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Re-evaluating the microbiology of the enhanced biological phosphorus removal process

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We have critically assessed some of the dogmas in the microbiology of enhanced biological phosphorus removal (EBPR) and argue that the genus *Tetrasphaera* can be as important as *Ca.* Accumulibacter for phosphorus removal; and that proliferation of their competitors, the glycogen accumulating organisms, does not appear to be a practical problem for EBPR efficiency even under tropical conditions. An increasing number of EBPR-related genomes are changing our understanding of their physiology, for example, their potential to participate in denitrification. Rather than trying to identify organisms that adhere to strict phenotype metabolic models, we advocate for broader analyses of the whole microbial communities in EBPR plants by iterative studies with isolates, lab enrichments, and full-scale systems.

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Introduction

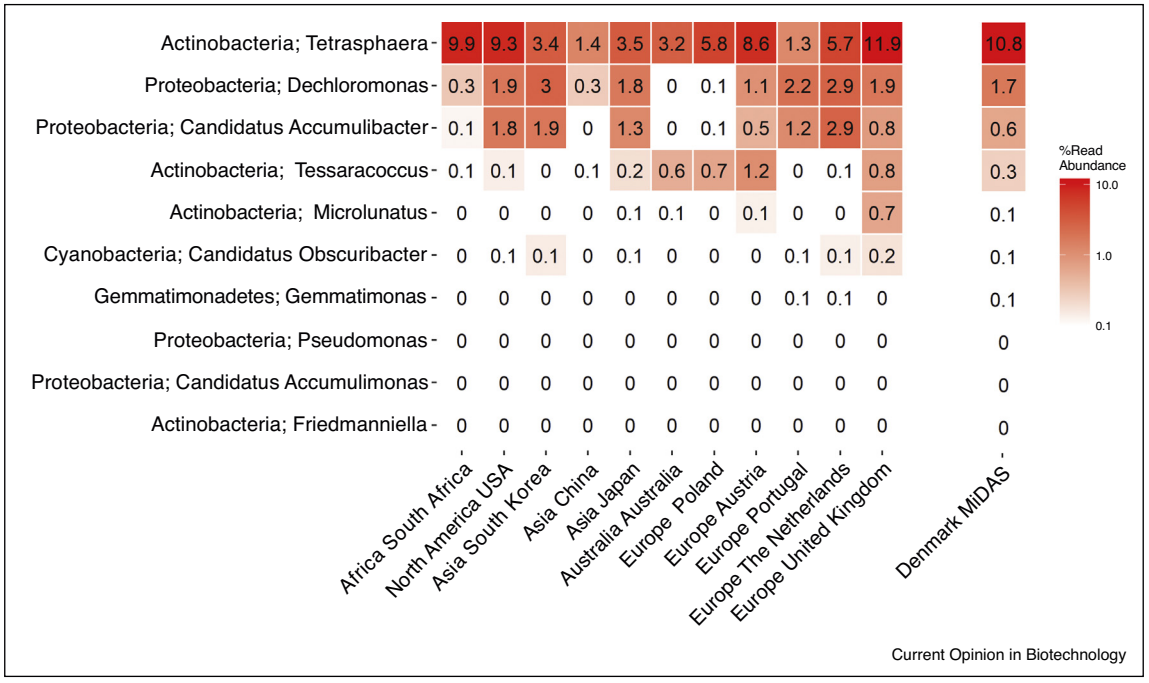
Wastewater treatment using the enhanced biological phosphorus removal (EBPR) process is becoming increasingly popular because the demand for achieving low effluent phosphorus (P) concentrations without addition of chemical precipitants is increasing across the world. The EBPR process relies on polyphosphate accumulating organisms (PAOs) which take up and store excessive amounts of P, and compared to chemical precipitation, EBPR is a sustainable, effective, and economical process [1–4]. Furthermore, it is a suitable process for recovery of P, which is a limited non-renewable resource, particularly important as fertilizer [5]. The P-rich biomass can be used directly as a fertilizer in agriculture or the P can be released as orthophosphate, often in an anaerobic digester, and precipitated, for example, as struvite, and applied as fertilizer [4].

Ever since the EBPR process was first described by James Barnard in South Africa in the 70s [6], the PAOs and their proposed competitors, the glycogen accumulating organisms (GAOs), have been intensively studied and their microbiology considered reasonably well described. Studies on organisms thought to be important for EBPR have historically focused on populations that appear to adhere to the canonical PAO and GAO phenotypes that are enriched in volatile fatty acids (VFA) fed lab-scale systems where the community composition is often not determined. However, full-scale systems provide substantially more complex substrate compositions and growth environments, providing diverse niches for organisms. Many of the proposed PAO and GAO are found to be absent or present only in low abundance in full-scale systems [7•]. Moreover, several variations of the classical phenotypes have been observed that question our common understanding of the microbiology and ecology of the EBPR process. The aim of this review is to outline the new studies and critically assess some common dogmas in EBPR microbiology related to the role of PAOs and GAOs in full-scale EBPR plants.

Which PAOs are important to full-scale EBPR?

Most studies in the field of EBPR microbiology have concentrated on the canonical model PAO *Candidatus* Accumulibacter. This genus is commonly found in full-scale plants and is relatively easy to enrich in VFA fed lab-scale reactors. As such, *Ca.* Accumulibacter has long been assumed to be the most important of the known PAOs, yet very few studies have concurrently assessed the abundance of the several populations proposed to possess the phenotype in full-scale plants [8•,9,10]. Our comprehensive surveys of more than 20 full-scale Danish wastewater treatment plants (WWTP) over several years with both Fluorescence *in situ* Hybridization (FISH) and 16S rRNA gene amplicon sequencing [7•,11] clearly show that although *Ca.* Accumulibacter is common (mean abundance 0.5%), members of the actinobacterial *Tetrasphaera* PAO are often observed in higher abundances. Our subsequent survey of 32 full-scale EBPR plants in 12 countries (covering 5 continents) (Figure 1) supports a higher abundance of *Tetrasphaera* in most plants globally. There are examples of plants where *Tetrasphaera* are absent or present in low abundance, primarily pilot-scale experiments [8•], but this could partly be due to extraction bias as this genus is underestimated by standard DNA

Figure 1



Relative read abundance of all putative PAOs [7•] in full-scale activated sludge WWTPs across 12 countries from 5 continents. Average values for each country (except Denmark) are based on 3 to 9 samples from 2 to 4 EBPR plants collected in years 2011-2014. PAO abundance in Denmark is an average of 18 full-scale WWTPs; data sourced from MiDAS survey [64].

extraction procedures [12]. Interestingly, the dominant OTUs (97% identity) from both *Tetrasphaera* and *Ca.* Accumulibacter were the same in most plants across the world. However, there may be significant microdiversity within the dominant OTUs as use of the *ppk1* gene as phylogenetic marker for *Ca.* Accumulibacter generally reveals large diversity in studies around the globe [10,13–15]. Other putative PAOs consistently found in a relatively high abundance in some plants included the genera *Dechloromonas* and *Tessaracoccus*, although their abundance appears to be overestimated by sequencing based methods (possibly due to known biases with extraction efficiency, primer choice, and copy number [12]), and they likely play a minimal role in P removal [7•]. To date, the genera *Tetrasphaera* and *Ca.* Accumulibacter appear to be the only known PAOs to be consistently found in abundances where they could be considered critical to P removal performance. However, several unknown bacteria are observed to contain excess polyphosphate (poly-P) reserves and future work should focus on their identification and characterization [16••].

Critically, P-removal in EBPR plants is not only a matter of abundance of different PAO phylotypes but also about their capability to store intracellular P. Raman microspectroscopy can be applied to identify intracellular polymers such as poly-P at a single cell level [17,18], and we recently coupled this with FISH for the absolute quantification of the intracellular content of poly-P, polyhydroxyalkanoates (PHA), and glycogen [16••]. Interestingly, the cellular content of poly-P in *Ca.* Accumulibacter was approx. 3 times higher than in *Tetrasphaera*, when considered per cell, but very similar per biovolume ($\approx 0.7 \times 10^{-13}$ gP μm^{-3}), as the cell volume of *Tetrasphaera* is 3–5 times smaller. A P-mass balance on biomass from the aeration tank, which contained the highest amount of intracellular P, showed that both *Tetrasphaera* and *Ca.* Accumulibacter made a significant contribution to P-removal. Also, an enriched lab-scale culture of *Tetrasphaera* was able to carry out at least 80% of the P-removal [19], supporting the suggestion that both *Ca.* Accumulibacter and *Tetrasphaera* are important for P-removal in full-scale EBPR plants.

Are GAOs really problematic in full-scale EBPR plants?

GAOs are thought to be a cause of EBPR failure in full-scale plants because they outcompete PAOs in lab-scale systems under a range of operational conditions (i.e. substrate type, pH, temperature, etc.) [1,3,20]. However, it is not clear if GAOs are of practical importance in full-scale systems. To the best of our knowledge, there is no well-documented case of GAO-induced full-scale EBPR problems in the literature, although anecdotal cases exist. Many studies

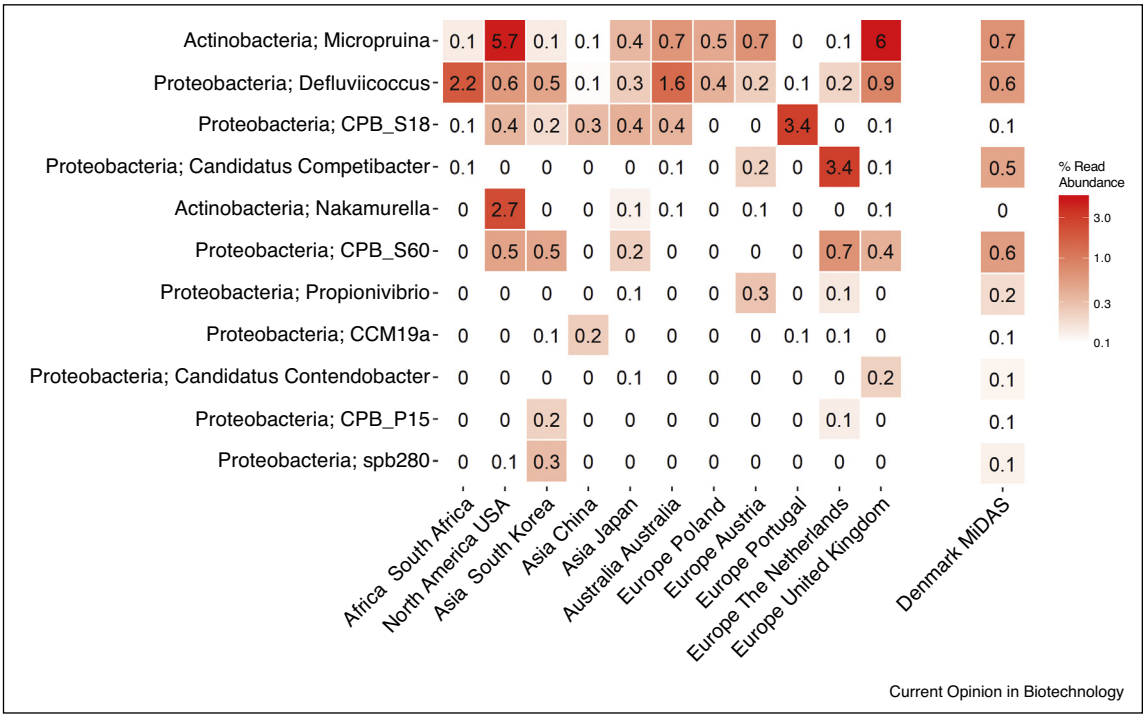
refer to the study of Saunders *et al.* [21]. However, they only showed that both *Ca.* Accumulibacter and the GAO *Ca.* Competibacter were present (the latter always in lower abundance) in 7 full-scale plants. Saunders *et al.* discuss the possibility that the GAO outcompete the PAO for available VFAs, but it is not directly shown. Several surveys have revealed that indeed some GAOs (*Ca.* Competibacter, *Defluviicoccus* and/or *Micropruina*) can be present at relatively high abundances in full-scale EBPR plants with stable performance [7•,21,22]. The work of Tu and Schuler [23] suggests that the importance of the GAOs has been overestimated due to the use of higher acetate feed concentrations in lab-scale studies compared to the concentrations present in full-scale systems. The occurrence of GAO populations may be controlled by the presence of carbon in excess of what is required for P removal, so we claim that a moderate amount of GAOs may be a good sign for efficient EBPR because they indicate a surplus of organics.

Despite the lack of evidence to suggest GAOs are responsible for the failure of EBPR, they can be abundant members of EBPR microbial communities and thus likely important in the overall carbon and nutrient transformations. Several GAOs are abundant in some plants, including members of the well-studied family *Competibacteraceae* [24] and the genus *Defluviicoccus* (Figure 2). Interestingly, the fermentative actinobacterial genus *Micropruina* was found to be the most abundant GAO in our Danish survey [7•] and is also found to be abundant globally (Figure 2).

EBPR in tropical conditions?

A well-known dogma among researchers, consultants, and the wastewater treatment industry has been that the EBPR process does not work under tropical conditions, mainly because it was believed that GAOs would outcompete PAOs. Several lab-scale studies have shown that temperatures around 30°C favor GAOs over PAOs (e.g. in Ref. [25]). For this reason, very few full-scale EPBR plants have been constructed in tropical areas. However, recent investigations have clearly shown that this assumption is incorrect. EPBR full-scale plants work very well at yearly temperatures of 28–32°C in Malaysia [14] and Singapore [26,27]. Some known GAOs co-existed in these EBPR plants but they were not present in high abundance and did not cause any recognizable problems. Moreover, studies of lab-scale reactors with biomass enriched from tropical EBPR plants support the feasibility of this process in warm climates [26,28], and show that EBPR can be used as sustainable technology in these regions.

Figure 2



Relative read abundance of all putative GAOs [7•] in full-scale activated sludge WWTPs across 12 countries from 5 continents. Average values for each country (except Denmark) are based on 3 to 9 samples from 2 to 4 EBPR plants collected in years 2011-2014. GAO abundance in Denmark is an average of 18 full-scale WWTPs; data sourced from MiDAS survey [64].

The role of meta-omic approaches in elucidating the physiology of PAOs and GAOs

Recent studies using genomics have resolved interesting features of the physiology of PAOs and GAOs. Several genomes have been recovered (Table 1) and a number of papers applying meta-omic approaches have enhanced our understanding of the PAO and GAO physiology. Genomic and expression analyses have largely confirmed the proposed classical PAO and GAO models. They have also allowed the identification of a novel quinol reductase putatively allowing anaerobic operation of the right branch of the TCA cycle by re-oxidizing quinones reduced by succinate dehydrogenase in the *Ca*. Accumulibacter [29] that is also possessed by the *Propionivibrio* GAO [30•]. Meta-omic approaches have also uncovered a possible role for hydrogenases annotated in *Ca*. Accumulibacter [31•], and lactate dehydrogenase in *Ca*. Competibacter [32], in redox balance under anaerobic conditions in these organisms.

A key question of interest is the genetic make-up of a PAO. Comparative genomics suggests the Pit transporter, involved in the generation of a proton motive force driving VFA uptake in *Ca*. Accumulibacter [33], to be vital, but not exclusive, to the PAO phenotype [32,34]. The key pathways that give rise to the PAO phenotype are likely to be explained by unresolved regulatory mechanisms, since the key pathways for glycogen, PHA, and polyphosphate cycling are widespread in many non-PAO bacteria, including some GAOs. Some PAO-specific traits seem to be related to horizontal gene transfer [35•]. We suggest that expression studies with *Ca*. Accumulibacter and the closely related non-PAO species, such as *Propionivibrio* GAO, provides an excellent opportunity to investigate this further [30•].

Genomic analyses have been particularly useful in determining the potential of PAOs and GAOs for denitrification. Several studies have tried to associate denitrification with different clades of the *Ca*. Accumulibacter [36,37]. However, the retrieval of several genomes and recent lab-scale experiments [38••,39] indicate that the use of nitrate to drive anoxic uptake of orthophosphate, cannot be assigned to specific clades, and that observed differences may also be the result of transcriptional control [40••] or physiological diversity that cannot be resolved by *ppk1* phylogeny. The *Tetrasphaera* PAO possess partial denitrification pathways and may be involved in the production of the greenhouse gas N_2O [41–43]. Several lab-scale and full-scale studies describe simultaneous anoxic N and P removal [22,37,39,44–46] and potential simultaneous aerobic denitrification and phosphorus removal by *Ca*. Accumulibacter [38••] or novel bacteria [47]. However, understanding of these processes is still limited and their importance in full-scale plants is unclear.

Among the GAO genomes sequenced thus far (Table 1), only *Ca*. Competibacter denitrificans has been shown to have the genetic potential for complete denitrification from nitrate [32]. *Ca*. Propionivibrio aalborgensis and *Micropruina glycogenica* possess the potential for both nitrate and nitrite reduction, but the latter could not utilize nitrite in pure culture studies [48,49•]. The available genomes for *Defluviicoccus* [34,50,69] and a lab-scale study with an enrichment of Cluster 1 [51], suggests an ability for some members of the genus to utilize nitrate as an electron acceptor. However, none of these genomes indicate an ability to denitrify, supporting MAR-FISH studies of full-scale systems [52].

Table 1

Complete or near-complete genomes from PAOs and GAOs from the EBPR process

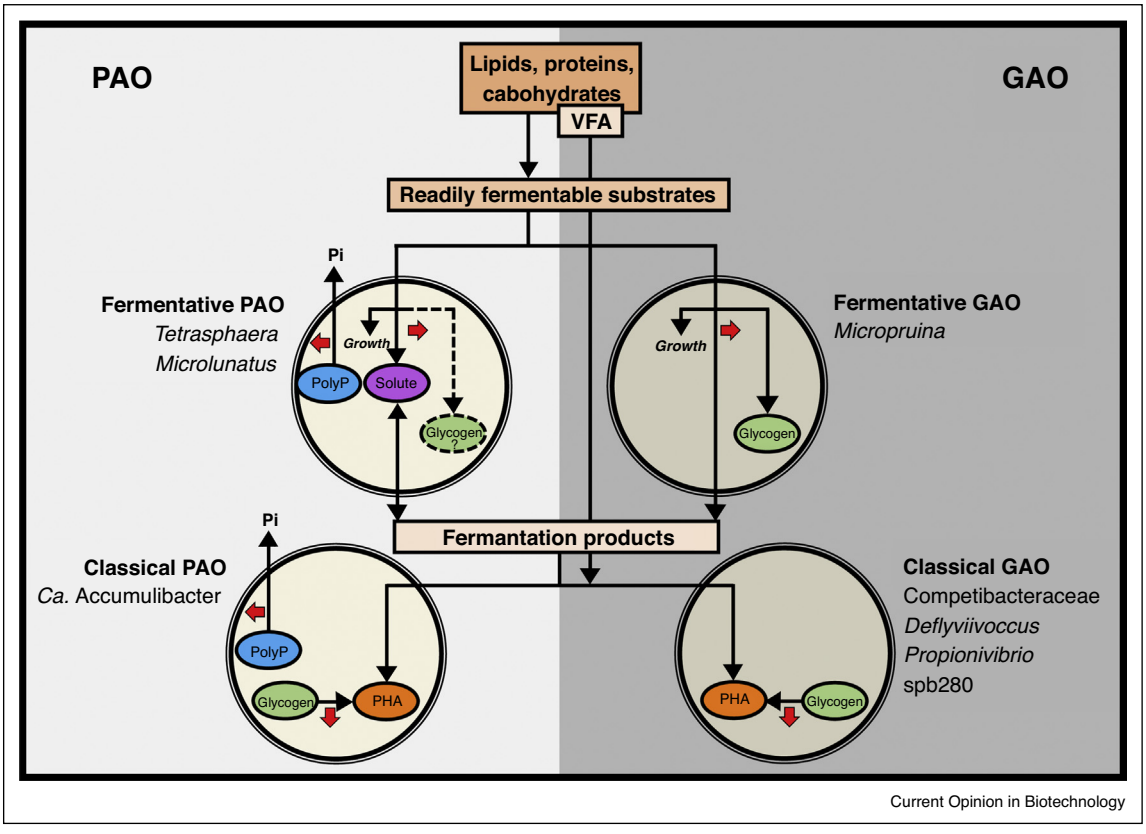
Group	Genus	Subgroup/Clade/Species	Reference
PAO	*Ca.* Accumulibacter	IIA	[29]
PAO	*Ca.* Accumulibacter	IA	[65]
PAO	*Ca.* Accumulibacter	IB	[66]
PAO	*Ca.* Accumulibacter	3xIIC, 3xIIF, IC (B), IA	[40••]
PAO	*Ca.* Accumulibacter	IIC	[67]
PAO	*Ca.* Accumulibacter	IIA	[30•]
PAO	*Ca.* Accumulibacter	IC	[38••]
PAO	*Ca.* Accumulibacter	IC	[39]
PAO	*Microlunatus*	*M. phosphovorus*	[68]
PAO	*Tetrasphaera*	*T. australiensis, T. japonica, T. elongata, T. jenkinsii*	[43]
GAO	*Ca.* Competibacter	*Ca.* C. denitrificans (formerly GB1)	[32]
GAO	*Ca.* Contendobacter	*Ca.* C. odensis (formerly GB5)	[32]
GAO	*Defluviicoccus*	*Ca.* D. tetraformis (Cluster I)	[34]
GAO	*Defluviicoccus*	Cluster II	[69]
GAO	*Defluviicoccus*	*Ca.* D. seviourii (Cluster III)	[50]
GAO	*Propionivibrio*	*Ca.* P. aalborgensis	[30•]
GAO	*Micropruina*	*M. glycogenica*	[49•]

The importance of fermenters and rethinking the interactions between PAOs and GAOs

Perhaps the biggest shift in our understanding of EBPR microbiology since the discovery of *Ca.* Accumulibacter [53–55] has been the observed importance of fermentative organisms, such as the *Tetrasphaera* PAO [43,56] and the *Micropruina* GAO [48,49•]. The *Tetrasphaera* PAO and *Micropruina* GAO possess a physiology markedly different from the canonical PAO and GAO models, respectively. Genomic and lab-studies have shown that they can grow anaerobically by fermenting amino acids and sugars without cycling PHAs, with the latter being the key storage compound of classical models [1]. The *Tetrasphaera* PAOs supplement their anaerobic energy requirements by the hydrolysis and release of aerobically stored poly-P, but do not appear to store glycogen *in situ*, despite possessing the metabolic potential for it [16••]. Instead, they are able to store high concentrations of amino acids and possibly fermentation products intracellularly in the anaerobic phase for subsequent use in the aerobic phase [57•]. This also challenges the dogma that carbon must be stored as polymers, given intracellular storage of solutes would create osmotic stress on the cell. Furthermore, *Tetrasphaera* seem well adapted to grow in dynamic conditions [58]. The *Micropruina* GAOs use the energy and carbon from fermentation for growth and the storage of glycogen. The stored glycogen may then be oxidized under aerobic conditions for growth. As they accumulate glycogen under anaerobic conditions, where excess substrate is available, without cycling poly-P, they are considered to behave as 'fermentative GAO' (fGAO) [49•]. Some classical GAOs may also ferment glycogen stores or glucose to lactate as an additional anaerobic energy source [24].

The historical understanding of EBPR microbiology has been that VFAs are made available to the PAOs and GAOs through the hydrolysis and fermentation of more complex substrates, such as carbohydrates, lipids, and proteinaceous material, by other heterotrophic organisms [59,60] (Figure 3). Instead, we have recently proposed that the fPAOs, fGAOs, and other abundant fermenters, such as the filamentous Chloroflexi '*Ca.* Promineofilum' [61] and '*Ca.* Amarolinea' [62], compete for sugars and amino acids, and the classical

Figure 3



Proposed interactions between fermentative and classical PAOs and GAOs. Other facultative anaerobic bacteria may also provide substrate to PAOs and GAOs. The PAO/GAO populations rarely exceed 5–10% of the entire community. In systems with little fermentable substrate and high VFA, the classical PAOs and GAOs will dominate. In systems with high fraction of organics (e.g. from some industries), the fermentative PAOs and GAOs may dominate. In systems with low C/P-ratio, the GAOs will be present in low abundance. Red arrows indicate production of energy. Adapted from Ref. [49•].

PAOs and GAOs compete for fermentation by-products, such as VFAs (see Figure 3). In addition, carbon stored by the fermentative organisms is not available to the classical PAOs under anaerobic conditions. Consequently, the ability of the fPAOs and fGAOs to grow under both aerobic and anaerobic conditions provides them with an advantage over classical PAO and GAO phenotypes, consistent with their higher relative abundances in Danish WWTPs [7•,63]. As discussed earlier, there are no reports to suggest that the GAOs should be a real problem in outcompeting PAOs. Instead we hypothesize that in real wastewater treatment systems, GAOs will only be abundant when VFAs or fermentable substrates are present in surplus (high C:P ratio), that is, more carbon than the highly competitive PAOs need – and that is largely determined by the amount of available phosphorus. A high proportion of VFAs in general will likely select for the classical PAO and GAO phenotypes.

Conclusion and perspectives

Several dogmas in the EBPR field have now been challenged revealing the need to rethink the EBPR microbiology in full-scale plants. Important recent shifts in our understanding include: the genus *Tetrasphaera* is as important as *Ca.* Accumulibacter for biological P removal; the diverse phylogeny and physiology of organisms storing excess P is likely not resolved; GAOs do not appear to be a real problem for the full-scale EBPR process; the process works very well under tropical conditions, and we need to rethink the PAO–GAO interactions in full-scale plants. A more robust approach going forward will be to focus on the community structures of full-scale EBPR WWTPs, identify the consistently abundant organisms, retrieve their genomes for metabolic reconstruction and omic-verification, and to track carbon flow/mass balance through these systems to identify potential points of competition for the organisms removing excess phosphorus. The MiDAS project has sought to identify the core members of EBPR [64] while methods, such as FISH-Raman, provide the ability to track carbon and P storage dynamics in individual populations *in situ*.

Conflict of interest statement

Nothing declared.

Acknowledgements

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- •• of outstanding interest

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