activated sludge WWTPs. Sequences representing genera with known fermentative members are in bold typeface. Sequences representing putative PAO and GAO are blue and green, respectively. Outer brackets to the right indicate the phylogenetic classification of sequences. Inner red brackets show probe coverage; broken lines indicate sequence information at the probe site is unavailable. The tree was prepared using the ARB software (Ludwig et al., 2004) from the MiDAS database (version 1.20), which is a version of the SILVA database (Release 119 NR99 (Quast et al., 2013)) curated for activated sludge organisms (McIlroy et al., 2015b). Sequences were aligned in the ARB software, trimmed and variable regions excluded with a custom filter (filter by base frequency, 40-100%) leaving 1100 aligned positions. *Short MiDAS OTU amplicon sequences (461-462 bp) were added after calculation of the tree with the ARB insert sequences function. Bootstrap values from 100 analyses are indicated when above 50%. The scale bar represents substitutions per nucleotide base.
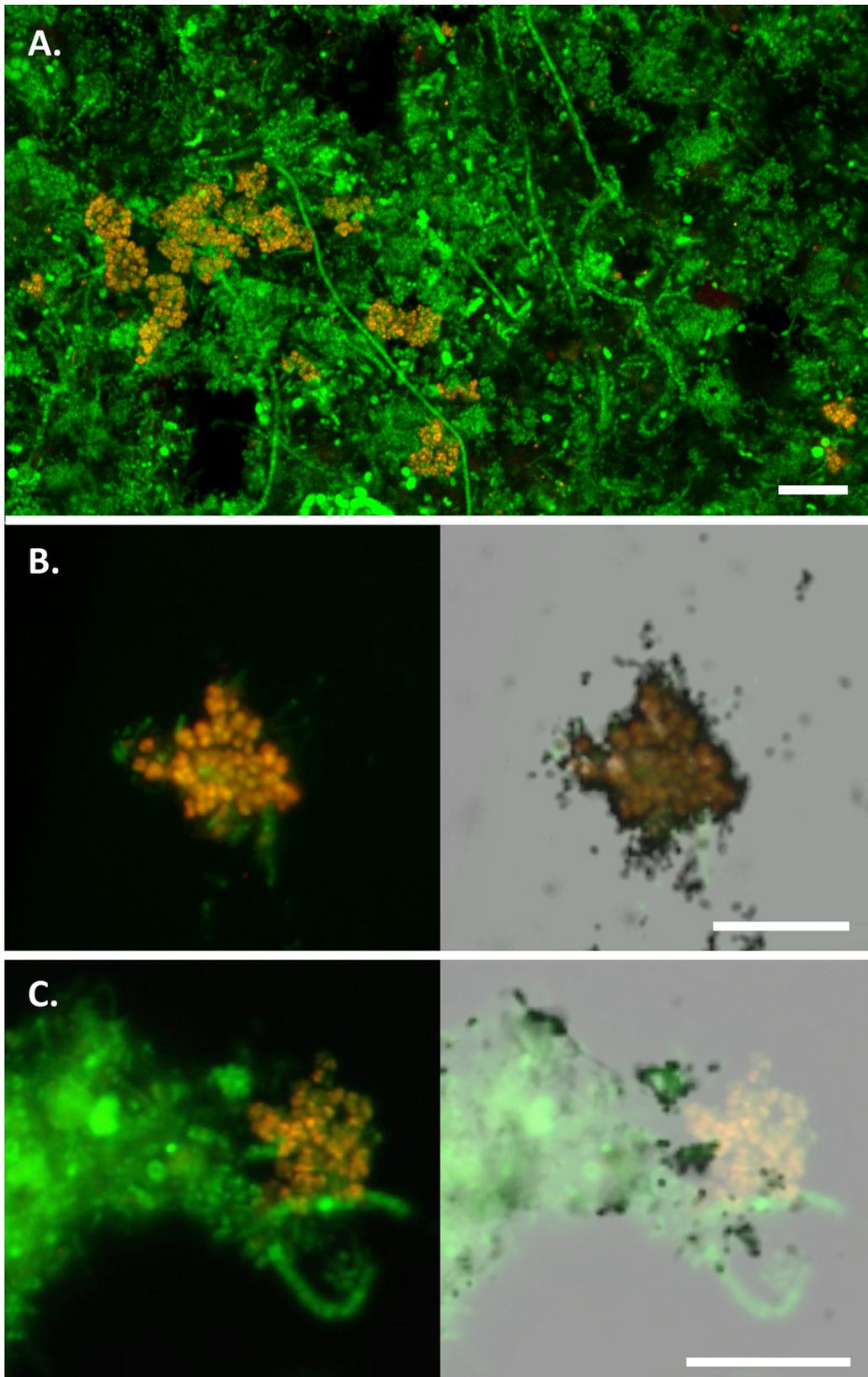
Figure 2. FISH micrographs of the *Micropruina* spp. in full-scale activated sludge. In all images, *Micropruina* appear yellow (MGL-67 (red) + EUBmix (green)) and other bacteria green (EUBmix alone). **A.** Composite fluorescence image of the Ejby Mølle WWTP sludge. **B. & C.** MAR-FISH images sets: composite fluorescence image alone (left) and with overlay of corresponding field of view with brightfield microscopy showing MAR signal (right). Cells with associated silver granules (black dots) are positive for uptake of the labelled substrate; **B.** Positive anaerobic uptake of ^{3}H-glucose; **C.** Negative aerobic uptake of ^{3}H- acetate. Scale bars indicate 10 μm.
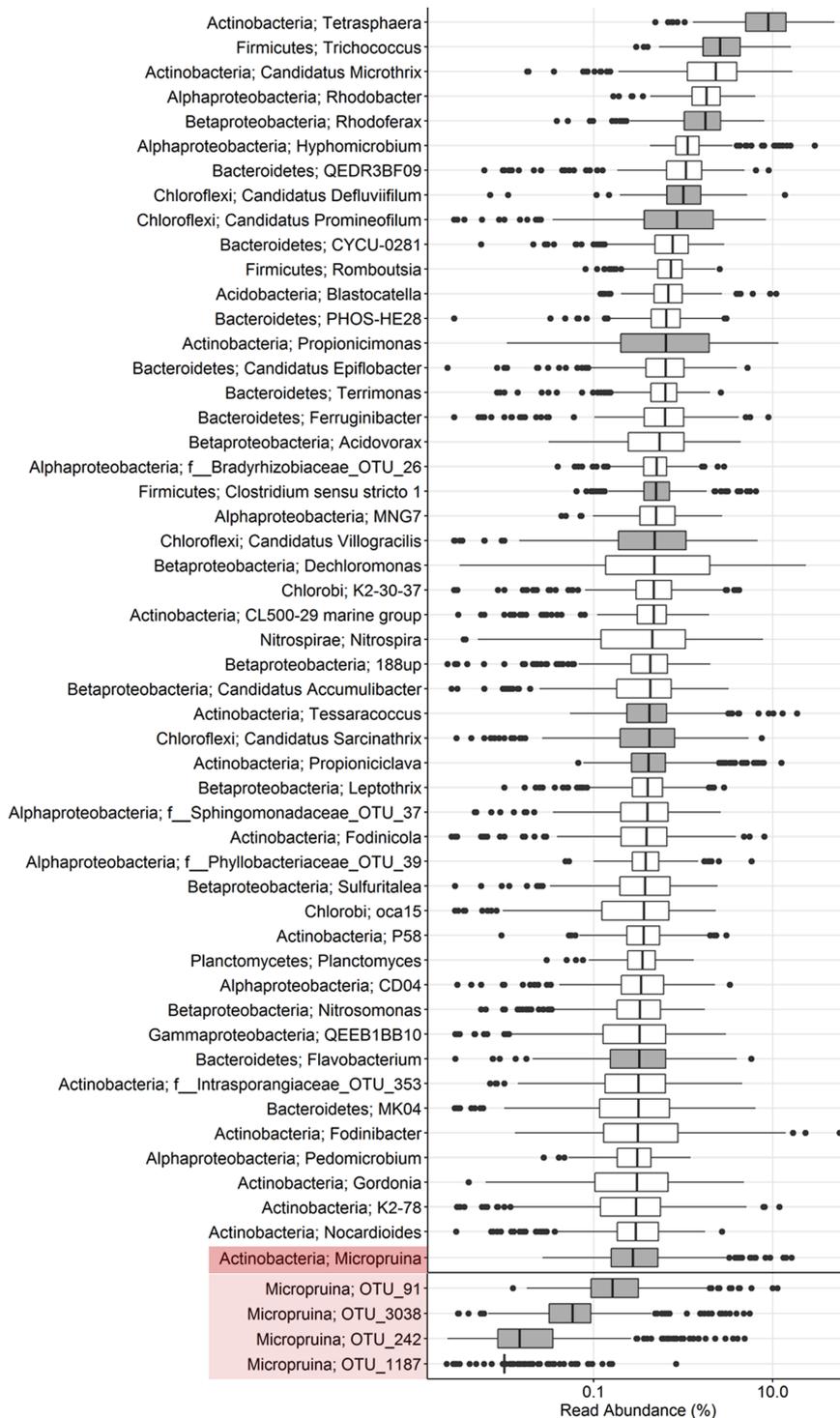
Figure 3. Boxplot showing the abundance distribution of the top genus-level phylotypes as well as individual OTUs belonging to the genus *Micropruina* in Danish EPBR plants. The data source is an extensive 16S rRNA gene amplicon sequencing (V1-3 region) survey of 18 plants over an 8-year period (2006-2014). For further details see Stokholm-Bjerregaard et al., (2017). Phylotypes with grey box plots are those known to be associated with a fermentative metabolism.
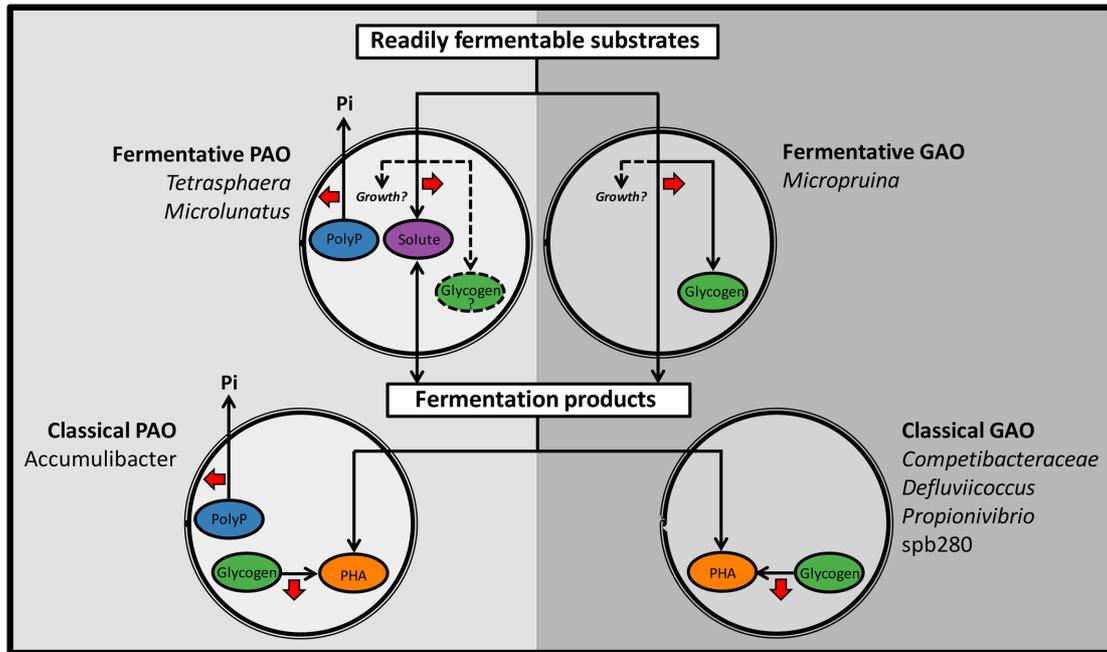
Figure 4. Schematic diagram summarising the important anaerobic transformations for known phenotypes with suggested importance to EBPR. Abundant phylotypes are included for each phenotype. Phenotypes to the left (PAO) favour EPBR and those to the right (GAO) are suggested to be detrimental to the process. Red arrows indicate net energy generation. In brief, the fermentative PAO (fPAO) compete with the fGAO and other fermenters for fermentable substrates (such as sugars and amino acids) and the classical PAO and GAO compete for fermentation by-products (such as VFAs). The *Micropruina* spp., and other potential fGAO, store some carbon as glycogen. The *Tetrasphaera* spp. and *Microlunatus* fPAO reportedly store some carbon as non-polymerised fermentation by-products and have the potential for glycogen storage (Santos *et al.*, 1999; Kristiansen *et al.*, 2013; Nguyen *et al.*, 2015; Nguyen, H. and Nielsen P.H., unpublished).

106

Genomic and *in situ* analyses reveal the *Micropruina* spp. as abundant fermentative glycogen accumulating organisms in enhanced biological phosphorus removal systems

Simon J. McIlroy, Cristobal A. Onetto, Bianca McIlroy, Florian-Alexander Herbst, Morten Simonsen Dueholm, Rasmus H. Kirkegaard, Eustace Fernando, Søren M. Karst, Marta Nierychlo, Jannie M. Kristensen, Kathryn L. Eales, Paul R. Grbin, Reinhard Wimmer and Per Halkjær Nielsen

FISH probe coverage analysis

Large scale surveys of full-scale activated sludge plants in Denmark revealed a relatively high abundance of the *Micropruina* spp. (Stokholm-Bjerregaard *et al.*, 2017). Quantitative FISH (qFISH) was applied in the current study to verify these abundances. Relatively high abundances of the genus were observed with qFISH analyses, where they constituted up to 6% of the biovolume, although lower values were determined relative to the amplicon read data for the same full-scale samples (**Figure S4**). This is explained by analyses of the abundant OTU sequences of the MiDAS survey (McIlroy *et al.*, 2015). There are three abundant OTUs and the MGL-67 probe target site is found in OTU-95 but not in the less frequently observed OTU-239 and OTU-3062 (**Figure 1, 3 & S4**). The latter two OTUs are abundant in the Randers and Hirtshals WWTPs, where a large difference was observed between the qFISH and amplicon abundance estimations (**Figure S4**). Coverage of the MGL-1223 probe could not be assessed, as the amplicons do not contain sequence information for the site. However good overlap for the MGL-67 and MGL-1223 probes was observed in all plants assessed in this study except Hirtshals (**Figure S5**), indicating that the latter probe does not cover these species either. The qFISH abundances correspond well with OTU-91 read abundance indicating that quantification with the amplicon method provides a good estimate of genus abundance. Therefore, the probes of this study appear to cover the abundant member of the genus, but not all members in full-scale systems, and their general use may underestimate *Micropruina* abundance.
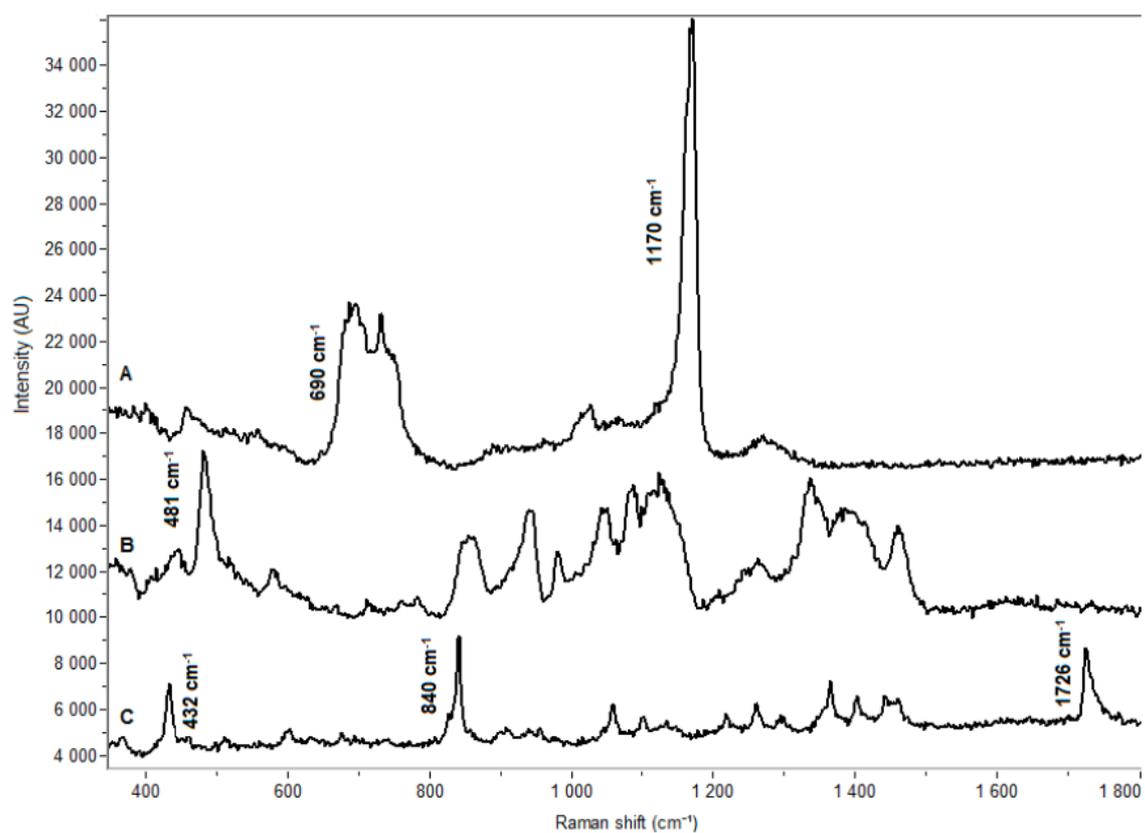
Figure S1. Raman spectra overlay of (A) polyphosphate (B) glycogen and (C) poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) standard compounds indicating their characteristic Raman marker peak positions.
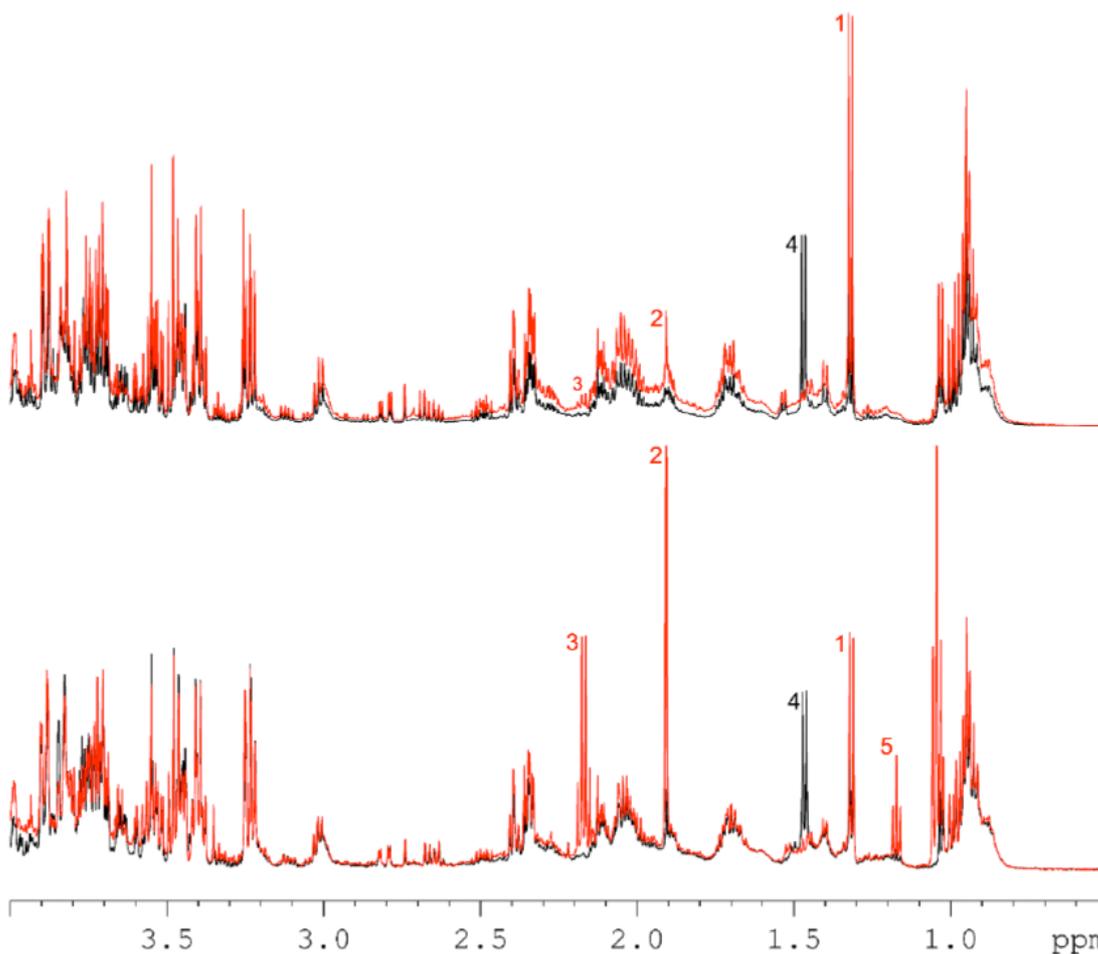
Figure S2. Example of NMR spectra for metabolite quantification. Original spectra range from 10 to -1 ppm and contain additional peaks for identification as well as quantification. One out of four biological replicates are chosen and shown before (black) and after cultivation (red) with (top) and without an additional concentration step (bottom). Numbers identify signature peaks for lactate (1), acetate (2), propionate (3), alanine (4), and ethanol (5). Example plots were created in Bruker TopSpin (v.3.5).
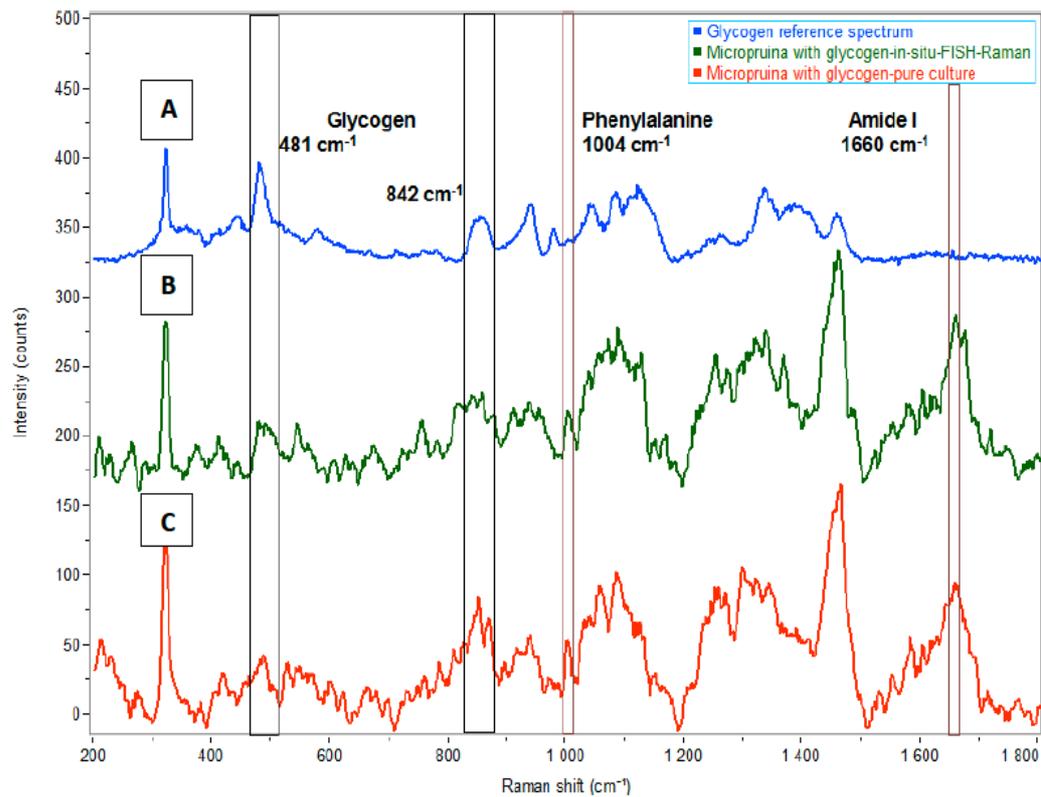
Figure S3. Overlay of the Raman spectra for (A) pure glycogen (B) *Micropruina* spp. FISH probe (MGL-67) and (C) *M. glycogenica* Lg2^T pure culture labelled cells indicating the characteristic Raman marker for glycogen at 481 cm^{-1}.
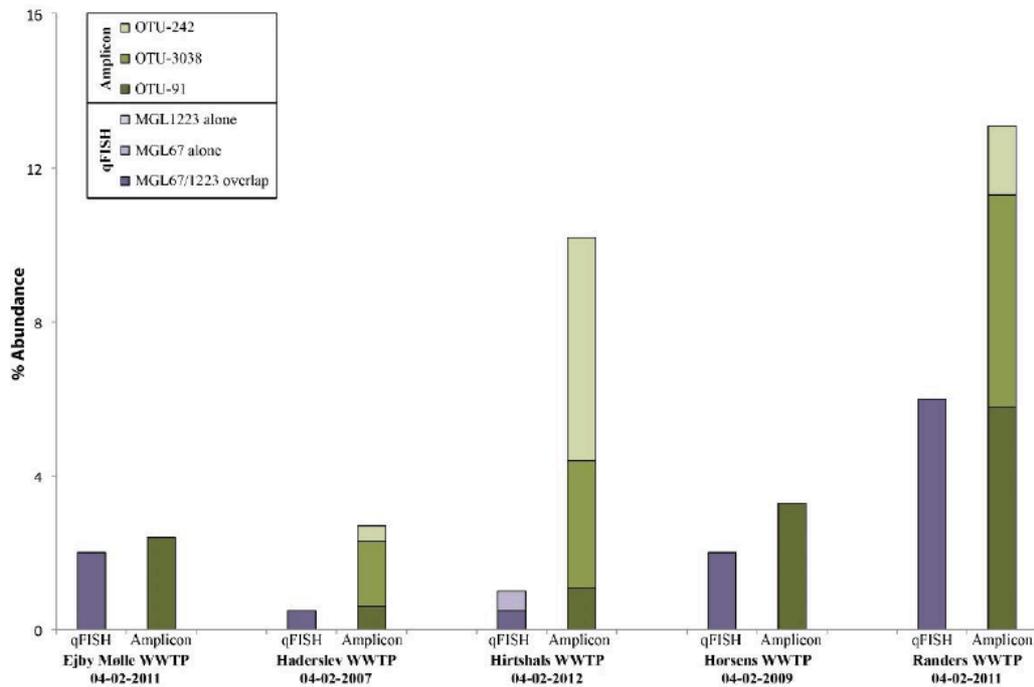
Figure S4. Comparison of FISH and 16S rRNA gene amplicon sequencing for estimating the relative abundance of *Micropruina* spp. in full-scale systems. Amplicon sequencing data is sourced from the MiDAS survey (McIlroy *et al.*, 2015).
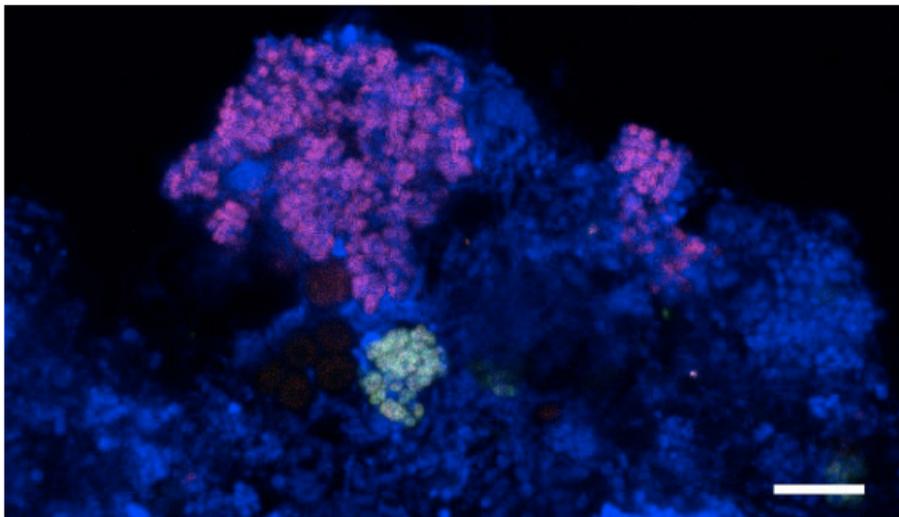
Figure S5. Composite FISH micrograph of biomass from the Hirtshals WWTP. Cells with a positive signal for both *Micropruina* spp. probes appear white (MGL-67 (green) + MGL-1223 (red) and EUBmix (blue)), cells hybridising only MGL-1223 appear magenta (MGL-1223 (red) + EUBmix (blue)) and those hybridising the EUBmix probe set only appear blue. Scale bar represents 10 μm.
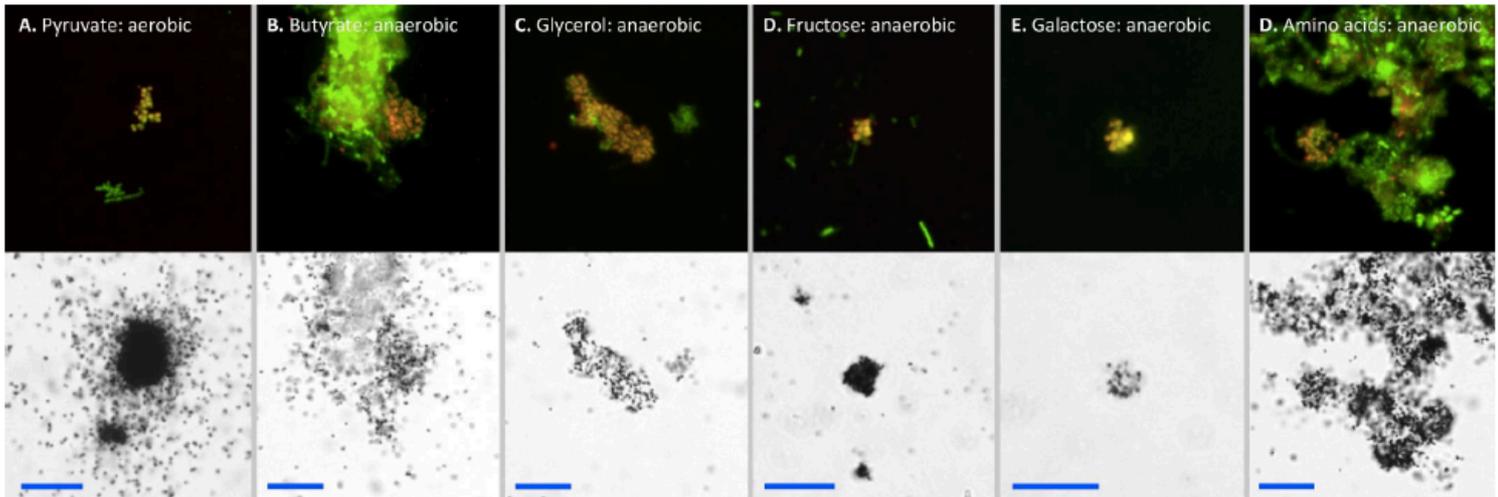
Figure S6. FISH and corresponding bright-field MAR micrographs depicting positive carbon source uptake for *Micropruina* spp. in sludge from full-scale WWTPs. Labelled substrates and electron acceptor conditions are indicated for each image. *Micropruina* spp. cells in FISH micrograph overlays appear yellow (MGL-67 (red) + EUBmix (green)) and non-target green (EUBmix only). Black silver granules indicate positive MAR signal. Scale bars represent 10 μm. Results are summarized in **Table S1**.
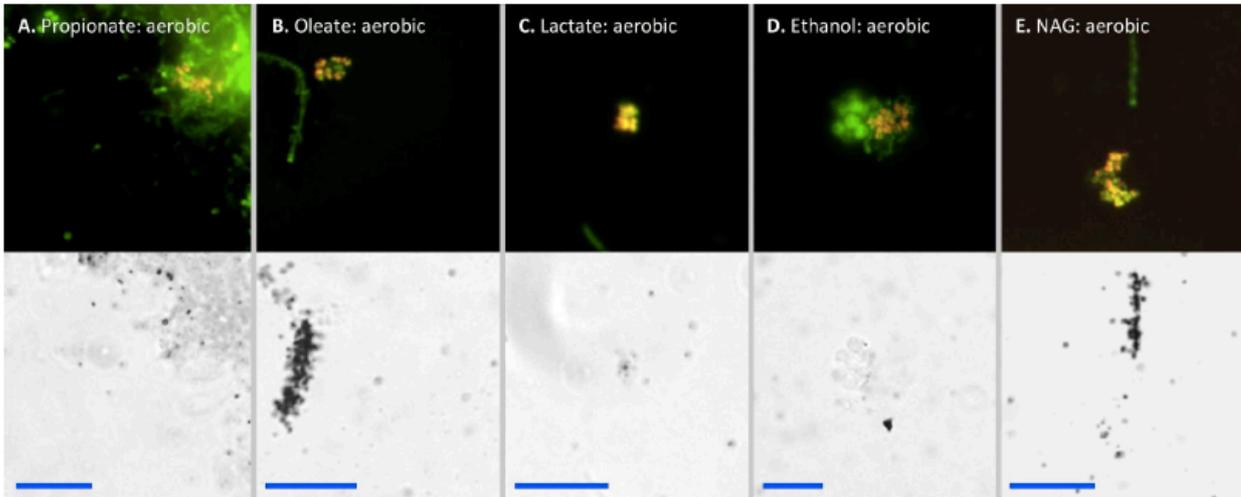
Figure S7. FISH and corresponding bright-field MAR micrographs depicting negative carbon source uptake for *Micropruina* spp. in sludge from full-scale WWTPs. Labelled substrates and electron acceptor conditions are indicated for each image. *Micropruina* spp. cells in FISH micrograph overlays appear yellow (MGL-67 (red) + EUBmix (green)) and non-target green (EUBmix only). Black silver granules indicate positive MAR signal. Scale bars represent 10 μm. Results are summarized in **Table S1**.

Table S1: Summary of MAR-FISH results for carbon uptake profiles.

Substrate	Aerobic	Anaerobic
Glucose	++	++
Fructose	++[1]	++[1]
Galactose	++[1]	++[1]
NAG	−	NA
Amino acids	++	+[2]
Pyruvate	++[2]	NA
Glycerol	++	++[2]
Ethanol	−	NA
Acetate	−	−[2]
Propionate	−	NA
Butyrate	+	++[2]
Oleate	−[2]	NA

Data represents the proportions of 20 FISH-positive cells that are also MAR positive: −, No MAR positive cells; +, 60-80%; ++, > 80%. NA= Not assessed. [1] Only determined for species in the Odense NW WWTP. [2] Only determined for species in the Ejby Mølle WWTP.

REFERENCES

McIlroy SJ, Saunders AM, Albertsen M, Nierychlo M, McIlroy B, Hansen AA, *et al.* (2015). MiDAS: the field guide to the microbes of activated sludge. *Database* **2015**: 1–8.

Stokholm-Bjerregaard M, McIlroy SJ, Nierychlo M, Karst SM, Albertsen M, Nielsen PH. (2017). A Critical Assessment of the Microorganisms Proposed to be Important to Enhanced Biological Phosphorus Removal in Full-Scale Wastewater Treatment Systems. *Front Microbiol* **8**: 718.

Chapter 6

Remediation of *Thiothrix* spp. associated bulking problems by raw wastewater feeding: A full-scale experience

Contextual statement

One of the WWW treatment plants (Plant A) surveyed in Chapter 2 showed an excessive and constant abundance of *Thiothrix* spp. causing bulking in codominance with GAOs belonging to the Competibacteraceae. The aim of the manuscript presented in this chapter is to investigate the effects of altering the wastewater feed composition with the ultimate objective of manipulating the microbial community and improve sludge settling.

Statement of Authorship

Title of Paper	Remediation of *Thiothrix* spp. associated bulking problems by raw wastewater feeding: A full-scale experience
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Onetto, CA, Eales, KL & Grbin, PR 2017, Remediation of *Thiothrix* spp. associated bulking problems by raw wastewater feeding: A full-scale experience. Systematic and applied microbiology, vol. 40, pp. 396-399.

Principal Author

Name of Principal Author (Candidate)	Cristobal Onetto		
Contribution to the Paper	Prepared data for sequencing, performed bioinformatics, chemical, *in situ* and data analyses, interpreted the data and drafted the manuscript		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22/02/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Kathryn Eales		
Contribution to the Paper	Designed experiments and revised the manuscript.		
Signature		Date	21/02/18

Name of Co-Author	Paul Grbin		
Contribution to the Paper	Oversaw experimental design and revised the manuscript.		
Signature		Date	22/02/208

Short Communication

Remediation of *Thiothrix* spp. associated bulking problems by raw wastewater feeding: A full-scale experience

Cristobal A. Onetto, Kathryn L. Eales, Paul R. Grbin *

Department of Wine & Food Science, University of Adelaide, Waite Campus, Australia

ABSTRACT

An industrial wastewater treatment plant (WWTP) in Australia has long suffered from bulking problems associated with the proliferation of *Thiothrix* spp. The WWTP consists of a covered anaerobic lagoon (CAL) followed by a sequencing batch reactor (SBR). The CAL functions as both an anaerobic digester and surge lagoon for the irregular flow of wastewater generated from the production of seasonal products. Chemical analysis of the raw influent showed it was composed of a mixture of organic acids, phenols and alcohols. The CAL effluent was characterised by high acetic acid and phenolic concentrations. An attempt was made to manipulate the SBR microbial community to improve settling by direct feeding small volumes of raw influent into the SBR. After raw feeding, the plant ceased bulking as the settled sludge volume reduced from 930 to 200 mL L^{-1}. 16S rRNA gene profiling and biovolumes of SBR samples revealed major changes in the microbial community. The *Thiothrix* spp. population decreased from 36.8% to 0.2%, and *Zoogloea* spp. dominated all samples after raw feeding. Therefore, direct feeding is proposed as a control method for industrial plants with surge/anaerobic lagoons in order to manage the bulking problems caused by *Thiothrix* spp. in downstream SBRs.

© 2017 Elsevier GmbH. All rights reserved.

Introduction

Bulking sludge is considered to be a frequent cause of operational problems in activated sludge systems [29]. This event is often attributed to the high growth of filamentous bacteria belonging to a diverse range of phylogenies, with more than 30 different morphotypes of filamentous bacteria characterised [23]. Several studies have been conducted in an attempt to understand the physiology of these groups of bacteria and the major factors triggering their proliferation in activated sludge communities, with the ultimate goal being to implement a targeted growth control strategy [17,24,26,28]. Common strategies employed are changes in aeration, sludge age and nutrient supplementation.

Type 021N is a well documented [12,18,19,21] and globally distributed group of filamentous bacteria comprising several species of the *Thiothrix* genus [10,16]. These species have been observed displaying multiple metabolisms depending on environmental conditions [26], which explains their ability to proliferate under various treatment plant configurations [29]. When observed in high

numbers, type 021N filaments can cause drastic bulking situations and a high sludge volume index (SVI) [26].

Wastewater composition is one of the major factors influencing the microbial community composition of activated sludge. The literature suggests that high concentrations of readily biodegradable substrates will favour the growth of filamentous bacteria, including Type 021N *Thiothrix* [29]. It is therefore plausible that changes in wastewater composition from mainly low molecular weight readily biodegradable substrates to a more diverse range of substrates will have an influence on the abundance of Type 021N *Thiothrix*.

In this study, a 3-year survey was performed on an industrial wastewater treatment plant (WWTP) in NSW Australia, treating wastewater derived from the production of beer, wine, cider and ready-to-drink beverages. The plant had long suffered from bulking which reduced efficiency and quality of the effluent. Periodic microscopic examinations indicated that *Thiothrix* spp. were the cause of poor settling. The WWTP included a large covered anaerobic lagoon (CAL) followed by a sequencing batch reactor (SBR). The CAL operated not only as an anaerobic digester but also as a surge lagoon for the irregular flow of wastewater generated from the production of seasonal products. From an understanding of the microbiology of the treatment plant and the chemical composition of the wastewater, an attempt was made to manipulate the microbial populations

---

* Corresponding author.
E-mail address: paul.grbin@adelaide.edu.au (P.R. Grbin).

				(25)	(31)		(1)	(0.6)	
Proteobacteria; Thiothrix	46.4	53.1	16.4	23.9	42.3	36.8	0.2	5.5	7.3	3.1
Proteobacteria; Zoogloea	0.1	1.1	1.3	0.2	0.3	7	15.1	18.6	23	15.8
Proteobacteria; f__Hyphomicrobiaceae	0	0	8	2.2	0.7	10.1	6.2	3.9	5.1	0.7
Planctomycetes; c__OM190	1	2	0	0	0	0	3.8	9.1	8.6	4.2
Proteobacteria; Defluviicoccus	0.2	0.1	0.6	0.2	0.1	0.7	3.9	8.9	0.5	11.4
Bacteroidetes; Paludibacter	0.4	0.4	0.3	0	0	0	6.5	5.9	0	12.1
Bacteroidetes; Ferruginibacter	0.3	0.2	7.2	8.2	2.3	1.7	1	0.4	0.3	0.1
Acidobacteria; Blastocatella	1.2	0.6	0.2	1.1	4.8	2.7	4.5	2.6	1.8	0.9
Bacteroidetes; Flavobacterium	1.3	2.2	0	0	0	0.1	0.9	7.3	5.6	1.6
Proteobacteria; CPB_S60	0.5	0.6	5.6	1.6	3.9	2.5	2.4	1	0.3	0.6
	Dec 2014	Jan 2015	Feb 2015	Mar 2015	Apr 2015	May 2015	Jun 2015	Jul 2015	Aug 2015	Sep 2015

Fig. 1. Heatmap representing the percentage read abundance of the 10 most abundant genera in SBR samples over a 10-month period. If no genus name could be assigned the best classification is provided. Numbers in parenthesis represent the estimated *Thiothrix* spp. biovolumes using the G123T and EUB338mix probes.

to improve settling in this full-scale system by direct feeding small volumes of raw influent into the SBR, bypassing the CAL.

Materials and methods

Raw feed trial and sampling

Samples were taken from a full-scale beverage treatment plant in NSW, Australia during the period December 2013–April 2016. Influent samples were taken post-screening where heavy particles and grit were removed *via* a composite sampler. Wastewater was continuously fed into a 30 ML CAL with a hydraulic retention time averaging 26 days. CAL samples were taken at the point of discharge into the SBR. Over the 10-month trial, the SBR (6 ML) processed three 450 kL batches/day with an average sludge retention time of 33 days. From 1st June, 2015, one batch/day contained 100 kL of raw influent bypassing the CAL. Raw influent and CAL samples were assessed by pH, electrical conductivity (EC), settled sludge volume (SSV60), chemical oxygen demand (COD) and total phenols using standard methods [27]. Organic acids, alcohols and sugars were determined by HPLC using an ion-exchange Aminex® HPX-87H HPLC column (Bio-Rad, USA) coupled to a refractive index detector (Agilent Technologies), as previously described [20]. Sulphur was analysed by ICP-MS at the CSIRO (Adelaide, South Australia) analytical services unit.

Fluorescence *in situ* hybridization (FISH)

SBR samples were assessed by FISH [9] using formaldehyde fixed cells and probe G123T [16] for the identification of *Thiothrix* spp. Biovolume fractions were estimated with the image analysis software DAIME v2.1 [8] using the G123T probe [16] as a specific target and the EUB338mix [2,7] probes for total biovolume. A minimum of 20 images at 400× magnification where analysed for each time point.

DNA extraction, sequencing and bioinformatics processing

Biomass DNA was extracted using a FastDNA® SPIN Kit for soil (MP Biomedicals, Santa Ana, CA) following the standard protocol, except for a four times increase in bead beating duration, as previously described [1]. Amplicon libraries and sequencing were performed by the Australian Centre for Ecogenomics (Brisbane, Australia) using primers targeting the V3-V4 16S rRNA gene region and the Miseq (Illumina) platform. Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32. [5]. The trimmed

Table 1
Composition of raw feed and covered anaerobic lagoon (CAL) during the period of April–August 2015 (values are means and SD in g L^{-1} unless otherwise stated).

Parameter	Raw feed	CAL
Tartaric acid	0.486 ± 0.27	n.d.
Succinic acid	0.073 ± 0.07	n.d.
Lactic acid	0.561 ± 0.29	n.d.
Acetic acid	0.214 ± 0.08	0.781 ± 0.61
Glycerol	0.033 ± 0.01	n.d.
Ethanol	1.132 ± 0.10	0.402 ± 0.30
Phenols	1.076 ± 0.10	0.940 ± 0.57
S (mg L^{-1})	4.75 ± 1.34	–
COD (mg L^{-1})	3970 ± 200	3435 ± 666
pH	6.1 ± 0.50	5.1 ± 0.70
EC (uS cm^{-1})	1081 ± 207	1485 ± 193

n.d. – not detected (<0.001 g L^{-1}).

forward and reverse reads were merged using FLASH v. 1.2.7 [22], then they were dereplicated and formatted for use in the UPARSE workflow [11]. Taxonomy was assigned with QIIME [6], using the MiDAS database v. 2.1 [25]. The results were analyzed with R [30] using the ampvis package v. 1.9.1 [1].

Results and discussion

Microscopic examination and application of 16S rRNA gene targeted probes to SBR samples before starting the raw feeding trial showed the community was dominated for six consecutive months by *Thiothrix* spp. with type 021N morphology (Figs. 1 and S1). Blastn analysis of 16S rRNA gene sequencing of the V3–V4 region (accession KY963627) revealed the species were 99% similar to *Thiothrix disciformis* (strain DSM 14473) [4]. Proliferation of this filamentous bacterium correlated with high SSV60 values and bulking problems (Fig. 2A), as previously reported [10,16].

Feeds mainly composed of a high concentration of readily biodegradable substrates have been suggested as a promoting growth factor for *Thiothrix* spp. [29]. Therefore, chemical analyses of the raw influent and CAL effluent were performed in order to assess the possible implications of the feed composition on the observed *Thiothrix* spp. associated bulking events. The COD revealed that only a small reduction in carbon (approximately 13%) was being achieved in the CAL during the period of the study, but significant chemical transformations were taking place (Table 1). The raw influent was characterised as being high in ethanol, phenols, tartaric acid and lactic acid. Exiting the CAL, tartaric acid and lactic acid were not detectable, ethanol was markedly reduced and acetic acid increased considerably, indicating the CAL was achieving acetoge-
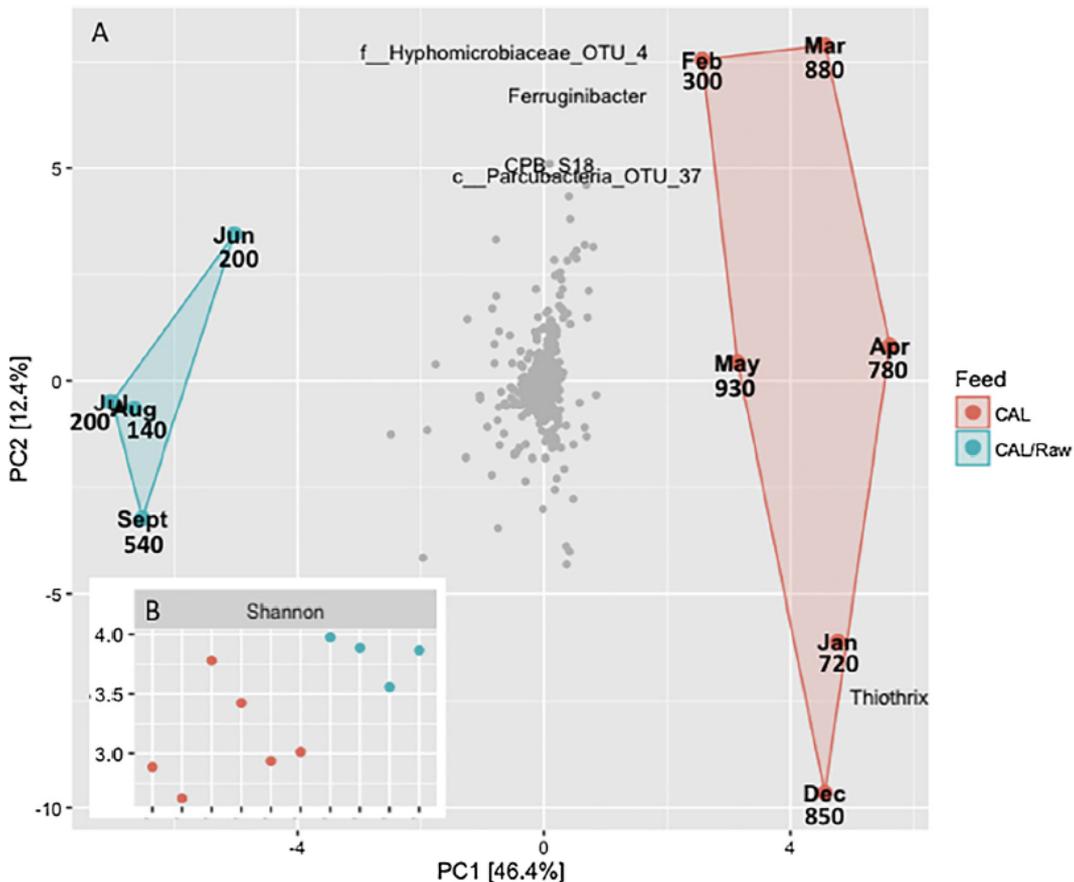
Fig. 2. (A) Principal component analysis (PCA) of the square root transformed OTU abundances (rarefied to 10000 counts per sample). Samples are represented as blue and red dots. Numbers underneath each sample correspond to the SSV60 (mL L^{-1}) values measured after sampling. OTUs are represented as grey dots, and the five most influential OTUs in the PCA are labelled to the genus level, but if no genus name could be assigned the best classification is provided. (B) Alpha diversity (Shannon) of samples before and after raw feeding.

nesis. Phenolic concentrations were unchanged, which indicated anaerobic degradation of phenolic compounds was often limited. The SBR was being fed with a less complex mixture of biodegradable substrates, composed mainly of acetic acid, ethanol and phenols. Therefore, it was speculated that changes in the feed composition to a more complex combination of substrates would facilitate the proliferation of a broader group of microorganisms and influence the growth of *Thiothrix* spp.

A trial was performed by feeding 100 kL/day of raw influent directly into the SBR. The influence of direct feeding on microbial populations was assessed by FISH microscopy and 16S rRNA gene amplicon analysis. Microscopy indicated dramatic changes in the community composition. Prior to direct feeding, samples had excessive growth of *Thiothrix* spp. (Figs. 1 and S1) with very few small (<150 μm), compact flocs (Fig. S2). However, after direct feeding, filament abundance was reduced significantly, flocs were larger (150–500 μm), firm and irregular with *Zoogloea* spp. dominating (Fig. S2). *Thiothrix* spp. biovolume estimates reduced from 31 to 1% (Fig. 1). Strong floc formation is frequently observed when *Zoogloea* spp. are present in high numbers due to their extracellular polymeric substances production capacity [3] that improves settling, as observed in the current study (Fig. 2A).

16S rRNA gene analysis was consistent with microscopic observations, since *Thiothrix* OTU's were reduced from 36.8 to 0.2%, similar to the biovolume estimates. *Zoogloea* spp. abundance had fluctuated prior to direct feeding but increased markedly afterwards (Fig. 1). Ecogenomics revealed notable changes in the

microbial community, with increased abundance of *Proteobacteria* (*Defluviicoccus*), *Bacteroidetes* (*Paludibacter* and *Flavobacterium*) and *Planctomycetes* (class OM190) (Fig. 1). *Zoogloea* and *Defluviicoccus* are both members of the *Betaproteobacteria*, which is routinely observed as the most abundant class in wastewater treatment and is largely responsible for organic and nutrient removal [15]. *Bacteroidetes* is also regarded as a core phylum in activated sludge, with varied metabolic roles, including floc forming properties [13,14,31]. The increase in these organisms may also have had a positive impact on the settling properties of the floc. Little is known about *Planctomycetes* Class OM190 in activated sludge yet visually the heat map suggested that co-occurrence and disappearance of species was occurring (Fig. 1). It can be speculated that these populations had been stimulated by changes in the feed composition, therefore, the consequential effect of bacterial interactions and their influence on bacterial community assembly would be worth pursuing in future research.

Shifts in the whole microbial community composition were assessed by principal component analysis (PCA) using square root transformed OTU counts of the 16S rRNA genes. PCA showed a clear separation between samples before and after raw feeding, which was mainly represented by the reduction of *Thiothrix* spp. (Fig. 2A). Changes in the feed composition had an impact on the whole microbial community, favouring the growth of a different set of microorganisms and inducing a shift in the microbial community. This shift was reflected in higher alpha diversity values (Fig. 2B) and improved settling properties of the flocs (Fig. 2A).

Conclusion

In this study, by changing the chemical characteristics of the SBR feed the metabolic selection of organisms was altered, and biodiversity and settling were improved. Whilst there would still be a need to send most of the raw influent into the CAL, introducing a relatively small volume of raw influent with a mixture of carbon substrates may be an easy and economical strategy for industrial plants with large surge lagoons to manage their microbial communities and control filamentous bulking caused by *Thiothrix* spp.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2017.06.004.

References

[1] Albertsen, M., Karst, S.r.M., Ziegler, A.S., Kirkegaard, R.H., Nielsen, P.H. (2015) Back to basics? The influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities. PLoS One 10, e0132783.

[2] Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.

[3] An, W., Guo, F., Song, Y., Gao, N., Bai, S., Dai, J., Wei, H., Zhang, L., Yu, D., Xia, M., Yu, Y., Qi, M., Tian, C., Chen, H., Wu, Z., Zhang, T., Qiu, D. (2016) Comparative genomics analyses on EPS biosynthesis genes required for floc formation of *Zoogloea resiniphila* and other activated sludge bacteria. Water Res. 102, 494–504.

[4] Aruga, S., Kamagata, Y., Kohno, T., Hanada, S., Nakamura, K., Kanagawa, T. (2002) Characterization of filamentous Eikelboom type 021N bacteria and description of *Thiothrix disciformis* sp. nov. and *Thiothrix flexilis* sp. nov. Int. J. Syst. Evol. Microbiol. 52, 1309–1316.

[5] Bolger, A.M., Lohse, M., Usadel, B. (2014) Trimmomatic: a flexible trimmer for illumina sequence data. Bioinformatics btu170.

[6] Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I. (2010) QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336.

[7] Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., Wagner, M. (1999) The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22, 434–444.

[8] Daims, H., Lücker, S., Wagner, M. (2006) Daime, a novel image analysis program for microbial ecology and biofilm research. Environ. Microbiol. 8, 200–213.

[9] Daims, H., Stoecker, K., Wagner, M. (2005) Fluorescence *in situ* hybridization for the detection of prokaryotes. Mol. Microb. Ecol. 213, 239.

[10] Dumonceaux, T.J., Hill, J.E., Pelletier, C.P., Paice, M.G., Van Kessel, A.G., Hemmingsen, S.M. (2006) Molecular characterization of microbial communities in Canadian pulp and paper activated sludge and quantification of a novel *Thiothrix eikelboomii*-like bulking filament. Can. J. Microbiol. 52, 494–500.

[11] Edgar, R.C. (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10, 996–998.

[12] Eikelboom, D. (1977) Identification of filamentous organisms in bulking activated sludge. Prog. Water Technol.

[13] Endo, T., Nakamura, K., Takahashi, H. (1976) Pronase-susceptible floc forming bacteria: relationship between flocculation and calcium ion. Agric. Biol. Chem. 40, 2289–2295.

[14] Hantula, J., Bamford, D.H. (1991) The efficiency of the protein-dependent flocculation of *Flavobacterium* sp. is sensitive to the composition of growth medium. Appl. Microbiol. Biotechnol. 36, 100–104.

[15] Ju, F., Zhang, T. (2015) Bacterial assembly and temporal dynamics in activated sludge of a full-scale municipal wastewater treatment plant. ISME J. 9, 683–695.

[16] Kanagawa, T., Kamagata, Y., Aruga, S., Kohno, T., Horn, M., Wagner, M. (2000) Phylogenetic analysis of and oligonucleotide probe development for Eikelboom Type 021N filamentous bacteria isolated from bulking activated sludge. Appl. Environ. Microbiol. 66, 5043–5052.

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

[18] Kristensen, G.H., Jørgensen, P.E., Nielsen, P.H. (1994) Settling characteristics of activated sludge in Danish treatment plants with biological nutrient removal. Water Sci. Technol. 29, 157–165.

[19] Levantesi, C., Beimfohr, C., Geurkink, B., Rossetti, S., Thelen, K., Krooneman, J., Snaidr, J., van der Waarde, J., Tandoi, V. (2004) Filamentous *Alphaproteobacteria* associated with bulking in industrial wastewater treatment plants. Syst. Appl. Microbiol. 27, 716–727.

[20] Li, S., Bindon, K., Bastian, S.E.P., Jiranek, V., Wilkinson, K.L. (2017) Use of winemaking supplements to modify the composition and sensory properties of Shiraz wine. J. Agric. Food Chem. 65, 1353–1364.

[21] Madoni, P., Davoli, D., Gibin, G. (2000) Survey of filamentous microorganisms from bulking and foaming activated-sludge plants in Italy. Water Res. 34, 1767–1772.

[22] Magoč, T., Salzberg, S.L. (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics.

[23] Martins, A.M.P., Pagilla, K., Heijnen, J.J., van Loosdrecht, M.C.M. (2004) Filamentous bulking sludge—a critical review. Water Res. 38, 793–817.

[24] McIlroy, S.J., Karst, S.M., Nierychlo, M., Dueholm, M.S., Albertsen, M., Kirkegaard, R.H., Seviour, R.J., Nielsen, P.H. (2016) Genomic and *in situ* investigations of the novel uncultured *Chloroflexi* associated with 0092 morphotype filamentous bulking in activated sludge. ISME J. 10, 2223–2234.

[25] McIlroy, S.J., Saunders, A.M., Albertsen, M., Nierychlo, M., McIlroy, B., Hansen, A.A., Karst, S.M., Nielsen, J.L., Nielsen, P.H. (2015) MiDAS: the field guide to the microbes of activated sludge. Database 2015, bav062.

[26] Nielsen, P.H., De Muro, M.A., Nielsen, J.L. (2000) Studies on the *in situ* physiology of *Thiothrix* spp. present in activated sludge. Environ. Microbiol. 2, 389–398.

[27] Rice, E., Bridgewater, L. 2012 Standard Methods for the Examination of Water and Wastewater, American Public Health Association, Washington, DC.

[28] Seviour, E.M., Eales, K., Izzard, L., Beer, M., Carr, E., Seviour, R. (2006) The *in situ* physiology of *Nostocoida limicola* II, a filamentous bacterial morphotype in bulking activated sludge, using fluorescence *in situ* hybridization and microautoradiography. Water Sci. Technol. 54, 47–53.

[29] Seviour, R.J., Nielsen, P.H. 2010 Microbial Ecology of Activated Sludge, IWA Publishing.

[30] Team, R.C. 2015 R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, 2013, Vienna, Austria, Document freely available on the internet at: http://www.r-project.org.

[31] Tezuka, Y. (1969) Cation-dependent flocculation in a *Flavobacterium* species predominant in activated sludge. Appl. Microbiol. 17, 222–226.

Supplementary material

Remediation of *Thiothrix* spp. associated bulking problems by raw wastewater feeding: A full-scale experience

Cristobal A.Onetto[†], Kathryn L.Eales[†], Paul R.Grbin[†]

[†] Department of Wine & Food Science, University of Adelaide, Waite Campus, Australia
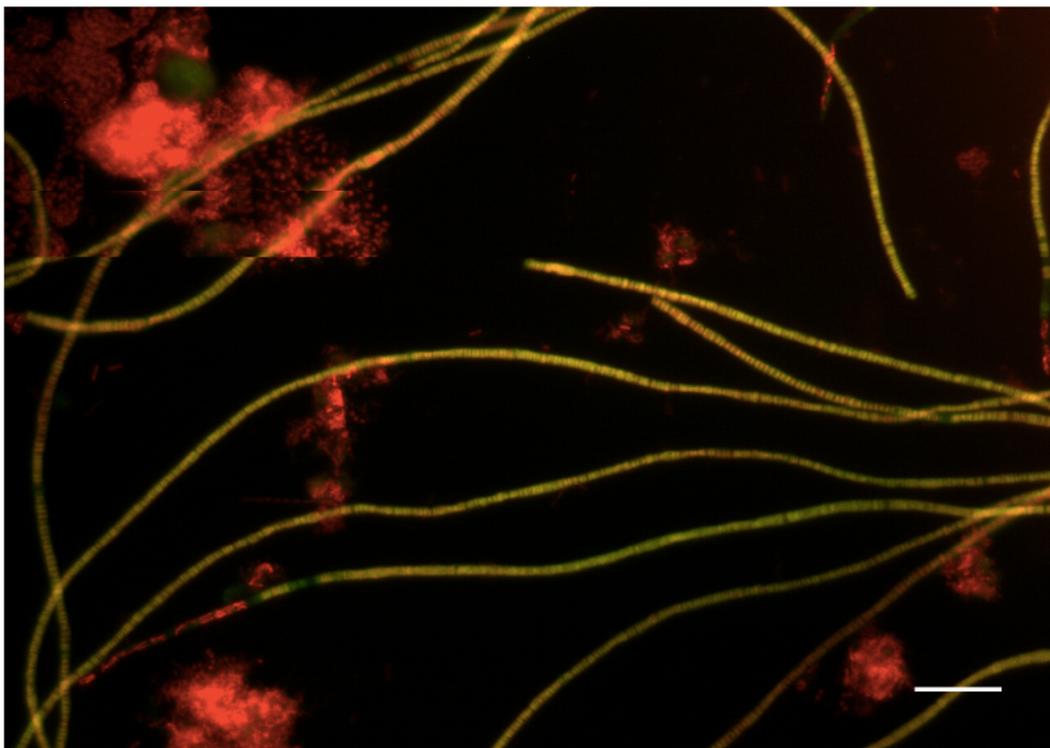
Figure S1. Composite FISH micrograph of *Thiothrix* spp. in a sample taken in May 2015. FISH probes applied include G123T (Fluos-label, green) and EUBmix (Cy3-label, red). *Thiothrix* spp. appear yellow/orange while all other cells appear red. Scale bar represents 20 μm.
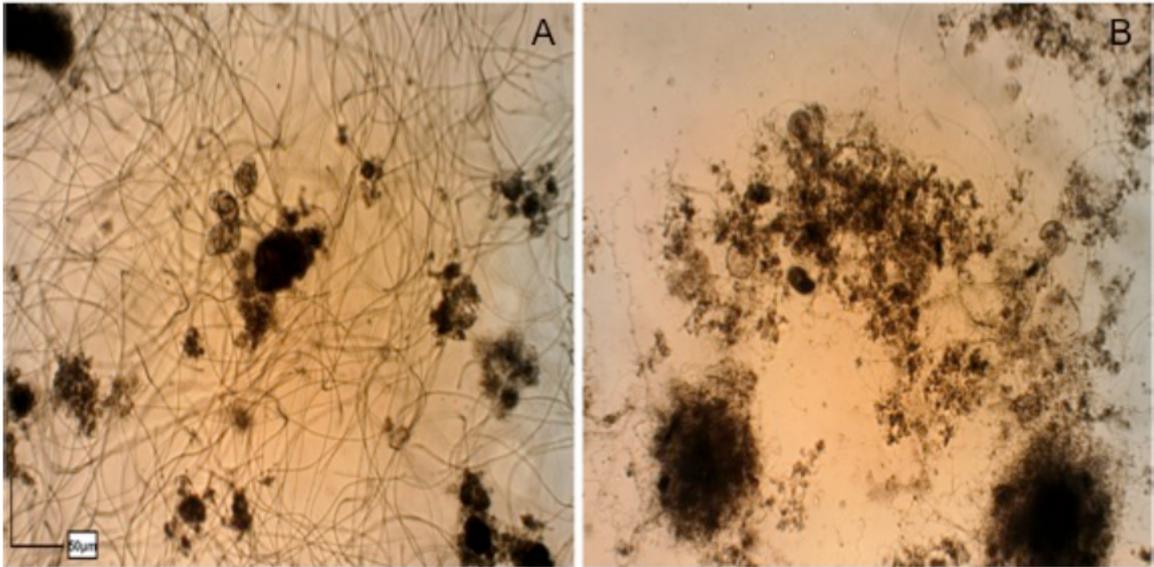
Figure S2. Light micrographs showing floc structure of samples taken before the feeding trial, May 2015 (A) and after July 2015 (B). Scale bar represents 50 μm.

Chapter 7

7.1. Conclusions

The identity and physiology of GAOs in WWW is now understood. Phylogenetic analyses of multiple WWW treatment plants revealed that the GAO community is diverse with members belonging to the Alphaproteobacteria (*Defluviicoccus*), Gammaproteobacteria (CPB_S18, CPB_S60 and CCM19a) and Actinobacteria (*Micropruina* and *Nakamurella*).

DF2 and DF3 were the most widely distributed and abundant GAO in WWW. From these, DF2 has been investigated thoroughly, with a genome available and several *in situ* physiology studies reported. Their excessive proliferation has been previously attributed to low nutrient levels, and nutrient supplementation has been suggested as a control measure. Manipulating the COD:N ratio of wastewater is a commonly used practice in the industry in order to troubleshoot microbial related issues, with little regard to how individual microbial populations are affected. FISH-NanoSIMS enabled the effect of different COD:N ratios on DF2 at the single cell level to be investigated. From the results, it was concluded that the nitrogen available in the activated sludge has an impact on the carbon assimilation of the DF2 community, with lower concentrations (100:1 and no nitrogen added) enhancing the carbon uptake of DF2. Higher nitrogen concentrations were shown to enhance the growth and nitrogen uptake of the whole microbial community.

The genome of a filamentous DF3 was extracted from a WWW activated sludge metagenome using the differential coverage principle (Albertsen et al. 2013) resulting in a 98% complete genome. Annotation showed the presence of the common genes associated with GAO metabolism and interesting metabolic pathways not previously described in other *Defluviicoccus* genomes. The genetic potential to cycle trehalose through glycogen, nitrogen fixation, hydrogenase activity and urea uptake appeared as adaptive strategies of DF3 and helped to understand the proliferation of this strain in the WWW nitrogen limited environment. From an overview of Chapter 3 and 4 it can be concluded that nitrogen deficiency is one of the major factors enhancing the proliferation of the two main groups of GAOs present in WWW. Monitoring and measuring carbon and nitrogen levels in influent streams routinely, taking measures to reduce excessive carbon load upstream or supplementing with nitrogen should effectively reduce the problems associated to the excessive proliferation of *Defluviicoccus* GAOs.

Actinobacteria *Micropruina* spp. were observed in low numbers; they are uncharacterised putative GAOs that have been reported as an important GAO for EBPR treatment plants (Stokholm-Bjerregaard et al. 2017). Pure culture *Micropruina glycogenica* str. Lg2T genomic and metabolomic studies were compared with the *in situ* physiology of the genus. *Micropruina* spp. and strain Lg2T were observed to take up carbon substrates including sugar and amino acids under anaerobic conditions, which were partly fermented to lactic acid, acetate, propionate and ethanol by strain Lg2T and partly stored as glycogen without any detectable PHA storage. This fermenting metabolism is different to the classical GAO phenotype. Pure culture and

in situ experiments confirmed their inability to utilise acetate, yet they utilised sugars and amino acids and fermented them to simple organic acids. This could explain why their low abundance in WWW, where acetate is one of the main carbon sources. It can be speculated that their role in WWW is rather beneficial as they release organic acids to be utilised by the microbial community without an excessive proliferation as observed for *Defluviicoccus* GAOs.

The microbial community of Plant A unlike the other plants surveyed had an excessive and constant abundance of *Thiothrix* spp. with GAOs belonging to the CPB_S60 clade as the second most abundant population. A full-scale trial was performed in order to manipulate the microbial community composition and improve sludge settling by raw wastewater feeding. Modifying the chemical characteristics of the SBR feed from mainly acetate to a more diverse range of substrates altered the metabolic selection of organisms and improved biodiversity and settling. *Zoogloea* spp. and *Defluviicoccus* GAOs belonging to cluster II formed part of the microbial communities stimulated by the raw feeding trial. Ecophysiology investigations on DF2 have shown the ability of this cluster to uptake several carbon substrates (see Chapter 1) which may help to explain the observed increase in abundance. Despite showing abundances of up to 11% during this trial, DF2 was not associated with bulking events. It can be speculated that the proliferation of non-filamentous GAOs is not necessarily detrimental when they form part of a more balanced microbial community, but rather beneficial as they play a central role in carbon removal.

7.2. Future directions

The data presented in this thesis enhances the knowledge associated to GAOs in WWW. Although these microorganisms can be identified as a core component of the microbial community of WWW activated sludge, it is clearly observed from the metagenomic surveys that several uncharacterised genera are also important members of this microbial community. More research should be performed to fully characterise the unique microbial community of WWW along with ecophysiology studies to understand the role of the different microbial communities.

It can be concluded that nutrient limitation is one of the major factors enhancing the proliferation of the most significant GAOs, however, the physiological traits (e.g. EPS production) associated to their involvement in non-filamentous bulking problems are poorly comprehended. The continuous developments in sequencing technologies and bioinformatics tools are facilitating the extraction of genomes from complex metagenomes. Future *in situ* transcriptomics studies should be performed to accurately characterise the role of each microbial community in the activated sludge during the wastewater treatment process.