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

Per Halkjær Nielsen, Simon J McIlroy, Mads Albertsen and Marta Nierychlo

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

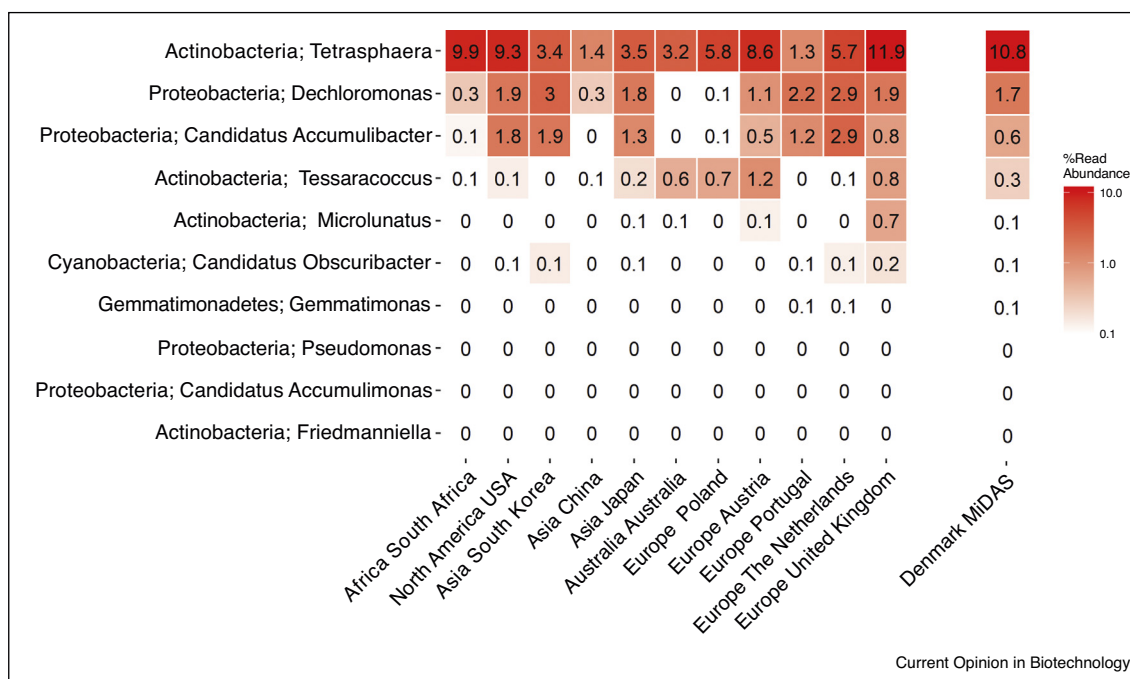
agriculture or the P can be released as orthophosphate, often in an anaerobic digester, and precipitated, for example, as struvite, and applied as fertilizer [4].

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 micro-spectroscopy 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.

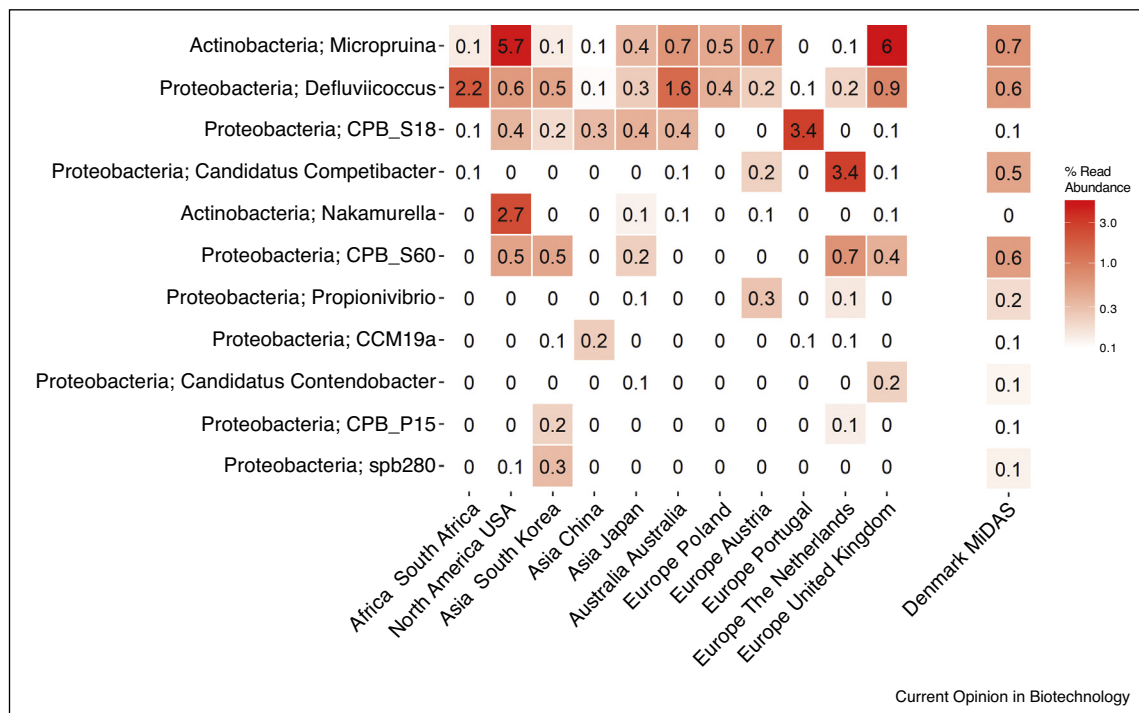
Despite the lack of evidence to suggest GAOs are responsible for the failure of EBPR, they can be abundant members of EBPR microbial communities and thus likely important in the overall carbon and nutrient transformations. Several GAOs are abundant in some plants, including members of the well-studied 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 EBPR plants have been constructed in tropical areas. However, recent investigations have clearly shown that this assumption is incorrect. EBPR 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 N₂O [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 *Deffluviococcus* [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	<i>Ca. Accumulibacter</i>	IIA	[29]
PAO	<i>Ca. Accumulibacter</i>	IA	[65]
PAO	<i>Ca. Accumulibacter</i>	IB	[66]
PAO	<i>Ca. Accumulibacter</i>	3xIIC, 3xIIF, IC (B), IA	[40 ^{**}]
PAO	<i>Ca. Accumulibacter</i>	IIC	[67]
PAO	<i>Ca. Accumulibacter</i>	IIA	[30 [*]]
PAO	<i>Ca. Accumulibacter</i>	IC	[38 ^{**}]
PAO	<i>Ca. Accumulibacter</i>	IC	[39]
PAO	<i>Microlunatus</i>	<i>M. phosphovor</i>	[68]
PAO	<i>Tetrasphaera</i>	<i>T. australiensis</i> , <i>T. japonica</i> , <i>T. elongata</i> , <i>T. jenkinsii</i>	[43]
GAO	<i>Ca. Competibacter</i>	<i>Ca. C. denitrificans</i> (formerly GB1)	[32]
GAO	<i>Ca. Contendobacter</i>	<i>Ca. C. odensis</i> (formerly GB5)	[32]
GAO	<i>Deffluviococcus</i>	<i>Ca. D. tetraformis</i> (Cluster I)	[34]
GAO	<i>Deffluviococcus</i>	Cluster II	[69]
GAO	<i>Deffluviococcus</i>	<i>Ca. D. seviourii</i> (Cluster III)	[50]
GAO	<i>Propionivibrio</i>	<i>Ca. P. aalborgensis</i>	[30 [*]]
GAO	<i>Micropruina</i>	<i>M. glycogenica</i>	[49 [*]]

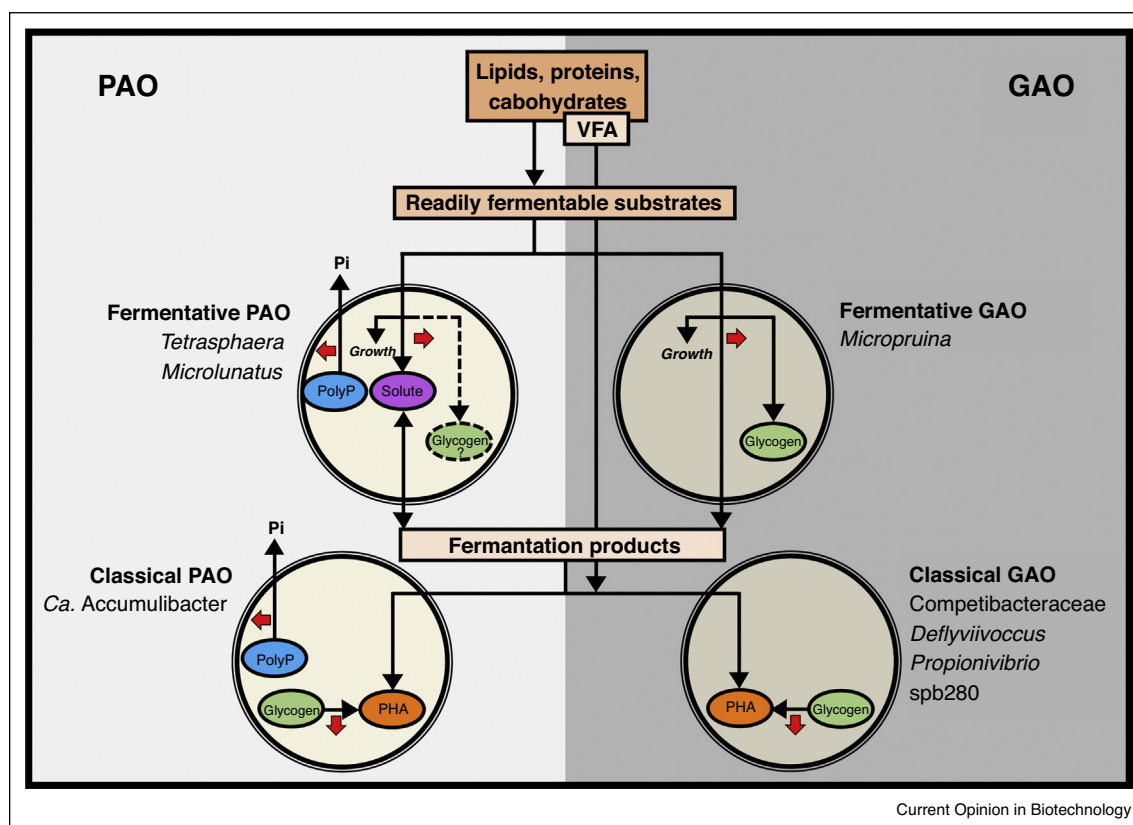
The importance of fermenters and rethinking the interactions between PAOs and GAOs

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.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Oehmen A, Lemos PC, Carvalho G, Yuan Z, Keller J, Blackall LL, Reis MAM: **Advances in enhanced biological phosphorus removal: from micro to macro scale.** *Water Res* 2007, **41**:2271–2300.

2. Seviour RJ, Mino T, Onuki M: **The microbiology of biological phosphorus removal in activated sludge systems.** *FEMS Microbiol Rev* 2003, **27**:99–127.
3. Welles L, Tian WD, Saad S, Abbas B, Lopez-Vazquez CM, Hooijmans CM, van Loosdrecht MCM, Brdjanovic D: **Accumulibacter clades Type I and II performing kinetically different glycogen-accumulating organisms metabolisms for anaerobic substrate uptake.** *Water Res* 2015, **83**:354–366.
4. Yuan Z, Pratt S, Batstone DJ: **Phosphorus recovery from wastewater through microbial processes.** *Curr Opin Biotechnol* 2012, **23**:878–883.
5. Gilbert N: **Environment: the disappearing nutrient.** *Nature* 2009, **461**:716–718.
6. Barnard JL: **A review of biological phosphorus removal in the activated sludge process.** *Water sa* 1976, **2**:136–144.
7. Stokholm-Bjerregaard M, McIlroy SJ, Nierychlo M, Karst SM, Albertsen M, Nielsen PH: **A critical assessment of the microorganisms proposed to be important to enhanced biological phosphorus removal in full-scale wastewater treatment systems.** *Front Microbiol* 2017, **8**:718.
8. Keene NA, Reusser SR, Scarborough MJ, Grooms AL, Seib M, Santo Domingo J, Noguera DR: **Pilot plant demonstration of stable and efficient high rate biological nutrient removal with low dissolved oxygen conditions.** *Water Res* 2017, **121**:72–85.
9. Law Y, Kirkegaard RH, Cokro AA, Liu X, Arumugam K, Xie C, Stokholm-Bjerregaard M, Drautz-Moses DI, Nielsen PH, Wurtz S *et al.*: **Integrative microbial community analysis reveals full-scale enhanced biological phosphorus removal under tropical conditions.** *Sci Rep* 2016, **6**:25719.
10. Zeng W, Zhang L, Fan P, Guo J, Peng Y: **Community structures and population dynamics of “Candidatus Accumulibacter” in activated sludges of wastewater treatment plants using *ppk1* as phylogenetic marker.** *J Environ Sci* 2018, **67**:237–248.
11. Mielczarek AT, Nguyen HT, Nielsen JL, Nielsen PH: **Population dynamics of bacteria involved in enhanced biological phosphorus removal in Danish wastewater treatment plants.** *Water Res* 2013, **47**:1529–1544.
12. Albertsen M, Karst SM, Ziegler AS, Kirkegaard RH, Nielsen PH: **Back to basics – the influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities.** *PLoS One* 2015, **10**:e0132783.
13. Zhang AN, Mao Y, Zhang T: **Development of quantitative real-time PCR assays for different clades of “Candidatus Accumulibacter”.** *Sci Rep* 2016, **6**:23993.
14. Ong YH, Chua ASM, Fukushima T, Ngho GC, Shoji T, Michinaka A: **High-temperature EBPR process: the performance, analysis of PAOs and GAOs and the fine-scale population study of “Candidatus Accumulibacter phosphatis”.** *Water Res* 2014, **64**:102–112.
15. Mao Y, Graham DW, Tamaki H, Zhang T: **Dominant and novel clades of “Candidatus Accumulibacter phosphatis” in 18 globally distributed full-scale wastewater treatment plants.** *Sci Rep* 2015, **5**:1–10.
16. Fernando EY, McIlroy SJ, Nierychlo M, Herbst F-A, Petriglieri F, Schmid MC, Wagner M, Lund Nielsen J, Nielsen PH: **Resolving the individual contribution of key microbial populations to enhanced biological phosphorus removal with Raman-FISH.** *ISME J* 2019 <http://dx.doi.org/10.1038/s41396-019-0399-7>.

A new method for quantification of intracellular polymers (poly-P, PHA, and glycogen) by Raman microspectroscopy combined with FISH reveals that both *Ca. Accumulibacter* and *Tetrasphaera* are important PAOs in 8 full-scale plants EBPR. The dynamics of intracellular pools of polymers were analysed in the different process tanks.

17. Majed N, Matthäus C, Diem M, Gu AZ: **Evaluation of intracellular polyphosphate dynamics in enhanced biological phosphorus removal process using Raman microscopy.** *Environ Sci Technol* 2009, **43**:5436-5442.
 18. Li Y, Cope HA, Rahman SM, Li G, Nielsen PH, Elfick APD, Gu AZ: **Towards better understanding of EBPR systems via linking Raman-based phenotypic profiling with phylogenetic diversity.** *Environ Sci Technol* 2018, **52**:8596-8606.
 19. Marques R, Santos J, Nguyen H, Carvalho G, Noronha JP, Nielsen PH, Reis MAM, Oehmen A: **Metabolism and ecological niche of *Tetrasphaera* and *Ca. Accumulibacter* in enhanced biological phosphorus removal.** *Water Res* 2017, **122**:159-171.
 20. Shen N, Zhou Y: **Enhanced biological phosphorus removal with different carbon sources.** *Appl Microbiol Biotechnol* 2016, **100**:4735-4745.
 21. Saunders AM, Oehmen A, Blackall LL, Yuan Z, Keller J: **The effect of GAOs (glycogen accumulating organisms) on anaerobic carbon requirements in full-scale Australian EBPR (enhanced biological phosphorus removal) plants.** *Water Sci Technol* 2003, **47**:37-43.
 22. Lanham AB, Oehmen A, Saunders AM, Carvalho G, Nielsen PH, Reis MAM: **Metabolic versatility in full-scale wastewater treatment plants performing enhanced biological phosphorus removal.** *Water Res* 2013, **47**:7032-7041.
 23. Tu Y, Schuler AJ: **Low acetate concentrations favor polyphosphate-accumulating organisms over glycogen-accumulating organisms in enhanced biological phosphorus removal from wastewater.** *Environ Sci Technol* 2013, **47**:3816-3824.
 24. McIlroy SJ, Nittami T, Kanai E, Fukuda J, Saunders AM, Nielsen PH: **Re-appraisal of the phylogeny and fluorescence *in situ* hybridization probes for the analysis of the *Competibacteraceae* in wastewater treatment systems.** *Environ Microbiol Rep* 2015, **7**:166-174.
 25. Lopez-Vazquez CM, Oehmen A, Hooijmans CM, Brdjanovic D, Gijzen HJ, Yuan Z, van Loosdrecht MCM: **Modeling the PAO-GAO competition: effects of carbon source, pH and temperature.** *Water Res* 2009, **43**:450-462.
 26. Cokro AA, Law Y, Williams RBH, Cao Y, Nielsen PH, Wuertz S: **Non-denitrifying polyphosphate accumulating organisms obviate requirement for anaerobic condition.** *Water Res* 2017, **111**:393-403.
 27. Qiu G, Zuniga-Montanez R, Law Y, Thi SS, Nguyen TQN, Eganathan K, Liu X, Nielsen PH, Williams RBH, Wuertz S: **Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources.** *Water Res* 2019, **149**:496-510.
 28. Shen N, Chen Y, Zhou Y: **Multi-cycle operation of enhanced biological phosphorus removal (EBPR) with different carbon sources under high temperature.** *Water Res* 2017, **114**:308-315.
 29. Martín HG, Ivanova N, Kunin V, Warnecke F, Barry KW, McHardy AC, Yeates C, He S, Salamov AA, Szeto E *et al.*: **Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities.** *Nat Biotechnol* 2006, **24**:1263-1269.
 30. Albertsen M, McIlroy SJ, Stokholm-Bjerregaard M, Karst SM, Nielsen PH: **"*Candidatus Propionivibrio aalborgensis*": a novel glycogen accumulating organism abundant in full-scale enhanced biological phosphorus removal plants.** *Front Microbiol* 2016, **7**:1033.
- The paper describes the discovery of a very close relative to *Ca. Accumulibacter* - *Ca. Propionivibrio*, and shows that it is a GAO that is abundant in some full-scale plants, and likely also many lab-scale reactors. Furthermore, the study shows that it is targeted by the commonly applied FISH probes for *Ca. Accumulibacter* - the PAOmix.
31. Oyserman BO, Noguera DR, Glavina Del Rio T, Tringe SG, McMahon KD: **Metatranscriptomic insights on gene expression and regulatory controls in *Candidatus Accumulibacter phosphatis*.** *ISME J* 2015, **10**:810-822.
- A time series metatranscriptome generated from enrichment cultures of *Ca. Accumulibacter* provided a basis and framework for detailed investigations of the metabolism and regulatory networks in *Ca. Accumulibacter* and other uncultured organisms.
32. McIlroy SJ, Albertsen M, Andresen EK, Saunders AM, Kristiansen R, Stokholm-Bjerregaard M, Nielsen KL, Nielsen PH: **"*Candidatus Competibacter*"-lineage genomes retrieved from metagenomes reveal functional metabolic diversity.** *ISME J* 2014, **8**:613-624.
 33. Saunders AM, Mabbett AN, McEwan AG, Blackall LL: **Proton motive force generation from stored polymers for the uptake of acetate under anaerobic conditions.** *FEMS Microbiol Lett* 2007, **274**:245-251.
 34. Nobu MK, Tamaki H, Kubota K, Liu WT: **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* 2014, **16**:2739-2751.
 35. Oyserman BO, Moya F, Lawson CE, Garcia AL, Vogt M, Heffernan M, Noguera DR, McMahon KD: **Ancestral genome reconstruction identifies the evolutionary basis for trait acquisition in polyphosphate accumulating bacteria.** *ISME J* 2016, **10**:2931-2945.
- A comprehensive investigation on the evolution of the PAO phenotype using *Ca. Accumulibacter phosphatis* showed that several key genes for the PAO phenotype are likely obtained by horizontal gene transfer. These were including genes involved in glycogen, polyhydroxyalkanoate, pyruvate and NADH/NADPH metabolism, as well as inorganic ion transport and regulatory mechanisms.
36. Saad SA, Welles L, Abbas B, Lopez-Vazquez CM, van Loosdrecht MCM, Brdjanovic D: **Denitrification of nitrate and nitrite by '*Candidatus Accumulibacter phosphatis*' clade IC.** *Water Res* 2016, **105**:97-109.
 37. Rubio-Rincón FJ, Lopez-Vazquez CM, Welles L, van Loosdrecht MCM, Brdjanovic D: **Cooperation between *Candidatus Competibacter* and *Candidatus Accumulibacter* clade I, in denitrification and phosphate removal processes.** *Water Res* 2017, **120**:156-164.
 38. Camejo PY, Oyserman BO, McMahon KD, Noguera DR: **Integrated omic analyses provide evidence that a "*Candidatus Accumulibacter phosphatis*" strain performs denitrification under microaerobic conditions.** *mSystems* 2019, **4**:e00193-18.
- A lab-scale omic study of *Ca. Accumulibacter* with a complete denitrifying pathway provides strong evidence for simultaneous use of oxygen and nitrate as electron acceptors under micro-aerobic conditions. A transcriptional level regulation of the expression of both denitrifying and respiratory pathways seems to take place.
39. Rubio-Rincón FJ, Lopez-Vazquez CM, Welles L, Weissbrodt DG, Abbas B, Albertsen M, Nielsen PH, van Loosdrecht MCM, Brdjanovic D: ***Candidatus Accumulibacter* sp. IHE1: a novel PAO without denitrifying activity on nitrate.** *Water Res* 2018. in press.
 40. Skennerton CT, Barr JJ, Slater FR, Bond PL, Tyson GW: **Expanding our view of genomic diversity in *Candidatus Accumulibacter* clades.** *Environ Microbiol* 2015, **17**:1574-1585.
- Several novel population genomes of *Ca. Accumulibacter* were recovered, and comparative genomics revealed a core of shared genes involved primarily in carbon and phosphorus metabolism. A major difference between the clades was the type of nitrate reductase encoded and the capacity to perform subsequent steps in denitrification.
41. Marques R, Ribera-Guardia A, Santos J, Carvalho G, Reis MAM, Pijuan M, Oehmen A: **Denitrifying capabilities of *Tetrasphaera* and their contribution towards nitrous oxide production in enhanced biological phosphorus removal processes.** *Water Res* 2018, **137**:262-272.
 42. Ribera-Guardia A, Marques R, Arangio C, Carvalheira M, Oehmen A, Pijuan M: **Distinctive denitrifying capabilities lead to differences in N₂O production by denitrifying polyphosphate accumulating organisms and denitrifying glycogen accumulating organisms.** *Bioresour Technol* 2016, **219**:106-113.
 43. Kristiansen R, Nguyen HTT, Saunders AM, Nielsen JL, Wimmer R, Le VQ, McIlroy SJ, Petrovski S, Seviour RJ, Calteau A *et al.*: **A metabolic model for members of the genus *Tetrasphaera* involved in enhanced biological phosphorus removal.** *ISME J* 2013, **7**:543-554.

44. Wang Y-K, Pan X-R, Geng Y-K, Sheng G-P: **Simultaneous effective carbon and nitrogen removals and phosphorus recovery in an intermittently aerated membrane bioreactor integrated system.** *Sci Rep* 2015, **5**:16281.
 45. Vieira A, Ribera-Guardia A, Marques R, Barreto Crespo MT, Oehmen A, Carvalho G: **The link between the microbial ecology, gene expression, and biokinetics of denitrifying polyphosphate-accumulating systems under different electron acceptor combinations.** *Appl Microbiol Biotechnol* 2018, **102**:6725-6737.
 46. Figdore BA, Stensel HD, Winkler MKH: **Comparison of different aerobic granular sludge types for activated sludge nitrification bioaugmentation potential.** *Bioresour Technol* 2018, **251**:189-196.
 47. Liu S, Chen Q, Ma T, Wang M, Ni J: **Genomic insights into metabolic potentials of two simultaneous aerobic denitrification and phosphorus removal bacteria, *Achromobacter* sp. GAD3 and *Agrobacterium* sp. LAD9.** *FEMS Microbiol Ecol* 2018, **94**.
 48. Shintani T, Liu WT, Hanada S, Kamagata Y, Miyaoka S, Suzuki T, Nakamura K: ***Micropruina glycogenica* gen. nov., sp. nov., a new Gram-positive glycogen-accumulating bacterium isolated from activated sludge.** *Int J Syst Evol Microbiol* 2000, **50**:201-207.
 49. Mclroy SJ, Onetto CA, Mclroy B, Herbst FA, Dueholm MS, Kirkegaard RH, Fernando E, Karst SM, Nierychlo M, Kristensen JM *et al.*: **Genomic and *in situ* analyses reveal the *Micropruina* spp. as abundant fermentative glycogen accumulating organisms in enhanced biological phosphorus removal systems.** *Front Microbiol* 2018, **9**:1004.
- Microorganisms belonging to the genus *Micropruina* were the most abundant GAO in Danish EBPR plants. Genomic and metabolomic studies were performed on *Micropruina glycogenica* str. Lg2T showing that the physiology is markedly different from the classical GAO model and, as fermenters, they compete for substrates with the *Tetrasphaera* PAO.
50. Onetto CA, Grbin PR, Mclroy SJ, Eales KL: **Genomic insights into the metabolism of '*Candidatus Defluviicoccus seviourii*', a member of *Defluviicoccus* cluster III abundant in industrial activated sludge.** *FEMS Microbiol Ecol* 2019, **95**.
 51. Wang X, Zeng RJ, Dai Y, Peng Y, Yuan Z: **The denitrification capability of cluster 1 *Defluviicoccus vanus*-related glycogen-accumulating organisms.** *Biotechnol Bioeng* 2008, **99**:1329-1336.
 52. Burow LC, Kong Y, Nielsen JL, Blackall LL, Nielsen PH: **Abundance and ecophysiology of *Defluviicoccus* spp., glycogen-accumulating organisms in full-scale wastewater treatment processes.** *Microbiology* 2007, **153**:178-185.
 53. Zhou Y, Pijuan M, Zeng RJ, Yuan Z: **Involvement of the TCA cycle in the anaerobic metabolism of polyphosphate accumulating organisms (PAOs).** *Water Res* 2009, **43**:1330-1340.
 54. Hesselmann RPX, Werlen C, Hahn D, van der Meer JR, Zehnder AJB: **Enrichment, phylogenetic analysis and detection of a bacterium that performs enhanced biological phosphate removal in activated sludge.** *Syst Appl Microbiol* 1999, **22**:454-465.
 55. Crocetti GR, Hugenholtz P, Bond PL, Schuler A, Keller J, Jenkins D, Blackall LL: **Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation.** *Appl Environ Microbiol* 2000, **66**:1175-1182.
 56. Kong Y, Nielsen JL, Nielsen PH: **Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants.** *Appl Environ Microbiol* 2005, **71**:4076-4085.
 57. Nguyen HTT, Kristiansen R, Vestergaard M, Wimmer R, Nielsen PH: **Intracellular accumulation of glycine in polyphosphate-accumulating organisms in activated sludge, a novel storage mechanism under dynamic anaerobic-aerobic conditions.** *Appl Environ Microbiol* 2015, **81**:4809-4818.
- The study revealed that *Tetrasphaera* is able to take up and accumulate glycine under anaerobic conditions without producing PHA. Subsequent oxidation took place along with uptake of orthophosphate in both pure culture of *T. elongata* and FISH probe-defined *Tetrasphaera* in activated sludge.
58. Herbst F-A, Dueholm MS, Wimmer R, Nielsen PH: **The proteome of *Tetrasphaera elongata* is adapted to changing conditions in wastewater treatment plants.** *bioRxiv* 2019 <http://dx.doi.org/10.1101/513143>.
 59. Kong Y, Xia Y, Nielsen PH: **Activity and identity of fermenting microorganisms in full-scale biological nutrient removing wastewater treatment plants.** *Environ Microbiol* 2008, **10**:2008-2019.
 60. Henze M, International Water Association: **Task group on mathematical modelling for design and operation of biological wastewater treatment.** In *Activated Sludge Models ASM1, ASM2, ASM2d and ASM3*. Edited by Gujer W, Mino T, van Loosdrecht MCM. IWA Pub; 2000.
 61. Mclroy SJ, Karst SM, Nierychlo M, Dueholm MS, Albertsen M, Kirkegaard RH, Seviour RJ, Nielsen PH: **Genomic and *in situ* investigations of the novel uncultured Chloroflexi associated with 0092 morphotype filamentous bulking in activated sludge.** *ISME J* 2016, **10**:2223-2234.
 62. Andersen MH, Mclroy SJ, Nierychlo M, Nielsen PH, Albertsen M: **Genomic insights into *Candidatus Amarolinea aalborgensis* gen. nov., sp. nov., associated with settleability problems in wastewater treatment plants.** *Syst Appl Microbiol* 2019, **42**:77-84.
 63. Nielsen JL, Nguyen H, Meyer RL, Nielsen PH: **Identification of glucose-fermenting bacteria in a full-scale enhanced biological phosphorus removal plant by stable isotope probing.** *Microbiol (United Kingdom)* 2012, **158**:1818-1825.
 64. Mclroy SJ, Saunders AM, Albertsen M, Nierychlo M, Mclroy B, Hansen AA, Karst SM, Nielsen JL, Nielsen PH: **MidAS: the field guide to the microbes of activated sludge.** *Database* 2015, **2015**:bav062.
 65. Flowers JJ, He S, Malfatti S, del Rio TG, Tringe SG, Hugenholtz P, McMahon KD: **Comparative genomics of two '*Candidatus Accumulibacter*' clades performing biological phosphorus removal.** *ISME J* 2013, **7**:2301-2314.
 66. Mao Y, Yu K, Xia Y, Chao Y, Zhang T: **Genome reconstruction and gene expression of '*Candidatus Accumulibacter phosphatis*' Clade IB performing biological phosphorus removal.** *Environ Sci Technol* 2014, **48**:10363-10371.
 67. Mao Y, Wang Z, Li L, Jiang X, Zhang X, Ren H, Zhang T: **Exploring the shift in structure and function of microbial communities performing biological phosphorus removal.** *PLoS One* 2016, **11**:e0161506.
 68. Kawakoshi A, Nakazawa H, Fukada J, Sasagawa M, Katano Y, Kamagata Y, Nakamura K, Yamazaki S, Fujita N: **Deciphering the genome of polyphosphate accumulating Actinobacterium *Microtholunatus phosphovorius*.** *DNA Res* 2012, **19**:383-394.
 69. Wang Z, Guo F, Mao Y, Xia Y, Zhang T: **Metabolic characteristics of a glycogen-accumulating organism in *Defluviicoccus* cluster II revealed by comparative genomics.** *Microb Ecol* 2014, **68**:716-728.