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Review

Demystifying polyphosphate-accumulating organisms relevant to wastewater treatment: A review of their phylogeny, metabolism, and detection



Lucia Ruiz-Haddad ^{a, b}, Muhammad Ali ^c, Mario Pronk ^d, Mark C.M. van Loosdrecht ^d, Pascal E. Saikaly ^{a, b, *}

^a Environmental Science and Engineering Program, Biological and Environmental Science and Engineering (BESE) Division, King Abdullah University of Science and Technology (KAUST), Thuwal, 23955-6900, Saudi Arabia

^b Water Desalination and Reuse Center, Biological and Environmental Science and Engineering (BESE) Division, King Abdullah University of Science and Technology (KAUST), Thuwal, 23955-6900, Saudi Arabia

^c Department of Civil, Structural & Environmental Engineering, Trinity College Dublin, The University of Dublin, Dublin, 2, Ireland

^d Department of Biotechnology, Delft University of Technology, Delft, 2629 HZ, the Netherlands

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ABSTRACT

Currently, the most cost-effective and efficient method for phosphorus (P) removal from wastewater is enhanced biological P removal (EPBR) via polyphosphate-accumulating organisms (PAOs). This study integrates a literature review with genomic analysis to uncover the phylogenetic and metabolic diversity of the relevant PAOs for wastewater treatment. The findings highlight significant differences in the metabolic capabilities of PAOs relevant to wastewater treatment. Notably, *Candidatus* Dechloromonas and *Candidatus* Accumulibacter can synthesize polyhydroxyalkanoates, possess specific enzymes for ATP production from polyphosphate, and have electrochemical transporters for acetate and C4-dicarboxylates. In contrast, *Tetrasphaera*, *Candidatus* Phosphoribacter, *Knoellia*, and *Phycococcus* possess PolyP-glucokinase and electrochemical transporters for sugars/amino acids. Additionally, this review explores various detection methods for polyphosphate and PAOs in activated sludge wastewater treatment plants. Notably, FISH-Raman spectroscopy emerges as one of the most advanced detection techniques. Overall, this review provides critical insights into PAO research, underscoring the need for enhanced strategies in biological phosphorus removal.

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1. Introduction

Before the Industrial Revolution, erosion was the main source of phosphorus (P) in water bodies. However, as time progressed, wastewater and agricultural run-off have become the primary source of phosphorus pollution due to the disposal of fertilizers, detergents, and human waste [1]. Unfortunately, this has led to eutrophication, where dissolved P stimulates the growth of microorganisms and aquatic plant life, reducing dissolved oxygen

(DO) and biodiversity [2]. To prevent eutrophication, regulatory limits have been established for the amount of P present in the effluent of wastewater treatment plants (WWTPs) discharged into water bodies. For instance, the European Environment Agency requires an 80% reduction in P release or a limit of 1–2 mg L⁻¹ in the effluent of WWTPs [3]. Meanwhile, the U.S. Environmental Protection Agency mandates an effluent P level of less than 1 mg L⁻¹ in WWTPs [4]. To achieve these standards, various chemical and biological methods have been developed and implemented for the removal of P from wastewater [5].

Currently, the most cost-effective and efficient method for P removal from wastewater is enhanced biological P removal (EPBR) [6,7]. In the EBPR process, bacteria uptake P from their environment for growth and metabolism [8]. This process, known as P assimilation, contributes to nearly 50% of the total P removal in WWTPs

* Corresponding author. Environmental Science and Engineering Program, Water Desalination and Reuse Center, Biological and Environmental Science and Engineering Division, King Abdullah University of Science and Technology, Thuwal, 23955 Saudi Arabia

E-mail address: pascal.saikaly@kaust.edu.sa (P.E. Saikaly).

[8]. However, to achieve higher removal of P, the presence of bacteria possessing the ability to not only assimilate P but also to uptake an excess amount of P beyond their assimilatory demand is essential. In this regard, polyphosphate-accumulating organisms (PAOs) play a critical role in P removal due to their capacity to store P in the form of polyphosphate (PolyP). Traditionally, the activity of PAOs encompasses P uptake under aerobic conditions to synthesize PolyP, and in the anaerobic phase, PolyP is hydrolyzed, and P is released for energy generation and substrate uptake [9].

The relevance of PAOs in EBPR has led to extensive research focusing on uncovering new PAOs and gaining a deeper understanding of their metabolic capacities and phylogenetic relationships [10–15]. Furthermore, several review articles have provided valuable insights into various aspects of PAOs, including their distribution in diverse ecosystems [1], the influence of operational conditions on their performance [16], the mechanisms by which they derive reducing power in anaerobic metabolism [17], and a general overview of the metabolic capabilities of *Ca. Accumulibacter*, *Ca. Dechloromonas*, and *Tetrasphaera* [16]. Additionally, other reviews have explored techniques for enriching and isolating PAOs [18], methods for detecting PolyP in microbial cells [19], and methodologies for characterizing P species in natural environments [20]. However, existing reviews do not cover the discovery of novel PAOs, such as *Ca. Phosphoribacter*, nor do they properly discuss the reclassification of *Tetrasphaera* into *Ca. Phosphoribacter*, *Knoellia*, and *Phycococcus*. Furthermore, there is a noticeable lack of emphasis on the existing methodologies for detecting and targeting PAOs in sludge from WWTPs.

Thus, it is crucial to have an updated review that thoroughly covers the phylogenetic and metabolic diversity of all existing wastewater treatment (WWT)-relevant PAOs, along with the latest detection methods. To address these gaps, this review integrates for the first time existing literature with metagenome-assembled genomes (MAGs) analysis of WWT-relevant PAOs, including *Ca. Accumulibacter*, *Ca. Dechloromonas*, *Tetrasphaera*, *Ca. Phosphoribacter*, *Knoellia*, and *Phycococcus*. This approach allows for exploring functional capabilities not explicitly described in previous reviews and facilitates comparisons of relevant metabolisms across different species and genera. Moreover, the study provides a comprehensive discussion of past and recent detection methods for PAOs, including a detailed list of probes designed for targeting PAOs, PolyP detection methods, and methodologies for identifying PAO activity in complex samples. The key motivation behind this review is to fill the knowledge gaps in WWT-relevant PAOs by comprehensively exploring their phylogenetic diversity, metabolic capabilities, and the latest detection methods.

2. Phylogenetic diversity of PAOs

PAOs have a remarkable ability to store unusually large quantities of PolyP, making them crucial for efficiently removing P in EBPR WWTPs. Among all reported PAOs (Fig. 1), *Ca. Accumulibacter*, *Ca. Dechloromonas*, and *Tetrasphaera* were identified as the most significant PAOs, primarily due to their high frequencies and abundance in a global sampling of WWTPs [21].

Ca. Accumulibacter was the first PAO discovered, and subsequently, this genus was further classified into type I or II, with clades ranging from A to C in type I and A to G in type II (Fig. 1), based on the *ppk1* gene [22,23]. Later, in 2023, the novel *Ca. Accumulibacter* genomes were classified as Type III [24]. Although these organisms have been reported exclusively in lab-scale bioreactors and thus are currently not considered in the analysis of WWT-relevant PAOs, it is essential to highlight their remarkable ability to utilize dimethyl sulfoxide for energy production [24]. Consequently, there is a potential to intentionally introduce or

naturally enrich Type III *Ca. Accumulibacter* organisms in full-scale WWTPs that manage high concentrations of both dimethyl sulfoxide and P [24].

In 2000, Maszenan and colleagues discovered three *Actinobacteria* isolates capable of accumulating PolyP, giving *Tetrasphaera* as their genus name [23]. *Tetrasphaera* was initially composed of eight species: *australiensis* (strains Ben 109 and Ben 110), *japonica* (strain T1-X7) [23], *elongata* (strain Lp2 and ASP12) [25,26], *jenkinsii*, *veronensis*, *vanveenii* [27], *duodecadis* [23], and *remsis* [28]. Later, during the study of WWTPs, novel species of *Tetrasphaera* were described, and as a result, this genus was classified into three clades [29]. Clades 1 and 2 encompassed the eight species mentioned above, while clade 3 comprised only environmental sequences [29].

However, recent phylogenetic analyses resulted in the reclassification of the different species, with *Tetrasphaera elongata* and *Tetrasphaera duodecadis* being moved into the genus *Phycococcus*, and *Tetrasphaera remsis* is now classified as *Knoellia remsis* [30,31]. Furthermore, the *Tetrasphaera* classified as clade 3, now belong to two distinct genera: *Ca. Phosphoribacter* (*Ca. Phosphoribacter baldrii* and *Ca. Phosphoribacter hodrii*) and *Ca. Lutibacillus* [14]. Lastly, two new species were introduced to clades 1 and 2, with *Tetrasphaera hjor* (*Tetrasphaera midas_s_299*) being integrated into clade 1, and *Tetrasphaera aved* (*Tetrasphaera midas_s_1404*) becoming part of clade 2 [14]. Fig. 1 illustrates the most recent categorization of these organisms; where green lines represent all the taxa previously categorized as *Tetrasphaera*.

Finally, the ability of some members of the genus *Dechloromonas* to accumulate PolyP was first reported in 2005 [32]. Recently, strains belonging to two species of *Dechloromonas* are well-identified as PAOs; these include *Ca. Dechloromonas phosphoritropha* and *Ca. Dechloromonas phosphorivorans* [12]. Notably, most WWT-relevant PAOs are classified as Candidatus since they have not been cultured yet, encompassing *Ca. Accumulibacter*, *Ca. Phosphoribacter*, and the two *Dechloromonas* species (*Ca. Dechloromonas phosphoritropha* and *Ca. Dechloromonas phosphorivorans*) [12,14].

3. Metabolic diversity of PAOs

In this section, we gathered publicly available amino acid sequences of MAGs from WWT-relevant PAOs. These sequences were collected from the National Center for Biotechnology Information (NCBI) database as of May 2023 (Table S1). Subsequently, functional gene annotation for each MAG was performed on these sequences using BlastKoala [33], which assigned Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) identifiers based on sequence similarity. Finally, all MAGs were analyzed to assess the presence or absence of a specifically targeted KEGG ID in each PAO. However, it is important to know that such analyses may be affected by various biological and bioinformatic factors that could lead to conflicting information. Examples of biological errors include DNA loss due to sample collection, storage, and DNA extraction procedures. While bioinformatic errors can result from sequencing, assembly, binning, and annotation [34]. Moreover, it is worth noting that our analysis aligns with the findings reported in existing literature, where researchers have examined the presence and absence of some studied genes. This literature includes *Ca. Accumulibacter* [13], *Ca. Dechloromonas* [12], *Tetrasphaera* [14], *Ca. Phosphoribacter* [14], and *Phycococcus* [14].

In terms of general metabolism, *Ca. Accumulibacter*, *Ca. Dechloromonas*, and *Ca. Phosphoribacter* possess the capacity for Glycolysis through the Embden-Meyerhof pathway. In contrast, *Tetrasphaera*, *Knoellia*, and *Phycococcus* lack the 6-phosphofructokinase or ADP-dependent phosphofructokinase

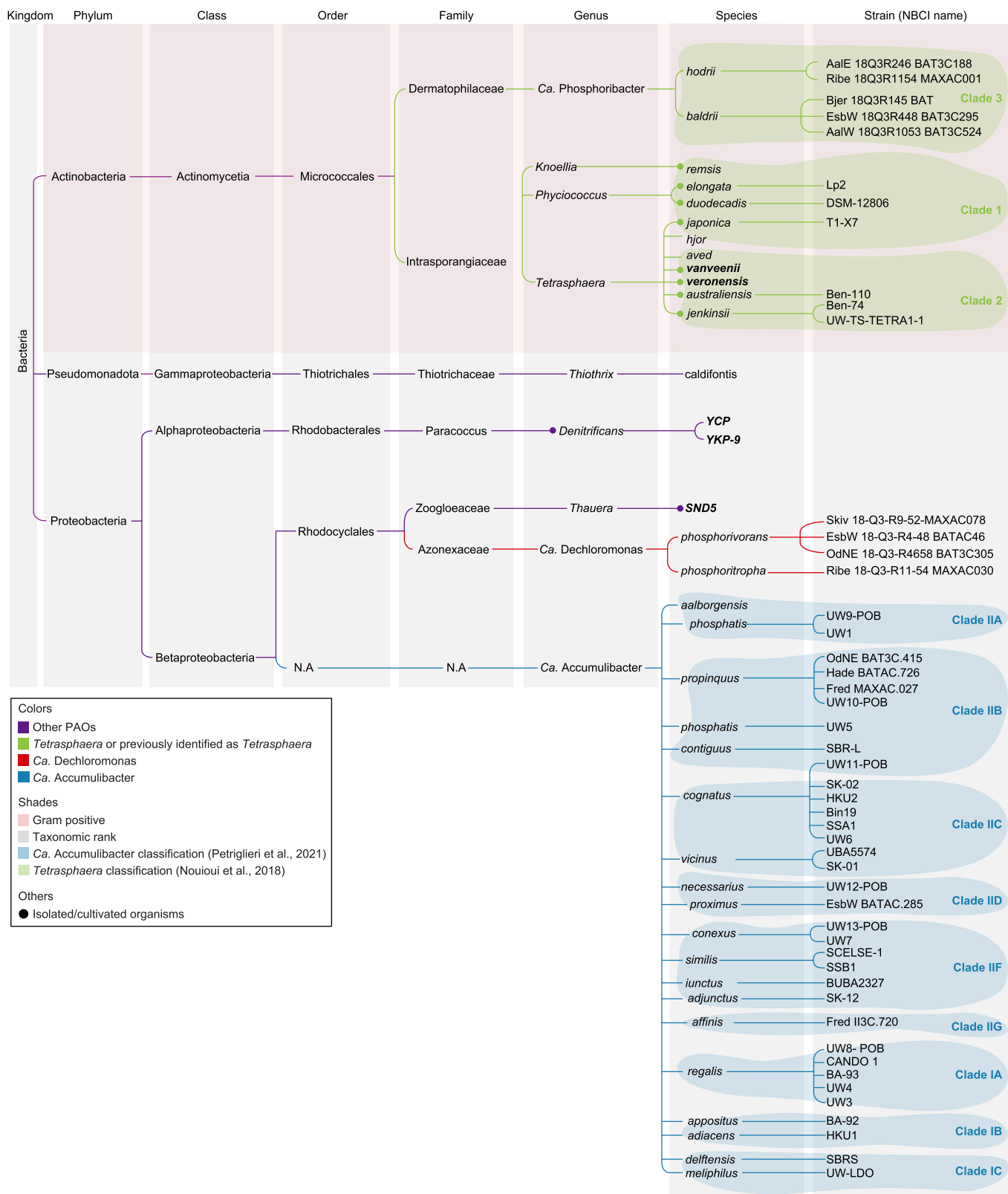


Fig. 1. This tree visually represents the taxonomic classification of organisms displaying PAO activity (nine genera). This classification is based on information from the NCBI taxonomy database. Isolated and cultured organisms are marked with dots. Additionally, most genomes are accessible on NCBI, except for those highlighted in bold text, which only have a partial 16S rRNA gene available.

enzyme that is essential for phosphorylating D-Fructose-6P to D-Fructose-1,6P in the Embden-Meyerhof pathway. The fact that *Tetrasphaera*, *Knoellia*, and *Phycococcus* lack this enzyme underscores the necessity for these organisms to evolve alternative pathways or mechanisms to compensate for this deficiency in carbon metabolism.

Furthermore, none of the PAOs can undergo assimilatory nitrate or nitrite reduction (Table S2), meaning they cannot incorporate the nitrogen from these molecules into cellular components for growth

and maintenance. However, all PAOs possess the metabolic capacity for pyruvate oxidation, citrate cycle (TCA cycle or Krebs cycle), pentose phosphate pathway, and ribose-P rypophosphokinase (PRPP) biosynthesis. Moreover, the following section will provide a more comprehensive analysis of the relevant metabolism for PAOs, encompassing PolyP synthesis and hydrolysis, transport capabilities, glycogen metabolism, PHA (Polyhydroxyalkanoates) metabolism, fermentation, and denitrification capability.

Table 1
Genes involved in PolyP metabolism in WWT-relevant PAOs. Green indicates the presence of a gene, while red denotes its absence. Complete gene names and KEGG nomenclature are available in Supplementary Materials (Table S8); Microorganism accession numbers (NCBI) are available in Supplemental Materials (Table S1).

Enzymes					Transporters								
<i>ppk1</i>	<i>ppk2</i>	<i>ppx</i>	<i>pap</i>	<i>ppgk</i>	<i>pit</i>	<i>pstS</i>	<i>pstA</i>	<i>pstC</i>	<i>pstB</i>				
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	IIC	Ca. Accumulibacter phosphatis Bin19	Type and Clades classification for Ca. Accumulibacter	
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter phosphatis HKU2		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter cognatus SSA1		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter cognatus SK-02		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter UW6		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter UW11-POB		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter vicinus		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter vicinus UBA5574		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter contiguous SBR-L		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		IIG		Ca. Accumulibacter Fred-II3C-720
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		IID		Ca. Accumulibacter necessaries UW12-POB
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		IIB		Ca. Accumulibacter OdNE-BAT3C-415
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green				Ca. Accumulibacter UW10-POB
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		IIF		Ca. Accumulibacter Hade BATACT26
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green				Ca. Accumulibacter iunctus UBA232A107
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	Ca. Accumulibacter adjunctus SK-12			
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	Ca. Accumulibacter conexus UW7			
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	IA	Ca. Accumulibacter Conexus UW13-POB		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter similis SSB1		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter SCELSE-1		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	IB	Ca. Accumulibacter UW8-POB		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter UW4		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter regalis BA-93		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	IC	Ca. Accumulibacter regalis CANDO-1		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter BA-92		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	IIA	Ca. Accumulibacter phosphatis HKU1		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter phosphatis SBR-S		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	3	Ca. Accumulibacter meliphilus UW-LDO		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter aalborgensis		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	2	Ca. Accumulibacter UW5		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Accumulibacter UW9-POB		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	1	Ca. Dechloromonas phosphorivorans Skiv		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Dechloromonas phosphorivorans EsbW		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Dechloromonas phosphorivorans OdNE		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Ca. Dechloromonas phosphoritropha Ribe		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		Formerly classified as Tetrasphaera	Ca. Phosphoribacter baldrii Bjer	
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	Ca. Phosphoribacter baldrii EsbW			
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	Ca. Phosphoribacter baldrii AalW			
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	Ca. Phosphoribacter hodrii i AalE			
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	Ca. Phosphoribacter hodrii Ribe			
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	2	<i>Tetrasphaera jenkinsii</i> Ben-74		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		<i>Tetrasphaera jenkinsii</i> UW-TS-TETRA1-1		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		<i>Tetrasphaera aved</i>		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		<i>Tetrasphaera australiensis</i> Ben-110		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		<i>Tetrasphaera japonica</i> T1-X7		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	1	<i>Tetrasphaera hijor</i>		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		<i>Knoellia remis</i>		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	1	<i>Phycococcus duodecadis</i> DSM-1806		
Green	Green	Green	Green	Red	Green	Green	Green	Green	Green		<i>Phycococcus elongatus</i> Lp2		

3.1. PolyP synthesis and hydrolysis, and P transport

The PolyP metabolic process involves a set of genes responsible for PolyP synthesis, hydrolysis, and P transport (Fig. S1 and Table S3). PolyP synthesis predominates in aerobic or denitrifying conditions and is catalyzed by the polyphosphate kinases PPK1 (gene *ppk1*) and PPK2 (gene *ppk2*) [35]. PPK1 and PPK2 differ in function, structure, and equilibrium preference. PPK1 is a soluble enzyme found in various bacteria and eukaryotic organisms, while PPK2 is a membrane-bound enzyme found primarily in bacteria. Although both enzyme families can catalyze PolyP synthesis, PPK1

preferentially synthesizes PolyP from nucleoside triphosphates, and PPK2 preferentially uses PolyP to phosphorylate nucleoside mono- or di-phosphates, such as adenosine monophosphate (AMP), dimethyl phosphate (DMP), and guanosine diphosphate (GDP) [36,37].

PolyP hydrolysis in PAOs occurs under anaerobic conditions and can be catalyzed by various enzymes, including PPKs, exopolyphosphatases, and endopolyphosphatases [38,39]. As previously stated, the PPKs reverse reaction contributes to the synthesis of adenosine triphosphate (ATP) and guanosine triphosphate (GTP) from PolyP [39]. Besides, exopolyphosphatases (gene *ppx*)

Table 2
Genes for transporting various carbon substrates in WWT-relevant PAOs. Green indicates the presence of a gene, while red denotes its absence. Complete gene names and KEGG nomenclature are available in Supplementary Materials (Table S8); Microorganism accession numbers (NCBI) are available in Supplementary Materials (Table S1).

	<i>nupABC</i>	<i>bmpABC</i>	<i>gguAB</i>	<i>chvE</i>	<i>ssSPA</i>	<i>msSPP₁</i>	<i>sugC</i>	<i>livKHMGF</i>	<i>dppFD</i>	<i>paSPA</i>	<i>aotJMQP</i>	<i>proXWV</i>	<i>tcyABC</i>		
	Nucleoside			Sugars				Amino Acids							
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	IIC	<i>Ca. Accumulibacter phosphatis</i> Bin19
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter phosphatis</i> HKU2
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter cognatus</i> SSA1
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter cognatus</i> SK-02
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> UW6
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> UW11-POB
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> vicinus
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> vicinus UBA5574
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> contiguous SBR-L
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		IIG <i>Ca. Accumulibacter</i> Fred-II3C-720
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	IID <i>Ca. Accumulibacter</i> necessaries UW12-POB	
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	IIB	<i>Ca. Accumulibacter</i> OdNE-BAT3C-415
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> UW10-POB
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	IIF	<i>Ca. Accumulibacter</i> Hade BATA726
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> iunctus UBA232A107
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> adjunctus SK-12
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> connexus UW7
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	IA	<i>Ca. Accumulibacter</i> Connexus UW13-POB
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> similis SSB1
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	IB	<i>Ca. Accumulibacter</i> SCELSE-1
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> UW8-POB
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> UW4
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	IC	<i>Ca. Accumulibacter</i> regalis BA-93
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> regalis CANDO-1
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	IIA	<i>Ca. Accumulibacter</i> BA-92
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> phosphatis HKU1
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	IIA	<i>Ca. Accumulibacter</i> phosphatis SBR-S
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> meliphilus UW-LDO
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	IIA	<i>Ca. Accumulibacter</i> aalborgensis
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Accumulibacter</i> UW5
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	IIA	<i>Ca. Accumulibacter</i> UW9-POB
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Dechloromonas</i> phosphorivorans Skiv
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	3	<i>Ca. Dechloromonas</i> phosphorivorans EsbW
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Dechloromonas</i> phosphorivorans OdNE
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Dechloromonas</i> phosphoritropha Ribe
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Phosphoribacter</i> baldrii Bjer
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	2	<i>Ca. Phosphoribacter</i> baldrii EsbW
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Phosphoribacter</i> baldrii AalW
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	1	<i>Ca. Phosphoribacter</i> hodrii AalE
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Ca. Phosphoribacter</i> hodrii Ribe
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	Formerly classified as <i>Tetrasphaera</i>	<i>Tetrasphaera</i> <i>jenkinsii</i> Ben-74
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Tetrasphaera</i> <i>jenkinsii</i> UW-TS-TETRA1-1
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	Formerly classified as <i>Tetrasphaera</i>	<i>Tetrasphaera</i> <i>aved</i>
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Tetrasphaera</i> <i>australiensis</i> Ben-110
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	Formerly classified as <i>Tetrasphaera</i>	<i>Tetrasphaera</i> <i>japonica</i> T1-X7
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Tetrasphaera</i> <i>hvor</i>
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	Formerly classified as <i>Tetrasphaera</i>	<i>Knoellia</i> <i>remis</i>
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red		<i>Phycococcus</i> <i>duodecadis</i> DSM-1806
	Red	Red	Red	Red	Red	Red	Red	Green	Green	Green	Green	Red	Red	Formerly classified as <i>Tetrasphaera</i>	<i>Phycococcus</i> <i>elongatus</i> Lp2

hydrolyze PolyP from polymer ends, releasing free phosphate [40]. Lastly, endopolyphosphatases break down the PolyP chain within the polymer, releasing shorter PolyP chains or PPi molecules [39]. As observed in Table 1, genes encoding for polyphosphate kinases (genes *ppk1* and *ppk2*) and exopolyphosphatase (gene *ppx*) are well conserved among all genera. However, endopolyphosphatase was not found in any organism using KEGG. The absence of endopolyphosphatase in the studied PAOs, as indicated by the KEGG database, could be attributed to two potential reasons: (1) the endopolyphosphatase in these PAOs may differ from the annotated endopolyphosphatase in the KEGG database, leading to its non-recognition in genomic analyses, and (2) this enzyme is actually absent in WWT-relevant PAOs.

Additionally, PolyP degradation can occur by transfer of the terminal P groups to an acceptor molecule, facilitated by a phosphotransferase. This process conserves the energy of the energy-rich phosphoanhydride bond in the newly formed phosphoanhydride or phosphoester bond at the acceptor molecule [39]. For example, a PolyP-AMP phosphotransferase (gene *pap*) transfers the P group from PolyP to AMP, while a PolyP glucokinase (gene *ppgk*) phosphorylates glucose using PolyP [7]. Although PPGK primarily targets glucose as its substrate, it has also demonstrated the potential to phosphorylate other sugars [41]. According to Table 1, phosphotransferase in *Ca. Accumulibacter* and *Ca. Dechloromonas* occurs via PolyP-producing ADP, whereas in *Tetrasphaera*, *Ca. Phosphoribacter*, *Knoellia*, and *Phycococcus*, it is achieved through the phosphorylation of a sugar.

P transporters play a crucial role in regulating the uptake and utilization of P within cells. There are different P transporters in bacteria, including low-affinity transporters (genes *pit*) and high-affinity transporters (genes *pstSCAB*), where *PSTSCAB* is likely to perform better in low P concentrations than *PIT*. The *PSTSCAB* proteins comprise a periplasmic P-binding protein (gene *pstS*), integral membrane proteins (genes *pstC* and *pstA*), and an ATP-binding protein (gene *pstB*) [42]. In contrast, the P transporter encoded by gene *pit* is a periplasmic single-component symporter that facilitates the transport of P or sulfate using either Na⁺ or H⁺ ions [43]. In anaerobic environments, PAOs employ the PIT to excrete P and cations simultaneously, generating an electrochemical gradient that facilitates carbon uptake [44]. For example, in organisms like *Ca. Accumulibacter* and *Ca. Dechloromonas*, the ACTP symporter uses an electrochemical gradient to drive acetate uptake [44]. As observed in Table 1, the *pit* gene is present in all PAOs, whereas the *pstSABC* cluster is not found in all species but exhibits high conservation within PAOs. Notably, the *pit* gene is not exclusive to PAOs and can also be found in organisms that do not show PAO activity.

3.2. Organic substrate transport

When examining the ABC transporters (Table 2), all genera show the potential for uptaking branched-chain amino acids (genes *livKHMGF*), polar amino acids (genes *paSPA*), suggesting a conserved capacity for uptaking different nitrogen sources. Additionally, *Ca. Accumulibacter*, *Ca. Dechloromonas*, and *Ca. Phosphoribacter*, possess the genes for lipopolysaccharide synthesis (genes *rfbAB*). However, it should be noted that *Ca. Phosphoribacter* lacks the potential for exporting lipopolysaccharides (genes *lptFGB*). On the other hand, all PAOs, except for *Ca. Accumulibacter* and *Ca. Dechloromonas*, possess genes responsible for the transport of nucleosides (genes *nupABC* and *bmpABC*), simple sugars (gene *ssSPA*), and multiple sugars (genes *gguAB*, *msSPA*, *chvE*, and *sugC*). These findings support previous reports on the ability of *Tetrasphaera* to utilize a diverse range of carbon sources, including yeast extract, peptone, glucose, and soluble starch [26,45]. Likewise, it is

in agreement with the potential of *Ca. Accumulibacter* to utilize and uptake glucose [45].

Moreover, all PAOs possess electrochemical potential-driven transporters, such as the cation/acetate (gene *actP*) and the malonate-proton symporter (gene *mdcF*), which allow these organisms to efficiently transport carbon compounds without requiring ATP. The presence of acetate transporters in all PAOs is consistent with the documented uptake of VFAs (acetate and propionate) by *Ca. Accumulibacter* and *Ca. Dechloromonas* during the anaerobic phase [46]. Similarly, a sludge enriched with *Tetrasphaera* and *Ca. Phosphoribacter* effectively removed acetate, though the specific genera accountable for this removal remains unidentified [45]. On the other hand, certain PAO species, such as *Tetrasphaera australiensis*, *Tetrasphaera japonica*, *Phycococcus elongata*, and *Tetrasphaera jenkinsii*, do not uptake acetate despite possessing the necessary transporters [47]. It has been hypothesized that the inability of these species to uptake acetate could be explained by the lack of the *phaC* gene and glyoxylate cycle genes required for processing acetate for PHA synthesis [29,44,45]. However, evidence suggests that ActP transporters have a broader range of substrates beyond acetate, such as glycolate [44]. Therefore, these species may utilize the ActP transporters to transport other molecules than acetate.

Moreover, *Ca. Accumulibacter* and *Ca. Dechloromonas* species feature an enrichment of electrochemical potential-driven transporters responsible for C4-dicarboxylate uptake (genes *dcuAB*, *dctA*, and *dctPQM*). This aligns with the reported ability of *Ca. Accumulibacter* to utilize fermentation products, such as succinate, as a carbon source [48]. In contrast, the *Ca. Dechloromonas* genus exclusively possesses the glutamate-sodium symporter (gene *gltS*), incorporating glutamate into its central carbon metabolism via glutamate dehydrogenase (gene *gdhA*) [12]. Conversely, *Tetrasphaera* exhibits a diverse array of specific electrochemical gradient transporters, including putrescine importer (gene *puuP*), the transport of sugars such as maltose/maltodextrin (gene *malY*), and the fucose-proton symporter (gene *fucP*), as well as amino acid transporters, such as L-asparagine permease (gene *ansP*) and glycine betaine transporter (gene *opuD* or *betL*). This agrees with the reported preference of these genera for consuming sugars and accumulating amino acids [49].

Certain transporters are exclusive to a particular species. For example, *Phycococcus duodecadiis* possesses the proline-proton symporter (gene *proP*), *Knoellia remsis* has the cytosine permease (gene *codB*) and the benzoate-proton symporter (gene *benK*) as specific transporters, *Tetrasphaera japonica* has the tartrate transporter (gene *ttuB*), galactose-proton symporter (gene *galP*), and lactate-proton symport (gene *jen*), *Tetrasphaera australiensis* Ben-110 has the ethanolamine permease (gene *eat*) and the oxalate-formate antiporter (gene *oxIT*).

3.3. Fermentation

Fermentation capabilities have not been reported in *Ca. Accumulibacter* and *Ca. Dechloromonas*. However, *Tetrasphaera* has been shown to have the capacity for anaerobic P release; it is also capable of anaerobic P uptake using the energy generated from the fermentation of various organic compounds [50]. It has been reported that *Tetrasphaera* can ferment glucose and amino acids to produce acetate, succinate, lactate, and ethanol [51–53]. Additionally, *Ca. Phosphoribacter* can ferment alanine to pyruvate, while *Ca. Phosphoribacter* possesses the complete fermentation pathway to convert pyruvate to acetate [14]. Likewise, it has been hypothesized that *Phycococcus elongata* can produce lactate as the end product of glucose fermentation.

The capability of these organisms to produce succinate and

Table 3
Genes involved in fermentation in WWT-relevant PAOs. Green indicates the presence of a gene, while red denotes its absence; Microorganism accession numbers (NCBI) are available in Supplemental Materials (Table S1).

Acetate Kinase	Acetyl-Coa-Synthetase	Alcohol Dehydrogenase	L-Lactate Dehydrogenase		
Green	Green	Green	Red	II C	Ca. Accumulibacter phosphatis Bin19
Green	Green	Green	Red		Ca. Accumulibacter phosphatis HKU2
Green	Green	Green	Red		Ca. Accumulibacter cognatus SSA1
Green	Green	Red	Red		Ca. Accumulibacter cognatus SK-02
Green	Green	Green	Red		Ca. Accumulibacter UW6
Green	Green	Green	Red		Ca. Accumulibacter UW11-POB
Green	Green	Green	Red		Ca. Accumulibacter vicinus
Green	Green	Green	Red		Ca. Accumulibacter vicinus UBA5574
Green	Green	Green	Red		Ca. Accumulibacter contiguous SBR-L
Green	Green	Green	Red		Ca. Accumulibacter Fred-II3C-720
Green	Green	Green	Red	II G	Ca. Accumulibacter necessities UW12-POB
Green	Green	Green	Red	II D	Ca. Accumulibacter OdNE-BAT3C-415
Green	Green	Green	Red	II B	Ca. Accumulibacter UW10-POB
Green	Green	Green	Red	Ca. Accumulibacter Hade BATAC726	
Green	Green	Green	Red	Ca. Accumulibacter iunctus UBA232A107	
Green	Green	Green	Red	II F	Ca. Accumulibacter adjunctus SK-12
Green	Green	Green	Red		Ca. Accumulibacter conexus UW7
Green	Green	Green	Red		Ca. Accumulibacter Conexus UW13-POB
Green	Green	Green	Red		Ca. Accumulibacter similis SSB1
Green	Green	Green	Red		Ca. Accumulibacter SCELSE-1
Green	Green	Green	Red	Ca. Accumulibacter UW8-POB	
Green	Green	Green	Red	IA	Ca. Accumulibacter UW4
Green	Green	Green	Red		Ca. Accumulibacter regalis BA-93
Green	Green	Green	Red	IB	Ca. Accumulibacter regalis CANDO-1
Green	Green	Green	Red		Ca. Accumulibacter BA-92
Green	Green	Green	Red	IC	Ca. Accumulibacter phosphatis HKU1
Green	Green	Green	Red		Ca. Accumulibacter phosphatis SBR-S
Green	Green	Green	Red	II A	Ca. Accumulibacter meliphilus UW-LDO
Green	Green	Green	Red		Ca. Accumulibacter aalborgensis
Green	Green	Green	Red		Ca. Accumulibacter UW5
Green	Green	Green	Red	Ca. Accumulibacter UW9-POB	
Green	Green	Green	Red	3	Ca. Dechloromonas phosphorivorans Skiv
Green	Green	Green	Red		Ca. Dechloromonas phosphorivorans EsbW
Green	Green	Green	Red		Ca. Dechloromonas phosphorivorans OdNE
Green	Green	Green	Red		Ca. Dechloromonas phosphoritropha Ribe
Green	Green	Green	Red	2	Ca. Phosphoribacter baldrii Bjer
Green	Green	Green	Red		Ca. Phosphoribacter baldrii EsbW
Green	Green	Green	Red		Ca. Phosphoribacter baldrii AalW
Green	Green	Green	Red		Ca. Phosphoribacter hodrii AalE
Green	Green	Green	Red		Ca. Phosphoribacter hodrii Ribe
Green	Green	Green	Red	1	<i>Tetrasphaera jenkinsii</i> Ben-74
Green	Green	Green	Red		<i>Tetrasphaera jenkinsii</i> UW-TS-TETRA1-1
Green	Green	Green	Red		<i>Tetrasphaera aved</i>
Green	Green	Green	Red	<i>Tetrasphaera australiensis</i> Ben-110	
Green	Green	Green	Red	<i>Tetrasphaera japonica</i> T1-X7	
Green	Green	Green	Red	<i>Tetrasphaera hjoer</i>	
Green	Green	Green	Red	<i>Knoellia remis</i>	
Green	Green	Green	Red	<i>Phycococcus duodecadis</i> DSM-1806	
Green	Green	Green	Red	<i>Phycococcus elongatus</i> Lp2	

acetate is supported by the presence of the TCA cycle and the acetate kinase (Table 3). However, it is crucial to note that a specific gene alone cannot conclusively predict acetate fermentation, as indicated by its occurrence in non-fermenting genomes. Lactate production in *Tetrasphaera* and *Phycococcus* is substantiated by the data presented in Table 3. This table shows that *Phycococcus elongata* and *Tetrasphaera Hjoer* contain the gene encoding lactate dehydrogenase. Moreover, *Tetrasphaera japonica* possesses alcohol dehydrogenase (Table 3), confirming the potential of this genus for alcohol production.

3.4. Glycogen

All WWT-relevant PAOs possess the capacity for glycogen synthesis but differ in the enzymes they use (Table 4). The classical biosynthetic pathway of glycogen involves three key enzymes: glucose-1-phosphate adenylyltransferase (gene *glgC*), ADP-glucose-specific glycogen synthase (gene *glgA*), and branching enzyme (gene *glgB*) [54]. GLGC is crucial in converting glucose-1-phosphate to ADP-glucose, serving as a substrate for GLGA to add glucosyl units to the growing glycogen chain, while GLGB further enhances glycogen complexity by cleaving portions of the chain and forming α -1,6 glycosidic bonds [54]. *Ca. Accumulibacter* and *Ca. Dechloromonas* exhibit the capability for glycogen production through the *glgCAB* pathway, as indicated by the data presented in Table 4 and corroborated by the current literature [12,16]. In contrast, *Tetrasphaera*, *Ca. Phosphoribacter*, *Knoellia*, and *Phycococcus* lack the GLGA enzyme required for glycogen elongation. Although, research has shown that the alpha-maltose-1-phosphate synthase (gene *glgM*) can employ the GLGC product to generate maltose, while the maltosyl-transferase (gene *glgE*) can synthesize glycogen through maltose elongation [55]. Given these insights, it can be inferred that *Tetrasphaera*, *Ca. Phosphoribacter*, *Knoellia*, and *Phycococcus* possess the potential to synthesize glycogen employing maltose as the monomer (Table 4).

On the other hand, during glycogen catabolism, two essential enzymes come into play: glycogen phosphorylase (gene *glgP*) and the debranching enzyme (gene *amy*) [56]. Glycogen phosphorylase helps remove glucose units from glycogen through phosphorolysis, generating glucose-1-phosphate. The debranching enzyme removes branch points in the glycogen chain, making it more accessible for further breakdown [56]. The presence of conserved GLGP enzymes across all genera indicates that all PAOs have the capacity to utilize glycogen as a carbon source (Table 4). Besides, *Ca. Phosphoribacter* is the only genus that possesses the alpha-amylase gene (*amyA*), which indicates its capacity to efficiently break down glycogen into shorter oligosaccharides, such as maltose, maltotriose, and maltotetraose (Table 4) [57]. Additionally, beta-amylase, responsible for breaking down glycogen into maltose, is exclusively found in *Tetrasphaera hjoer* [58].

3.5. Polyhydroxyalkanoates (PHA) metabolism

PHA production occurs during periods of abundant carbon and energy, often in the presence of PolyP, in the anaerobic phase. Subsequently, PHA can be hydrolyzed to serve as a carbon substrate under aerobic conditions for PAOs and anoxic conditions for DPAOs (Fig. 2) [36,59]. In PAOs, it has been demonstrated that both acetate and glycogen contribute to anaerobic PHA synthesis through their conversion to acetyl-CoA. The PHA generated through this process is subsequently utilized aerobically for glycogen production [60]. Furthermore, glycogen breakdown via the Entner-Doudoroff (ED) pathway allows it to serve as a considerable source of reducing power for PHA production. Regarding the proteins involved in PHA synthesis, they are encoded by the PHA gene cluster, which is composed of a set of genes that may differ based on the microorganism in question. The PHA gene cluster typically includes *phaC*, *phaA*, *phaB*, *phaG*, and *phaJ* [61]. The genes *phaA*, *phaB*, *phaG*, and *phaJ* encode for a group of enzymes that are essential for producing PHA monomers from different carbon sources, including acetyl-CoA (genes *phaA* and *phaB*), fatty acids (gene *phaG*), and lipids (gene *phaJ*) [62]. Then, these monomers are polymerized into PHA by the PHA synthase (gene *phaC*). As a result, *phaC* is regarded as a marker for determining microorganisms' potential for PHA synthesis [63].

Table 4 demonstrates that both *Ca. Accumulibacter* and *Ca.*

Table 4

Genes involved in PHA synthesis, PHA hydrolysis, glycogen synthesis, and glycogen degradation in WWT-relevant PAOs. Green indicates the presence of a gene, while red denotes its absence. Complete gene names and KEGG nomenclature are available in Supplementary Materials (Table S8); Microorganism accession numbers (NCBI) are available in Supplemental Materials (Table S1).

Glycogen synthesis					Glycogen degradation					PHA					Type and Clades classification for <i>Ca. Accumulibacter</i>	
<i>gigC</i>	<i>gigA</i>	<i>gigB</i>	<i>gigM</i>	<i>gigE</i>	<i>gigP</i>	<i>amyA</i>	<i>amyB</i>	<i>amyM</i>	<i>ma</i>	<i>phaA</i>	<i>phaB</i>	<i>phaJ</i>	<i>phaC</i>	<i>phaZ</i>		
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	IIC	<i>Ca. Accumulibacter phosphatis</i> Bin19
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Ca. Accumulibacter phosphatis</i> HKU2
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Ca. Accumulibacter cognatus</i> SSA1
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Ca. Accumulibacter cognatus</i> SK-02
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Ca. Accumulibacter</i> UW6
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Ca. Accumulibacter</i> UW11-POB
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Ca. Accumulibacter</i> vicinus
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Ca. Accumulibacter</i> vicinus UBA5574
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Ca. Accumulibacter</i> contiguous SBR-L
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		IIG <i>Ca. Accumulibacter</i> Fred-II3C-720
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	IID <i>Ca. Accumulibacter</i> necessaries UW12-POB	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> OdNE-BAT3C-415	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	IIB <i>Ca. Accumulibacter</i> UW10-POB	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> Hade BATACT26	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> iunctus UBA232A107	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> adjunctus SK-12	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	IIF <i>Ca. Accumulibacter</i> conexus UW7	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> Conexus UW13-POB	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> similis SSB1	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> SCELSE-1	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> UW8-POB	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	IA <i>Ca. Accumulibacter</i> UW4	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> regalis BA-93	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> regalis CANDO-1	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	IB <i>Ca. Accumulibacter</i> BA-92	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> phosphatis HKU1	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	IC <i>Ca. Accumulibacter</i> phosphatis SBR-S	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> meliphilus UW-LDO	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> aalborgensis	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	IIA <i>Ca. Accumulibacter</i> UW5	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Accumulibacter</i> UW9-POB	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Dechloromonas</i> phosphorivorans isolate Skiv	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Dechloromonas</i> phosphorivorans isolate EsbW	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Dechloromonas</i> phosphorivorans isolate OdNE	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Ca. Dechloromonas</i> phosphoritropha isolate Ribe	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	3	<i>Ca. Phosphoribacter</i> baldrii isolate Bjer
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Ca. Phosphoribacter</i> baldrii isolate EsbW
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Ca. Phosphoribacter</i> baldrii isolate AalW
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Ca. Phosphoribacter</i> hodrii isolate AalE
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Ca. Phosphoribacter</i> hodrii isolate Ribe
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	2	<i>Tetrasphaera</i> jenkinsii Ben-74
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Tetrasphaera</i> jenkinsii UW-TS-TETRA1-1
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Tetrasphaera</i> aved
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	1	<i>Tetrasphaera</i> australiensis Ben-110
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green		<i>Tetrasphaera</i> japonica T1-X7
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Tetrasphaera</i> hjor	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Knoellia</i> remis	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Phycococcus</i> duodecadis DSM-12806	
Green	Green	Green	Green	Green	Green	Red	Red	Red	Red	Green	Green	Green	Green	Green	<i>Phycococcus</i> elongatus Lp2	

Dechloromonas contain *phaA*, *phaB*, *phaJ*, and *phaC* genes, indicating they can potentially use acetate and lipids as substrates for PHA synthesis. This is consistent with studies showing that *Ca. Accumulibacter* and *Ca. Dechloromonas* can synthesize PHA using VFAs [12,46]. In contrast, *Tetrasphaera*, *Ca. Phosphoribacter*, *Knoellia*, and *Phycococcus* have been found to lack the *phaB*, *phaJ*, and *phaC* genes. This genetic profile aligns with the reported inability of *Tetrasphaera* to produce PHA [47]. Interestingly, *Ca.*

Phosphoribacter hodrii showed the potential for synthesizing PHA due to the presence of the *phaC* gene (Table 4). Moreover, it has been established that the synthesis of different PHAs (Table S4) depends on the types of VFA present. For example, polyhydroxy butyrate (PHB) can be synthesized from acetate only, polyhydroxy-2-methyl valerate (PH2MV) from propionate only, polyhydroxy valerate (PHV) and polyhydroxy-2-methyl butyrate (PH2MB) from acetate and propionate [64].

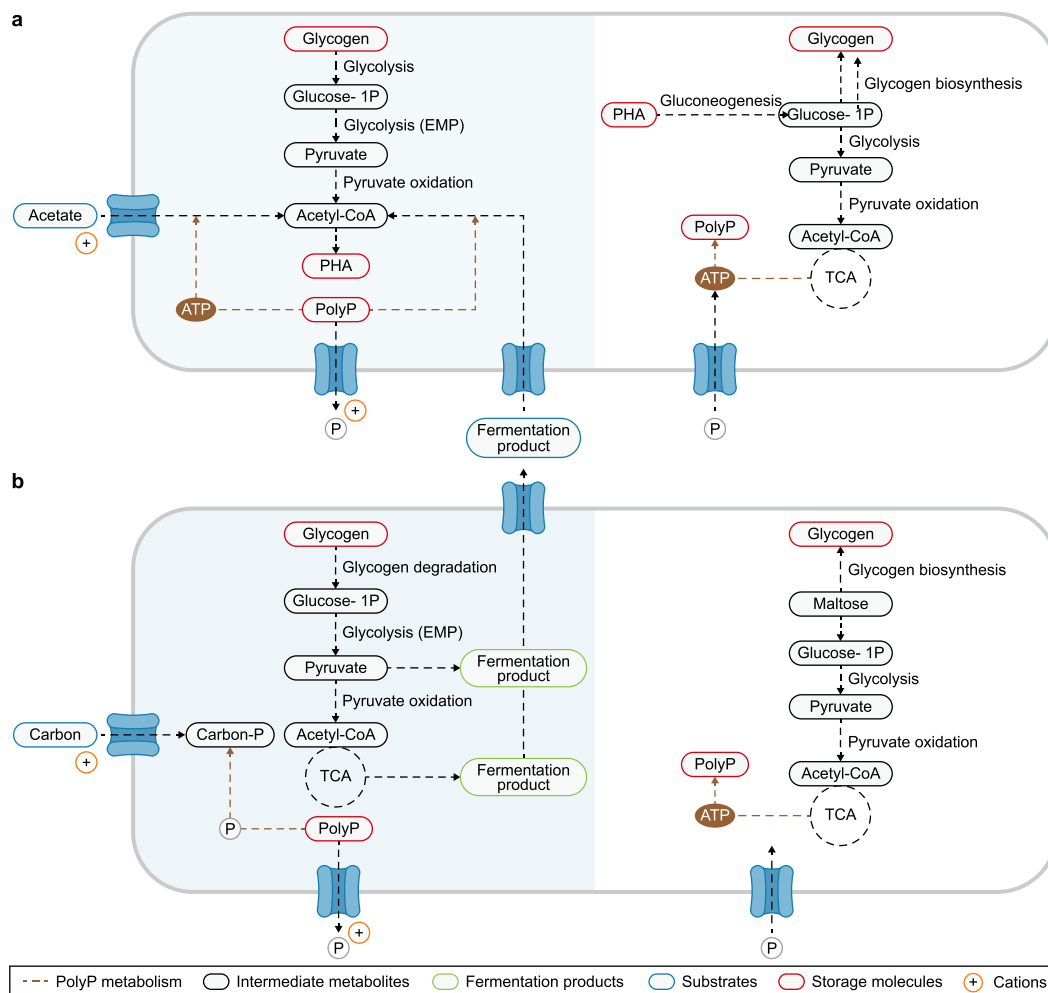


Fig. 2. Metabolism of WWT-relevant PAOs under anaerobic (shade) and aerobic conditions: **a**, Metabolic capabilities of *Ca. Accumulibacter* and *Ca. Dechloromonas*, encompassing substrate uptake, polyphosphate (PolyP), glycogen, and polyhydroxyalkanoates (PHA) synthesis. **b**, Metabolic characteristics of *Ca. Phosphoribacter*, *Tetrasphaera*, *Knoellia*, and *Phycococcus*, specifically addressing carbon uptake, storage, and PolyP and glycogen synthesis.

The enzyme PHA depolymerase, encoded by the *phaZ* gene, plays a vital role in hydrolyzing water-insoluble PHAs into soluble 3-hydroxyalkanoic acids [65]. These enzymes lack common sequences and substrate preferences [65,66]. Still, they share two consistent structural elements: the presence of the α/β -hydrolase fold and an active-site catalytic triad (composed of serine, histidine, and aspartic acid) [65,66]. Among the various *phaZ* genes reported in KEGG, only the poly(3-hydroxyoctanoate) depolymerase was present in the WWT-relevant PAOs. Furthermore, this enzyme was found exclusively in species within the *Ca. Accumulibacter* clade IIC (Table 4). The absence of the *phaZ* gene in *Ca. Dechloromonas* is consistent with previous reports [12]. However, it is essential to note that PHA degradation has been reported in these genera. Therefore, it is possible that other species of *Ca. Accumulibacter* and *Ca. Dechloromonas* may possess PHA depolymerases with significant differences in enzyme structure compared to those reported in KEGG, or they might utilize alternative pathways for PHA degradation.

3.6. Denitrification capabilities of PAOs

Denitrification is a respiratory process in which nitrogen compounds, such as nitrate or nitrite, can be used as electron acceptors [67]. The denitrification process involves several steps and

enzymes. The first step involves reducing nitrate (NO_3^-) to nitrite (NO_2^-) via nitrate reductases. There are three types of known nitrate reductases: periplasmic nitrate reductase (gene *nap*), respiratory nitrate reductase (gene *nar*), and assimilatory nitrate reductase (gene *nas*) [67]. After nitrate reduction, nitrite reductase (gene *nir*) catalyzes the conversion of NO_2^- into nitric oxide (NO) and plays an essential role in transforming N from liquid to gas. Nitrite reduction in denitrification could be done by two distinct nitrite reductase enzymes encoded by the *nirK* and *nirS* genes. The *nirK* gene encodes a copper-containing enzyme found in low-oxygen environments, whereas *nirS* encodes a cytochrome cd1-containing enzyme found in high-oxygen environments [68,69]. Next, nitric oxide reductase (gene *nor*) catalyzes the reduction of NO to nitrous oxide (N_2O) [70]. In the final step, the nitrous oxide reductase (gene *nos*) reduces N_2O to N_2 and completes denitrification [71].

PAOs that demonstrated complete denitrification capability (i.e., reducing NO_3^- to N_2) are called DPAOs [72]. Consequently, if present, DPAOs can remove P, hydrolyze PHA, and grow in anoxic conditions [71]. However, it is important to highlight that their ability to remove P in anoxic conditions is approximately half as fast and less efficient than aerobic conditions [73]. So far, identified DPAOs from WWTPs include *Paracoccus denitrificans* YCP [74], *Paracoccus* sp. YKP-9 [67], *Thauera* sp. SND5 [68] (Table S5). Besides the already identified DPAOs, certain species from WWT-relevant PAOs have

Table 5
 Genes involved in denitrification in WWT-relevant PAOs. Green indicates the presence of a gene, while red denotes its absence. Complete gene names and KEGG nomenclature are available in Supplementary Materials (Table S8); Microorganism accession numbers (NCBI) are available in Supplemental Materials (Table S1).

	<i>narGZA</i>	<i>narHYB</i>	<i>narIV</i>	<i>napA</i>	<i>napB</i>	<i>nirK</i>	<i>nirS</i>	<i>norB</i>	<i>norC</i>	<i>nosZ</i>		
	NO ₃ ⁻					NO ₂ ⁻		NO		N ₂ O		
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	IIC	<i>Ca. Accumulibacter phosphatis</i> Bin19
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red		<i>Ca. Accumulibacter phosphatis</i> HKU2
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red		<i>Ca. Accumulibacter cognatus</i> SSA1
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red		<i>Ca. Accumulibacter cognatus</i> SK-02
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red		<i>Ca. Accumulibacter</i> UW6
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red		<i>Ca. Accumulibacter</i> UW11-POB
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red		<i>Ca. Accumulibacter</i> vicinus
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red		<i>Ca. Accumulibacter</i> vicinus UBA5574
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red		<i>Ca. Accumulibacter</i> contiguous SBR-L
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red		IIG
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	IID	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> Fred-II3C-720	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> necessaryes UW12-POB	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> OdNE-BAT3C-415	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	IIB	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> UW10-POB	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> Hade BATAc726	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> iunctus UBA232A107	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> adjunctus SK-12	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	IIF	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> conexus UW7	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> Conexus UW13-POB	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> similis SSB1	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> SCELSE-1	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> UW8-POB	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	IA	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> UW4	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> regalis BA-93	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> regalis CANDO-1	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	IB	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> BA-92	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> phosphatis HKU1	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> phosphatis SBR-S	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	IC	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> meliphilus UW-LDO	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	IIA	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> aalborgensis	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> UW5	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Accumulibacter</i> UW9-POB	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Dechloromonas</i> phosphorivorans Skiv	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Dechloromonas</i> phosphorivorans EsbW	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Dechloromonas</i> phosphorivorans OdNE	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Dechloromonas</i> phosphoritropha Ribe	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	3	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Phosphoribacter</i> baldrii Bjer	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Phosphoribacter</i> baldrii EsbW	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Phosphoribacter</i> baldrii AalW	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Phosphoribacter</i> hodrii i AalE	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Ca. Phosphoribacter</i> hodrii Ribe	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	2	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Tetrasphaera</i> jenkinsii Ben-74	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Tetrasphaera</i> jenkinsii UW-TS-TETRA1-1	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Tetrasphaera</i> aved	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Tetrasphaera</i> australiensis Ben-110	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	1	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Tetrasphaera</i> japonica T1-X7	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Tetrasphaera</i> hjor	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Knoellia</i> remsis	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Phycococcus</i> duodecadis DSM-1806	
	Green	Green	Green	Red	Red	Red	Green	Red	Red	Red	<i>Phycococcus</i> elongatus Lp2	

Type and Clades classification for *Ca. Accumulibacter*

Formerly classified as *Tetrasphaera*

shown the potential for denitrification, and as a result, this section will focus on discussing their denitrification capabilities.

As observed in Table 5, several PAO species, such as *Knoellia remsis*, *Tetrasphaera aved*, *Ca. Accumulibacter adjunctus*, and *Ca. Accumulibacter cognatus*, were found to lack the genes necessary for denitrification. However, most PAOs possess the necessary genes for reducing nitrate and nitrite, indicating their potential to utilize these compounds as electron acceptors, while interesting distinctions exist among different genera regarding their enzyme

capabilities for nitrate and nitrite reduction. For instance, *Ca. Accumulibacter* and *Ca. Dechloromonas* have the ability to utilize either the NAP or NAR enzyme for nitrate reduction, whereas the other genera exclusively possess the NAR enzyme. On the other hand, when it comes to nitrite reduction, the *nirS* gene is found in *Ca. Dechloromonas* and *Ca. Accumulibacter* species, whereas the *nirK* gene is present in *Tetrasphaera*, *Phycococcus*, and *Ca. Phosphoribacter*. This distinction is interesting because the presence of the *nirK* gene suggests that these species may be more adept at

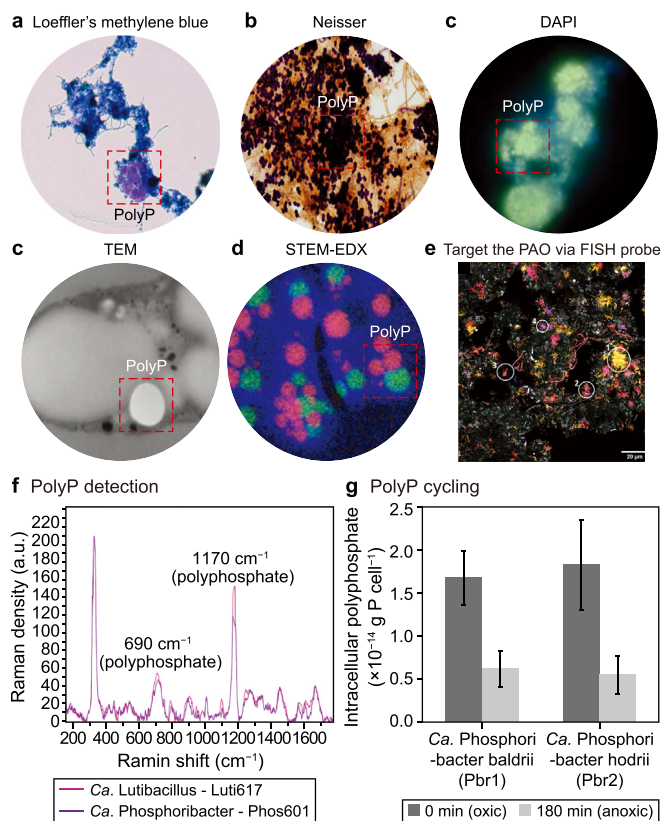


Fig. 3. Techniques for detecting PolyP and PAOs. **a–c.** The commonly used staining methods include Loeffler's Methylene Blue [102] (a), Neisser [103] (b), and 4',6-diamidino-2-phenylindole (DAPI) [104] (c). **d–e.** Staining-independent visualization techniques include Transmission electron microscopy (TEM) (d) [104] and scanning transmission electron microscopy (STEM) coupled with X-ray spectroscopy (STEM-EDX) (e) [79]. **f–h.** The methodology for identifying PAOs [14] includes FISH probes targeting PAOs (f), Raman for detecting the presence of PolyP in the targeted cell (g), and Raman for measuring the PolyP cycling in anaerobic and aerobic conditions (h).

denitrification in low-oxygen environments [75].

On the other hand, most PAO species lack the genes responsible for reducing nitric oxide (gene *nor*) and nitrous oxide (gene *nos*). The absence of these enzymes, which act as electron sinks and are crucial for reducing toxic intermediate products (i.e., NO), results in a diminished ability to utilize NO_3^- and NO_2^- as terminal electron acceptors [64]. Furthermore, it is worth noting that *Ca. Accumulibacter meliphilus* UW-LDO, *Ca. Accumulibacter contiguous* SBR-L, *Tetrasphaera australiensis*, and *Tetrasphaera japonica*, possess the *nor* gene and lack the *nos* gene. This genetic configuration could potentially produce nitrous oxide, a potent greenhouse gas, by these PAOs [69]. Nevertheless, in WWTPs, PAOs coexist with a diverse microbial community that can utilize PAOs' byproducts, effectively mitigating the aforementioned issues [70].

Lastly, certain PAOs, such as *Ca. Accumulibacter* (including BA-92, phosphatis SBR-S, UW5, UW9-POB, and all species in cluster IA) and *Ca. Dechloromonas phosphorivorans* Skiv and EsbW, possess all the necessary genes for complete denitrification. Interestingly, it has been observed that PAOs possessing all genes required for denitrification possess the NAP enzyme for nitrate reduction, which is considered more efficient than *nar* enzymes [64]. These findings align with the reported synergistic role of the NAP-NIR enzyme in generating a proton motive force to support denitrification, even in the absence of the NAR enzyme [64]. Furthermore, the complete denitrification ability of species from

clade IA has been experimentally demonstrated [71]. However, as the study utilized fluorescence *in situ* hybridization (FISH) probes targeting clade IA, it lacks the ability to differentiate whether the observed activity is shared by all species within this clade or limited to a specific subset. As a result, there is uncertainty about which particular species are prominently exhibiting DPAO activity.

4. Methods for the detection and quantification of PolyP and PAOs

4.1. Staining and visualization techniques for PolyP detection in cells

PolyP can be observed as an electron-dense material using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). However, electron microscopy may confuse PolyP with another molecule (Fig. 3) [76]. To provide a more accurate identification of PolyP clusters, a combination of electron microscopy and PolyP staining techniques can be used [77]. The most common PolyP stains are Loeffler's Methylene Blue, Neisser, and 4',6-diamidino-2-phenylindole (DAPI) [78] (Fig. 3). When using Loeffler's staining method, PolyP appears to be pink violet on a blue cell background, whereas Neisser's staining method colors the PolyP in purple-black in a yellowish-brown background of counterstained cells. The latter procedure is more effective than other light microscopic staining techniques since it yields the highest contrast between PolyP granules and cells [19,26]. DAPI staining also stains PolyP granules with a yellow fluorescence when used at high concentrations. However, DAPI is not specific because it also stains other polymeric ions, such as DNA and lipids, where DAPI-DNA fluorescence is blue, whereas both DAPI-PolyP and DAPI-lipids show a yellow fluorescence [78]. Additionally, PolyP can be visualized using scanning transmission electron microscopy (STEM) combined with energy-dispersive X-ray spectroscopy (STEM-EDX), enabling the detection of the X-ray emission spectrum of P [79]. On the other hand, Saito et al. developed a novel technique for PolyP detection using a recombinant PolyP binding domain (PPBD) from an exopolyphosphatase enzyme found in *Escherichia coli* [80]. The PPBD has a high affinity for long-chain PolyP and a low affinity for short-chain PolyP, which allows for the specific detection and isolation of long-chain PolyP molecules in a sample [80].

PolyP can also be identified and quantified within cells through flow cytometry, nuclear magnetic resonance (NMR) spectroscopy, and Raman spectroscopy [77]. Flow cytometry can be used to detect PolyP molecules by incorporating a staining technique and modifying the laser's excitation wavelength to detect the used dye. For example, in the case of DAPI-stained PolyP molecules, the fluorescence emitted can be detected at approximately 550 nm [76]. It is important to emphasize that achieving accurate separation of cluster cells into single cells for flow cytometry analysis requires crucial steps of homogenization, sonication, and filtration of sludge samples, especially for microorganisms like *Ca. Accumulibacter* that tend to grow in clusters [81]. On the other hand, Raman spectroscopy and NMR can achieve quantification without requiring the dyeing of PolyP, as they focus on the absorption/emission spectra or mass-to-charge ratio of P and PolyP [82]. In NMR, interferences may arise due to the similarity of linkages in PolyP and other molecules containing phosphorane bonds, such as nucleotides, resulting in limited accuracy in determining PolyP concentration [83]. Raman spectroscopy accurately identifies PolyP based on its unique spectral signature, though determining PolyP length is challenging due to the overlapping band [84,85]. Therefore, Raman spectroscopy is the simplest and most widely used method for accurately detecting PolyP in non-cultivated PAOs,

particularly in complex samples [14,49].

4.2. Molecular-based techniques for identifying PAOs

Initially, the taxonomic classification of PAOs relied on two criteria: DNA GC content and chemotaxonomic properties [22,30,86,87]. However, with the advent of molecular biology tools, the DNA sequencing of three genes (16S rRNA, PPK1, and 23S rRNA) has been used to reflect the phylogenetic relationships among different genera of PAOs [12,14,88]. For example, the full 16S rRNA gene sequence was used to classify PAOs from the genus *Dechloromonas* (*Ca. Dechloromonas phosphoritropha* and *Ca. Dechloromonas phosphorivorans*) [12]. Furthermore, the full-length 16S rRNA gene divides the *Tetrasphaera* genus into three clades [29]. Nevertheless, the utilization of the 23S rRNA gene has been demonstrated to be more effective in revealing the phylogenetic classification within this genus, preventing incorrect assignment of genera such as *Ca. Phosphoribacter*, *Phycococcus*, and *Knoellia* [14]. Finally, the 16S rRNA was used to identify *Ca. Accumulibacter*. However, to reveal finer-scale population structure in *Ca. Accumulibacter*, McMahon and coworkers used polyphosphate kinase 1 (gene *ppk1*) [89]. The *ppk1* gene encodes for PPK1 enzyme that participates in PolyP synthesis and is typically only present in one copy in *Ca. Accumulibacter* genome [89]. The *Ca. Accumulibacter ppk1* gene classification revealed the presence of two genotypes (types I and II) and five clades (one clade within type I and four within type II). The *ppk1* primers used to categorize type I and type II are shown in Table S6, along with improved primers for *Ca. Accumulibacter* type and clades identification [71,88,90]. The only consistent difference observed in the classification of clades is that clade IA exhibits the capacity to couple nitrate reduction with P uptake, whereas clade IIA does not possess this capability [71]. However, in addition to this distinction, relying solely on clade description is insufficient for accurately characterizing a specific metabolic capability [91,92].

The most used method for identifying PAOs in wastewater samples is sequencing the variable regions of the 16S rRNA gene and comparing the sequences to publicly available databases of 16S rRNA, such as MiDAS. The MiDAS guide is a well-known database for identifying PAOs in WWTPs, and it is based on the V1–V3 and V4 variable regions of the 16S rRNA gene. Of these regions, the V1–V3 region provides the most reliable taxonomic resolution for identifying PAOs in WWTPs [21]. However, it has a poor species-level resolution for PAOs. For instance, *Ca. Accumulibacter affinis*, *Ca. Accumulibacter proximus*, *Ca. Accumulibacter propinquus*, and *Ca. Accumulibacter phosphatis* will all be classified as *Ca. Accumulibacter phosphatis* despite being a different species [13]. Therefore, this limits MiDAS's ability to provide accurate species-level identification of PAOs in WWTPs.

Another commonly used method to identify known PAOs in WWTPs is FISH with 16S/23S rRNA-targeted oligonucleotide probes. Using FISH allows for identifying target organisms and provides a better understanding of the distribution and morphology of the targeted PAOs [93]. Out of all the FISH probes designed to target the 16S rRNA of *Ca. Accumulibacter*, the PAOmix (a combination of PAO462, PAO651, and PAO846) was considered the most sensitive and specific for targeting *Ca. Accumulibacter* [22,86]. However, subsequent research revealed that the traditionally used PAOmix also targets *Propionivibrio* GAO [94]. Consequently, it is now recommended to employ PAO651 alone to ensure accurate targeting of *Ca. Accumulibacter* [94]. Furthermore, a set of new species-level FISH probes (Table S7) were designed in 2022 using the full-length 16S rRNA, resulting in a more accurate phylogenetic classification [13].

The FISH probes for *Tetrasphaera* were initially discovered using

probes HGC69a and HGC236, which are specific to Actinobacteria [95,96]. In 2000, Maszenan and colleagues discovered three Actinobacteria isolates capable of accumulating PolyP, giving *Tetrasphaera* as their genus name. These isolates were classified into two species: *Tetrasphaera japonica* sp. (T1-X7T) and *Tetrasphaera australiensis* sp. (Ben 109 and Ben 110) [23]. In 2001, the FISH probe Actino-1011 was used to target *Tetrasphaera japonica* [97]. *Tetrasphaera elongata* was discovered in 2002, but no FISH probes were developed despite sequencing its 16S rRNA gene [25,26]. In 2005, two FISH probes, Actino-221 and Actino-658, were designed to specifically target new, uncultivated *Tetrasphaera* species (still unnamed) that exhibited PolyP accumulation, with Actino-658 targeting short and Actino-221 targeting tetrad-arranged cocci [98]. Actino-221 and Actino-658 do not target clade 1 and a significant portion of clade 2, while HGC69a and HGC236 fail to target branched rods and thin filaments, resulting in an underestimation of the total abundance of *Tetrasphaera* [29]. In 2006, McKenzie and coworkers proposed that *Ca. Nostocoida limicola* (NLIMIII175 probe) should be re-classified as three novel species belonging to the genus *Tetrasphaera*: *Tetrasphaera jenkinsii*, *Tetrasphaera veronensis*, and *Tetrasphaera vanveeni* [27,99]. Later, FISH probes were designed to target the different clades in *Tetrasphaera*: Tet1-266 for clade 1, Tet2-892 and Tet2-174 for clade 2, and Tet3-654 for clade 3 [29]. However, in this classification, *Tetrasphaera japonica* does not belong to the described clades [95,96,98].

With the discovery of other genera within the organisms initially classified as *Tetrasphaera*, new FISH probes were developed to improve accuracy and avoid unintentional targeting of these additional genera. Therefore, Tetra67 specifically targeted clade 1 *Tetrasphaera* species, such as *Tetrasphaera elongata*, *Tetrasphaera japonica*, and *Tetrasphaera Hjor*. Tetra732, on the other hand, is designed to target certain *Tetrasphaera* organisms from clade 2 [14]. Furthermore, Tetra183 has been developed to target a wide range of *Tetrasphaera*-related organisms, covering both clade 1 and clade 2. Specific FISH probes have also been developed to target the recently discovered *Ca. Phosphoribacter* genera, with Phos1260-23S-Pbr1 designed for *Ca. Phosphoribacter baldrii* and Phos601 for *Ca. Phosphoribacter hodrii* [14].

In 2007, the BET135 probe became the first FISH probe targeting *Dechloromonas* with the capability for PolyP accumulation [97]. Subsequently, those FISH probes were coupled with Raman spectroscopy to detect the P uptake and release cycles, elucidating the PAO activity in *Ca. Dechloromonas phosphoritropha* and *Ca. Dechloromonas phosphorivorans* [12]. However, these FISH probes are not species-specific and may hybridize with other *Dechloromonas* species not associated with PAO activity.

As mentioned above, WWTP-relevant PAOs typically reside within complex communities and cannot be readily isolated. For identifying known PAOs, the prevailing methods include utilizing specialized FISH probes to target the desired PAO(s) and subsequently detecting PolyP, mainly through Raman microspectroscopy [49]. In addition, metagenomics has proven valuable in discovering novel PAOs within complex microbial communities present in WWTPs. The procedure involves using high-quality MAGs to design FISH probes that target microorganisms carrying genes related to PolyP metabolism and P transport [12]. Then, Raman microspectroscopy is employed to verify the dynamic levels of PolyP during anaerobic-aerobic conditions in the cells targeted by the novel FISH probes (Fig. 3) [12].

Furthermore, novel approaches have been proposed for identifying novel PAOs. One suggestion involves employing a trait-based comparative omics approach, integrating genomics, transcriptomics, and proteomics data [100]. This method aims to identify gene expression patterns associated with specific traits, such as P-cycling. However, the specific trait accurately reflecting

the PAO activity is still uncertain [100]. Moreover, recent analysis suggests that proteomics may offer a potential avenue for identifying and measuring the abundance of PAOs [101].

To summarize, various methods exist for PAO detection, and the choice of the most suitable method depends on the specific objective. For the detection of known PAOs, sequencing methods enable their classification and abundance quantification. In this context, metagenomic analysis offers improved phylogenetic resolution, whereas 16S rRNA amplicon sequencing provides a faster and more cost-effective genus identification [12,13,21]. For visualizing the distribution of PAOs within sludge, it is recommended to utilize FISH probes designed for the desired genera in conjunction with Raman spectroscopy to confirm the presence of PolyP [12,49]. Lastly, the FISH-Raman method is recommended for identifying novel PAOs, as it enables simultaneous detection and quantification of PolyP and specific PAO genera [12,14,49].

5. Conclusions

Considering the significance of PAOs in WWT, this article reviews the literature and analyzes the MAG annotations from WWT-relevant PAOs to provide valuable insights into their metabolic capabilities. The main conclusions derived from this review are.

- *Ca. Accumulibacter* and *Ca. Dechloromonas* exhibit similar metabolic capabilities. Under anaerobic conditions, they can utilize PolyP to generate an electrochemical gradient for VFAs and C4-dicarboxylates uptake; likewise, they can use PolyP and glycogen for PHA synthesis. Under oxic conditions, they can use PHA for glycogen synthesis.
- *Tetrasphaera*, *Ca. Phosphoribacter*, *Knoellia*, and *Phycococcus* share similar metabolic capabilities. Under anaerobic conditions, they utilize PolyP to generate an electrochemical gradient, enabling the uptake of sugars and amino acids through electrochemical potential-driven transporters. Additionally, they can use PolyP for sugar phosphorylation. Furthermore, these genera have been reported to possess fermentation capacity, with *Tetrasphaera* exhibiting the ability to couple this process with P removal capabilities. Under aerobic conditions, they have the potential to produce glycogen via maltose. However, further research is essential to understand energy production mechanisms and unveil the yet unknown carbon storage molecules in these genera.
- This review describes the most relevant FISH probes developed for PAO detection and highlights the limitations of current methods in accurately reflecting the diversity of PAO subgenera and distinguishing PAOs from non-PAOs. It emphasizes the importance of developing comprehensive and reliable markers to better understand their diversity and identify PAO indicators to accurately forecast their activity.

CRediT authorship contribution statement

Lucía Ruiz Haddad: Conceptualization, Writing - Original Draft, Methodology, Formal Analysis. **Muhammad Ali:** Conceptualization, Supervision, Writing - Review & Editing. **Mario Pronk:** Writing - Review & Editing, Supervision. **Mark C.M. Van Loosdrecht:** Writing - Review & Editing, Supervision. **Pascal E. Saikaly:** Conceptualization, Project Administration, Supervision, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing

financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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