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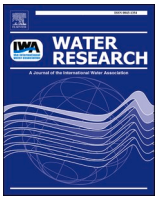

The phototrophic metabolic behaviour of *Candidatus* accumulibacter

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ABSTRACT

The phototrophic capability of *Candidatus* Accumulibacter (*Accumulibacter*), a common polyphosphate accumulating organism (PAO) in enhanced biological phosphorus removal (EBPR) systems, was investigated in this study. *Accumulibacter* is phylogenetically related to the purple bacteria *Rhodocyclus* from the family Rhodocyclaceae, which belongs to the class Betaproteobacteria. *Rhodocyclus* typically exhibits both chemoheterotrophic and phototrophic growth, however, limited studies have evaluated the phototrophic potential of *Accumulibacter*. To address this gap, short and extended light cycle tests were conducted using a highly enriched *Accumulibacter* culture (95%) to evaluate its responses to illumination. Results showed that, after an initial period of adaptation to light conditions (approximately 4–5 h), *Accumulibacter* exhibited complete phosphorus (P) uptake by utilising polyhydroxyalkanoates (PHA), and additionally by consuming glycogen, which contrasted with its typical aerobic metabolism. Mass, energy, and redox balance analyses demonstrated that *Accumulibacter* needed to employ phototrophic metabolism to meet its energy requirements. Calculations revealed that the light reactions contributed to the generation of, at least more than 67% of the ATP necessary for P uptake and growth. Extended light tests, spanning 21 days with dark/light cycles, suggested that *Accumulibacter* generated ATP through light during initial operation, however, it likely reverted to conventional anaerobic/aerobic metabolism under dark/light conditions due to microalgal growth in the mixed culture, contributing to oxygen production. In contrast, extended light tests with an enriched *Tetrasphaera* culture, lacking phototrophic genes in its genome, clearly demonstrated that phototrophic P uptake did not occur. These findings highlight the adaptive metabolic capabilities of *Accumulibacter*, enabling it to utilise phototrophic pathways for energy generation during oxygen deprivation, which holds the potential to advance phototrophic-EBPR technology development.

1. Introduction

Enhanced biological phosphorus removal (EBPR) processes have demonstrated their efficacy in wastewater treatment plants (WWTPs) for phosphorus (P) removal and recovery (Oehmen et al., 2007a; Yuan et al., 2012). These processes involve the growth of polyphosphate accumulating organisms (PAOs) in activated sludge through sequential anaerobic-aerobic zones (Smolders et al., 1995). *Candidatus* Accumulibacter (referred to as *Accumulibacter* hereafter) is one of the major groups of PAOs in EBPR systems. During the anaerobic phase, *Accumulibacter* metabolise organic carbon, typically volatile fatty acids (VFAs), and store it as polyhydroxyalkanoates (PHA) (Smolders et al., 1994a). This conversion relies on the energy obtained from the hydrolysis of glycogen and polyphosphate (poly-P). Subsequently, in the aerobic phase, *Accumulibacter* oxidise PHA to generate energy for replenishing glycogen reserves, supporting biomass growth, and obtaining ATP for P uptake (Oehmen et al., 2007a; Smolders et al., 1994b).

Accumulibacter is a member of the order Rhodocyclales and family Rhodocyclaceae, and it is phylogenetically closely related to *Rhodocyclus* (Crocetti et al., 2000; Hesselmann et al., 1999). *Rhodocyclus* is known to exhibit phototrophic growth within the group of purple phototrophic bacteria (PPB), however, the light impact on *Accumulibacter* remains largely unexplored. Hesselmann et al. (1999) attempted to differentiate *Accumulibacter* from *Rhodocyclus* using a simple light test that indicated that *Accumulibacter* did not demonstrate growth in the presence of light. Nevertheless, subsequent research over the last 15+ years (Camejo et al., 2016; Kolakovic et al., 2021; Skennerton et al., 2015) have demonstrated that *Accumulibacter* is a diverse group of organisms,

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consisting of two types, each with multiple clades and sub-clades. One possible explanation could be that the selected *Accumulibacter* in Hesselmann et al. (1999) did not possess phototrophic capabilities.

PPB perform anoxygenic photosynthesis and synthesise light harvesting complexes (LHC) containing light-absorbing pigments such as bacteriochlorophyll (Bchl) *a* or *b* and carotenoids (Madigan and Jung, 2009). Previous metagenomic studies have identified phototrophic genes in *Accumulibacter*, suggesting the potential for phototrophic growth. These genes include those involved in the calvin-benson-bassham (CBB) cycle and certain genes responsible for translation to produce LHC proteins such as *pucA* and *pucB* (García Martín et al., 2006; Skennerton et al., 2015). The presence of these genes suggests the need for further investigation to understand if *Accumulibacter* can perform phototrophic metabolic activity, or if these genes are effectively dormant within *Accumulibacter* cells.

Phototrophic EBPR systems involving *Accumulibacter* have been developed in combination with microalgae (Carvalho et al., 2019, 2018). These systems operate with alternating dark/light cycles to select a microbial consortium containing both PAOs and photosynthetic organisms, primarily microalgae, which contribute towards oxygen production in the system that can be utilised by *Accumulibacter* PAOs in their aerobic metabolism. However, the implementation of photo-EBPR could be complex and challenging, requiring the enrichment and maintenance of both bacteria and algae (Carvalho et al., 2023). Therefore, it is of interest for phototrophic EBPR system development to assess if light can activate phototrophic metabolism in *Accumulibacter*.

In addition to *Accumulibacter*, another group of PAOs is related to *Tetrasphaera* (note that some members of clades I and III have been reclassified as *Phycicoccus* and *Phosphoribacter*, respective, (Singleton et al., 2022) and are referred to as *Tetrasphaera*-related organisms here. These PAOs are also present in a high abundance in full scale EBPR plants (Fernando et al., 2019; Stokholm-Bjerregaard et al., 2017). Therefore, it is of relevance to explore the impact of light on their metabolism as well. *Tetrasphaera* is within the Intrasporangiaceae family (Hanada et al., 2002; Kong et al., 2005). Although this family has varying metabolic capabilities, ranging from aerobic to facultative anaerobic metabolism (Stackebrandt et al., 2014), there is no evidence regarding their capacity for phototrophic metabolism.

The aim of this study is to investigate if exposing *Accumulibacter* to light, without external oxygen supply, would have a quantitative impact on the performance and stoichiometry of *this* PAOs related microorganism. A highly *Accumulibacter* culture (inoculated from Kolakovic et al., 2021) was subjected to light exposure without additional aeration, and the microbial response to these conditions was monitored over time. An extended test was then performed to assess the long-term behaviour of the culture under dark/light conditions. A similar experimental approach was employed using an enriched culture of *Tetrasphaera* (inoculated from Nguyen et al., 2023) for comparative purposes. Furthermore, a metabolic model was assembled to compare the obtained experimental data with the theoretical metabolic pathways, allowing for a deeper analysis of *Accumulibacter's* potential for phototrophy. To the best of our knowledge, this is the first study designed to examine the impact of light on *Accumulibacter*, moreover it quantitatively assesses the stoichiometry of *Accumulibacter* during the light phase, thereby enhancing our understanding of its capabilities under illumination.

2. Materials and methods

2.1. Accumulibacter EBPR reactor

A sequencing batch reactor (SBR) with 2 L working volume was inoculated with sludge from a WWTP in Lisbon, Portugal. The SBR was operated with 6-hour cycles, including an anaerobic phase (2 h), aerobic phase (2.5 h), settling and decanting (1.5 h) phases and argon sparging to guarantee anaerobic conditions before the anaerobic feed. The reactor was fed in the beginning of the anaerobic phase with 0.750 L of synthetic phosphate medium, whereas 0.250 L of synthetic carbon and nutrient solution was fed continuously during 50 min of the anaerobic phase, after the phosphate feed. This slow carbon feed strategy, which mimics continuous feed in WWTPs, has previously been shown to confer a competitive advantage to *Accumulibacter* over glycogen-accumulating organisms (GAOs) (Tu and Schuler, 2013) and is suitable for *Accumulibacter* enrichment, as indicated by Kolakovic et al. (2021). The reactor was operated with a hydraulic retention time (HRT) of 12 h and a sludge retention time (SRT) of 10 days. Temperature was controlled at 20 ± 1 °C and pH at 7.5 using 0.1 M HCl. Argon and air were continuously bubbled to achieve anaerobic or aerobic conditions, respectively (Kolakovic et al., 2021). The chemical oxygen demand (COD) in the feed was 300 mg/L, where the carbon source was a mixture of acetate and propionate (75% / 25% of COD) to promote the proliferation of PAOs over GAOs (Lopez-Vazquez et al., 2009). P concentration in the feed medium was 44 mg P/L, leading to an initial P concentration in the reactor of 20 mg/L. Allylthiourea (ATU) was also added to the feed (2.93 mg/L) as a nitrification inhibitor. See Kolakovic et al. (2021) for further details on the SBR operation and the feed composition.

2.2. Phototrophic tests

2.2.1. Accumulibacter enriched biomass

The light tests were performed using a highly enriched *Accumulibacter* biomass, with around 10% of Type I and 85% of Type II *Accumulibacter* (Kolakovic et al., 2021). The remaining sludge composition included 2% of GAOs (*Competibacter*) and ammonia and nitrate oxidizing organisms (AOB & NOB) respectively (Table S1). Moreover, other GAOs including *Defluviicoccus* and *Propionivibrio*, were never found at levels exceeding 1 % (Kolakovic et al., 2021). With the aim to explore the metabolic response of *Accumulibacter* under non-aerated illuminated conditions, a highly enriched sludge was exposed to light after a dark anaerobic phase. Triplicate batch tests were performed in a 500 mL SBR. 200 mL of *Accumulibacter* enriched biomass was taken from the parent SBR at the end of the anaerobic phase and was placed into a 500 mL SBR with argon sparging in the headspace, ensuring anaerobic conditions inside the reactor. Additionally, dissolved oxygen (DO) measurements confirmed anaerobic conditions in the reactor (See Fig. 1a). The DO probe utilised in this study was manufactured by Inpro, which offers an accuracy of ±1% for readings above 6 ppb.

The culture was subjected to a light intensity of 84 W/m^2, which corresponded to 5.6 W/L, for 19.5 h. Afterwards, in dark anaerobic conditions, the SBR was fed with phosphate and carbon medium (same conditions as in the parent SBR) and was kept for 3 h in dark anaerobiosis. Light was then turned on and the culture was subjected to light for 11.5 h. Control tests, where the same *Accumulibacter* enriched biomass was only subjected to 26 h dark conditions (with argon sparging in the headspace), were also carried out.

2.2.2. Extended test

To understand the long-term effects of illumination on the enriched *Accumulibacter* culture, a 21-day test was also performed. A 0.5 L SBR reactor was inoculated with 250 mL of sludge from the *Accumulibacter* SBR and operated with a 12-hour cycle, comprising 2 h of dark/anaerobic conditions, 8.5 h of light, a 1-hour settling period, 10 min of decantation, and a 20-minute idle period. During the dark phase, argon was sparged in the mixed liquor to guarantee that the culture was in anaerobiosis when carbon started to be fed. The culture was subjected to a light intensity of 84 W/m^2, which corresponded 5.6 W/L, with a SRT of 10 days and an HRT of 24 h. SRT was controlled through manually wasting sludge, accounting for the volume removed from the reactor during sampling campaigns. The feed conditions were the same as those used in the parent SBR. This test was repeated with a *Tetrasphaera* enriched sludge (Nguyen et al., 2023), where more information on the *Tetrasphaera* extended test can be found in supplemental information.

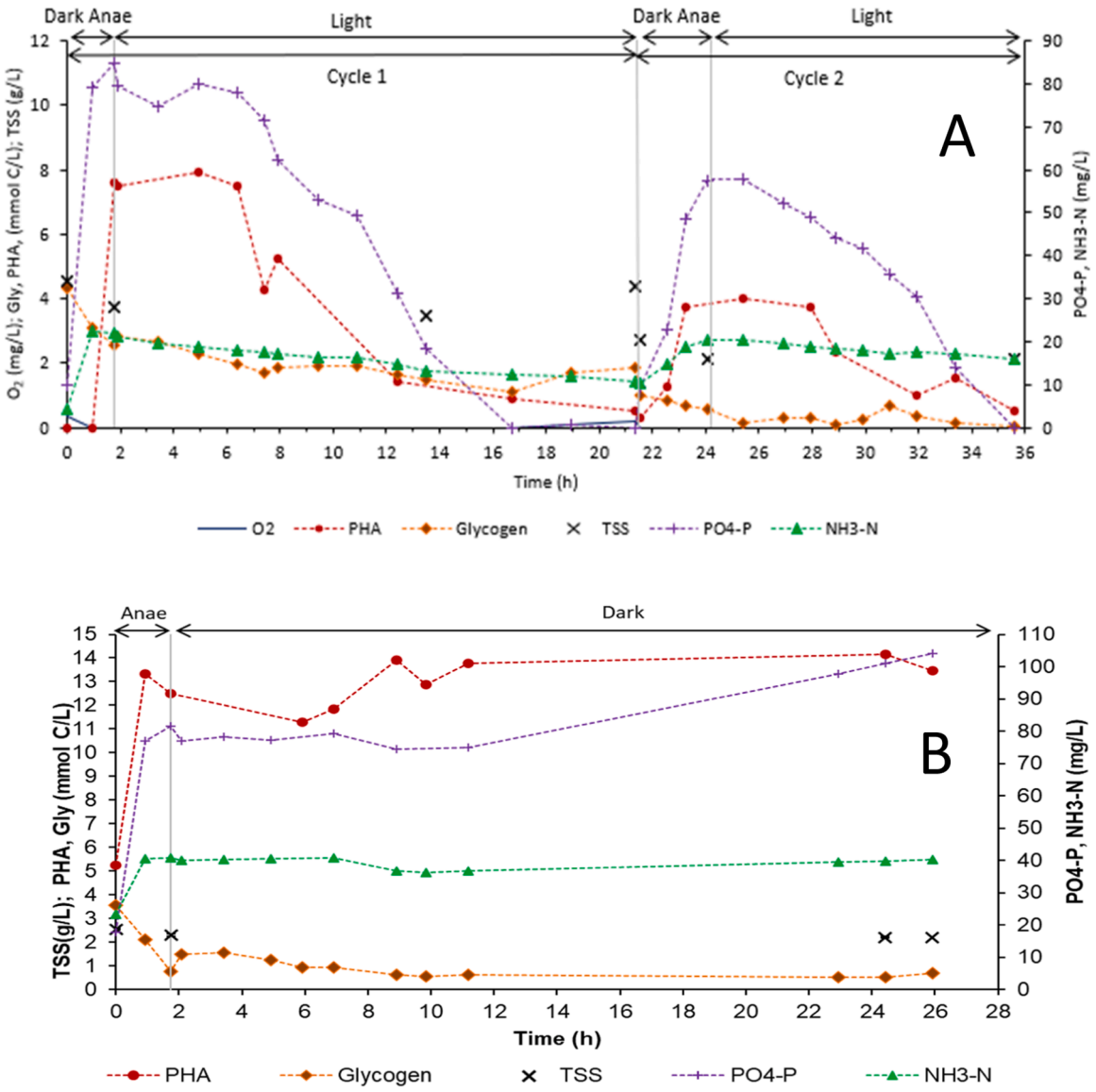

Fig. 1. The profiles of dark/light/dark/light anaerobic cycles of the enriched Accumulibacter biomass (A). The first dark anaerobic phase occurred in the parent reactor. Dark starvation test with enriched Accumulibacter biomass (B). VFAs were not detected throughout the tests, due to the slow carbon feed applied during the anaerobic period.

2.3. Analytical methods

Acetate and propionate were determined by high-performance liquid chromatography (HPLC), using a VWR Hitachi Chromaster with a Bio-rad Aminex HPX-87H 300 × 7.8 MM column and a DAD detector. 0.01 N sulfuric acid was used as eluent, with an elution rate of 0.5 mL/min and an operating temperature of 30 °C.

Phosphate and ammonia concentrations were determined by colorimetric methods implemented in a flow segmented analyser (Skalar 5100, Skalar Analytical, The Netherlands). For the cell poly-P content, acidic digestion of a sample from the end of the aerobic period was performed with 0.3 M H_2SO_4 and 400 mg of $K_2S_2O_8$ and analysed using the flow segmented analyser. PHAs were determined by gas chromatography (GC) using the method described by (Lanham et al., 2013b) using a Bruker 430-GC gas chromatograph equipped with a FID detector and a Restek column (60 m, 0.53 mm internal diameter, 1 μM df, crossbond). Standards for PHA, PHB, and PH2MV were consistently employed during sample analysis to confirm elution times. Carbohydrates were determined by acid digestion as described by (Lanham, 2012) with 0.9 M HCl during 3 h, where the supernatant was analysed by HPLC with a VARIAN Metacarb 87H column and a Merck Differential Refractometer RI-71 detector. 0.01 N sulfuric acid was used as eluent, with an elution rate of 0.5 mL/min and an operating temperature of 30 °C. When microalgae are present in the biomass, as in the case of the extended tests, carbohydrates correspond to the measurement of glycogen from bacteria and starch from the microalgae. total suspended solids (TSS) and volatile suspended solids (VSS) were calculated according to standard methods (APHA, 1998). The light intensity provided by the halogen lamp was measured using a LI-COR light meter (LI-250 A), equipped with a pyranometer sensor LI-200 SA.

To determine the presence of pigments in the *Accumulibacter* and *Tetrasphaera* cultures, chlorophyll and bacteriochlorophyll extraction were performed using the biomass pellet. 1 mL of ethanol (95% V/V) was added to the pellet of 1 mL of the biomass centrifuged, vortexed and incubated overnight at room temperature in dark conditions. Afterwards, samples were again centrifuged at 8000 g for 5 min and the absorbance spectrum of the supernatant was measured using an Ultrospec 2100 pro Amersham spectrometer. Chlorophyll and bacteriochlorophyll pigment concentrations were calculated according to Ritchie (2018).

2.3.1. Calculation of stoichiometric parameters

Phosphate release ($P_{release}$ in mg-P/L) was calculated as the difference between the phosphorus concentration in the end of the dark phase and the phosphorus concentration in the beginning of the dark phase. Total phosphate uptake (P_{uptake} in mg-P/L) was calculated as the difference between the phosphorus concentration in the end of the light period and the phosphorus concentration in the beginning of the light period. To determine the cell poly-P content, the supernatant phosphate concentration was subtracted from the total phosphate concentration obtained by sample digestion.

2.4. Microbiological analysis

2.4.1. Genomic database information

The information about the proteins and genes already detected and reported in the sequenced genomes of *Accumulibacter* and *Tetrasphaera* were downloaded from UNIPROT (https://www.uniprot.org/uniprotkb?query=accumulibacter for *Accumulibacter* and https://www.uniprot.org/uniprotkb?query=tetrasphaera for *Tetrasphaera*). Phototrophic proteins and genes from all available genomes were assessed.

2.4.2. FISH analysis

Phylogenetic analysis of the bacterial community was performed through fluorescence in situ hybridization (FISH) as previously described by (Amann, 1995), on fixed samples with 4 % paraformaldehyde or ethanol, according to (Nielsen, 2009). The oligonucleotide probes used were the fluorescein isothiocyanate (FITC)-labelled EUBmix (EUB338, EUB338II, EUB338III) for all bacteria, applied with the cyanine 3 (Cy3)-labelled probes: PAOmix (PAO651, PAO462, PAO846) for *Candidatus* Accumulibacter Phosphatis; Acc-I-444 which targets type I *Accumulibacter* PAOs and Acc-II-444 for *Accumulibacter* PAOs type II; CPB_654 for *Candidatus* Competibacter. Tet1–126, Tet2–892, Tet2–147 and Tet3–654 for *Tetrasphaera.* Grb for *Rhodobacter & Roseobacter* and RHC439 for *Rhodocyclus*. Nso1225 for AOB and Nit3 for NOB. More details are available at probeBase 2016. The biomass samples were visualized using a Zeiss Imager D2 epifluorescence microscope (Germany), at 1000 X amplification.

3. Results and discussion

3.1. Effects of light on Accumulibacter physiology

Illuminated tests were performed using a highly enriched *Accumulibacter* biomass, which illustrated a P uptake rate of 85 mg P/L.h during the aerobic phase, with concurrent PHA consumption and replenishment of glycogen (Figure S1) (Kolakovic et al., 2021), which is consistent with a PAO metabolism.

At the start of the test (Fig. 1a), the culture exhibited typical PAO activity under anaerobic conditions, characterised by the P release (resulting in decreasing TSS) and the formation of PHA. When the culture was exposed for the first time to light without aeration (cycle 1), glycogen was consumed even in the presence of high levels of PHA, and no P uptake was observed during the first 5 h hours of light exposure (Fig. 1a). Similar behaviour was observed in the other two repeated dark/light/dark/light anaerobic cycles batch tests, as illustrated in Figure S2, as well as another dark/light/dark cycle test (Figure S3), all indicating a lag time of 4 – 7 h before phosphorus uptake occurred. PHA is the preferred energy source for *Accumulibacter* PAOs under aerobic conditions (Lopez et al., 2006; Smolders et al., 1994b; Vargas et al., 2013). The glycogen hydrolysis, together with low PHA consumption and scant P uptake, suggested that glycogen was being utilised as an energy source for cell maintenance purposes as suggested in Lu et al. (2007). During this period, *Accumulibacter* was likely adapting to light exposure and activating its metabolic pathways to convert light to ATP. After 5 h of light exposure, PHA hydrolysis increased, along with P uptake (Fig. 1a). This suggests that after the initial adaptation period, P uptake was driven by light. It was hypothesised that *Accumulibacter* was able to harness ATP from light for P uptake, which was evaluated by metabolic model analysis (see Section 3.3). The ammonia consumption, without nitrite or nitrate production, indicated that the ATP was also consumed for cellular growth, as ATU inhibited nitrification in the SBR. The consistent measurement of DO at 0 mg/L throughout the light phase indicates limited oxygen availability. This scarcity is further suggested by the continuous consumption of glycogen during the light phase, implying a limitation in oxygen produced by any photosynthetic organisms present, which would then have led to energy production by *Accumulibacter*. After P being fully consumed, about 0.77 Cmmol/L of glycogen was formed, which is consistent with the oxygen concentrations of 0.2 mg/L detected at the end of the light phase. Additionally, this glycogen synthesis was correlated to the amount of PHA consumption during this period. However, the minimal replenishment of glycogen at the end of the light phase could lead to its depletion in further cycles.

A new cycle commenced (cycle 2) after the culture was exposed to light for more than 19 h (Fig. 1a). The anaerobic phase was extended to 3 h to guarantee that all the organic carbon was consumed. One hour after the light was turned on, P uptake commenced, coupled with PHA hydrolysis. However, in contrast to aerobic *Accumulibacter* -metabolism, there was no glycogen production. Considering only the period where P uptake occurred, a similar P uptake rate was achieved: 5.6 mg P/L.h in cycle 1 and 5.1 mg P/L.h in cycle 2 (Fig. 1a). Nevertheless, the second cycle exhibited a shorter lag time, with P uptake initiating after only approximately 1 hour, as opposed to the 5-hour delay observed in the first cycle. The study of Zeng et al. (2003a), though focused on PAO adaptation to denitrifying conditions instead of adaptation to illuminated conditions, also observed a similar lag time required for P uptake when they exposed an anaerobic/aerobic *Accumulibacter* culture to an extended anoxic phase for the first time. The culture took 5 h to begin consuming nitrate and performing P uptake during the first cycle after their operational change. In the second cycle of that study, no adaptation phase was needed, which was also consistent with our observations for phototrophic P uptake. Zeng et al. (2003a) indicated that the 5-hour lag phase preceding anoxic P uptake was necessary for enzyme synthesis/acclimatisation, which could potentially explain the observed lag phase prior to phototrophic P uptake in the initial cycle subjected to illumination.

Control tests were also performed, where the biomass was kept in the dark after the anaerobic phase (Fig. 1b) or after a light phase (Figure S3). PHA production, glycogen consumption and poly-P hydrolysis were observed in each case, without sulfate consumption over the light and dark phase. These results are expected when PAOs are exposed to anaerobic starvation conditions (Lopez et al., 2006; Lu et al., 2007; Vargas et al., 2013). Thus, light exposure was suggested to be the trigger leading to P uptake in the enriched *Accumulibacter* sludge in the illuminated tests.

The potential impact of light on *Accumulibacter* metabolism may find support in the data derived from the sequenced genomes. The published genomes (UNIPROT) of *Accumulibacter* were analysed to identify phototrophic proteins/genes. The essential genes required for light harvesting in *Rhodocyclus* are involved in the biosynthesis of pigments (*bch* genes, (Igarashi et al., 2001)), assembly of a phototrophic reaction centre and light-harvesting complexes (*puf/puc* genes, (Bélanger and Gingras, 1988; Tichy et al., 1989)). In addition, *RegA/RegB* have been identified to control the expression of genes involved in the synthesis of the phototrophic gene cluster (Alberti et al., 1995).

Some phototrophic genes were identified from sequenced *Accumulibacter* genomes (Table S4), such as *RegA/RegB*, and *puc* and proteins, including: Photosystem I P700 chlorophyll apoprotein A2 (EC 1.97.1.12), Purine catabolism protein *pucB*, Putative xanthine dehydrogenase subunit A protein (pucA), Photosystem I assembly protein Ycf3 and Photosystem reaction center subunit H. The *puf* gene encoding the RC-LH1 core unit (Bélanger and Gingras, 1988) was not sequenced in

the current *Accumulibacter* genomic database, which could be a sequencing gap from previous studies. Nonetheless, all the phototrophic genes identified and observed light activity in the experiment suggesting that *Accumulibacter* could possess the capability to perform phototrophic activity.

3.2. Long term phototrophic capability of Accumulibacter versus Tetrasphaera

To better understand *Accumulibacter's* ability to grow without the need for aeration in the presence of light over an extended period, an extended light test was conducted. A similar test was also performed with an enriched *Tetrasphaera* PAO culture, which serves as a point of comparison. The results obtained from day 1, as illustrated in Fig. 2, were consistent with those from the previous short-term light cycle tests. Glycogen and PHA were consumed along with the P uptake. However, the P uptake was not fully completed on day 1. One possible explanation for this is that the culture may not have fully adapted to phototrophic metabolism, as it was evident from the 5-hour delay observed in Fig. 1a that was needed prior to PHA consumption and P uptake after the culture was first exposed to light. It is plausible that the duration of the light phase was insufficient to fully adapt the *Accumulibacter* enriched culture towards phototrophic metabolism, resulting in limited ATP production for complete P uptake.

The initial low levels of ammonia consumption, measured at 3 mgN/L on the first day and 4 mgN/L on day 3, indicate that the ammonia was primarily utilised for growth purposes, similar to the short light cycle test in the previous section (See Fig. 1a). Throughout the extended light test, there was a gradual change in the reactor's colour to green, as shown in Figure S4, coinciding with the elevated chlorophyll (microalgae) content presented in Table 1. It is noteworthy that no algae inoculum was added to any of the tests, and the parent enriched *Accumulibacter* reactor was covered with fabric material to prevent light penetration and algae growth, as detailed by Kolakovic et al. (2021). Hence, the observed algae growth in the test occurred naturally. Although microalgae proliferated in the test, the P uptake by microalgae was minimal. As mentioned in Powell et al. (2011) the highest observed P content in microalgae is 3.85%, with an average of 1%. Considering the maximum P content in microalgae, the minimal uptake by microalgae would only account for 0.27 mgP/L and 1.43 mgP/L on day 1 and 3, respectively, which was lower than observed total P uptake of 14 mgP/L and 48 mgP/L. Thus, we conclude that the majority of the P uptake was executed by *Accumulibacter*, rather than microalgae.

By measuring the changes in chlorophyll concentration, it was possible to estimate the amount of oxygen generated by microalgae (See 3.3 for more details on this calculation). Taking into account the total ATP consumption, including P uptake, cell growth and aerobic cell maintenance, the oxygen requirements during each illuminated day can be estimated, as shown in Table 1. Despite the continuous increase in chlorophyll and oxygen production throughout the test, the available oxygen proved insufficient to meet the demand of *Accumulibacter's* aerobic metabolism on days 1 and 3. This suggests the need for an alternative energy source to fulfil the ATP requirements of *Accumulibacter*, where we investigated the impact of phototrophic metabolism, see Section 3.3 for further elaboration.

Given that PPB like *Rhodocyclus* and *Rhodobacter* are anoxygenic and do not produce oxygen during phototrophic metabolism (Madigan and Jung, 2009; Wang et al., 2020) and considering the phylogenetic relationship of *Accumulibacter* with *Rhodocyclus*, we hypothesised *Accumulibacter* would similarly perform an anoxygenic photosynthesis phototrophic metabolism and that microalgae to would be the producer of oxygen in this system. The available oxygen in the system was abundant compared to the required amount, indicating that *Accumulibacter* had sufficient oxygen for its aerobic metabolism, from day 7 onwards (See Table 1). This suggests the successful formation of photo-EBPR, where a combination of PAOs and algae were enriched, similar to previous studies (Carvalho et al., 2019, 2018). Nevertheless, the selection of photo-EBPR in this study occurred much faster than in both previously reported studies, which took 29 to 14 days for photo-EBPR selection. This accelerated selection process could be

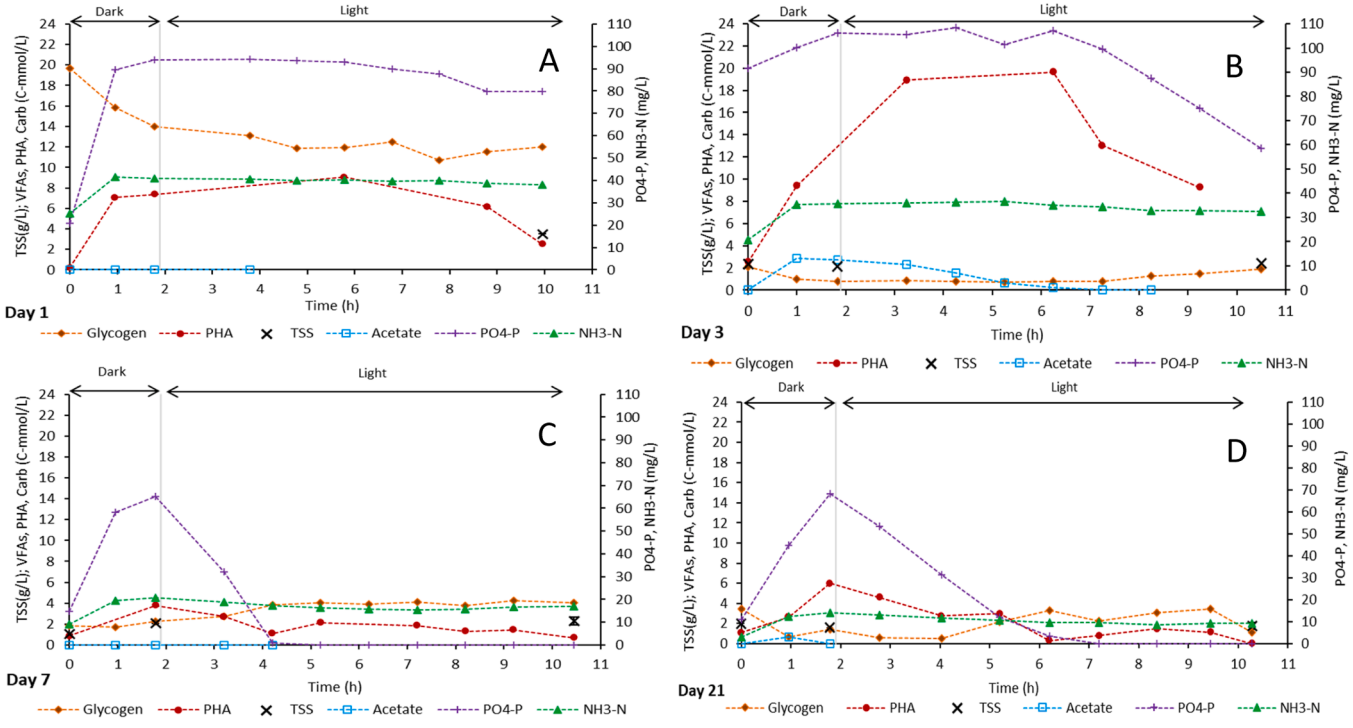

Fig. 2. Dark and light transformations during a photo-EBPR cycle during the 21-day test enriched Accumulibacter biomass. Day 1 (A), Day 3 (B), Day 7 (C), Day 21 (D).

Table 1
P release and uptake transformations during the 21-day illuminated test as shown in Fig. 2, with the corresponding chlorophyll quantity and microbial community composition.

	$P_{release}$	P_{uptake}		P content Biomass	Chlorophyll	Bacterio-chlorophyll	Microalgae DO production Max (Min)[1]	*Accumulibacter* DO requirement[2]	FISH			
	mg P/L	mg P/L	mg P/L.h	%	mgChl/gVSS	mgChl/gVSS	$mmolO_2$/L		PAO mix	CPB 654	GRb	RHC439
Day 1	73	14	2.7	11	0.10	0.02	0.81 (0.10)	2.56	+++	+	+	++
Day 3	15	47	7.8	5.3	0.66	0.02	4.45 (0.56)	3.42				
Day 7	50	64	26	9.3	2.9	n.d.	18.11 (2.26)	3.43				
Day 10	58	74	24	12	3.1	n.d.	18.21 (2.28)	3.57	++	++	+	++
Day 14	58	78	13	10	5.1	n.d.	23.02 (2.88)	3.64				
Day 21	57	68	15	12	9.2	0.03	22.65 (5.03)	3.08	++	+	+	++

n.d. not detected. (-) non-existent; (+-) almost non-existent; (+) present; (++) abundant; (+++) dominant. Probes: PAOmix (PAO651, PAO462, PAO846) for *Candidatus* Accumulibacter *phosphatis*; CPB_654 for *Candidatus Competibacter phosphatis*; GRb for *Rhodobacter* and *Roseobacter*; RHC 439 for Rhodocyclus.
[1] The DO production by microalgae, using the SOPR of microalgae Max: 400 and low: 50 $molO_2$/mole chl.hr as suggested in Myers and Graham (1971), through R7;.
[2] DO requirement estimated for *Accumulibacter* aerobic metabolism, using R6.

attributed to the high abundance of *Accumulibacter* and their phototrophic capability. From day 7 onwards, the chlorophyll concentration and the potential oxygen production increased significantly, reaching abundant levels that could support the conventional metabolism of *Accumulibacter*. Consequently, it is likely that *Accumulibacter* would cease its phototrophic metabolism and transition back to its aerobic metabolism during the light phase of the cycle in this scenario.

Tetrasphaera, another abundant group of PAOs in full-scale WWTPs (Lanham et al., 2013a; Stokholm-Bjerregaard et al., 2017), was also investigated to determine if it possesses the capability to develop phototrophic metabolism similar to *Accumulibacter* (for further details about the test conditions, see supplementary material, Section 2). However, after more than 150 days of operation, the enriched *Tetrasphaera* sludge did not exhibit notable phototrophic behaviour. Additionally, the selection of a culture capable of photo-EBPR from the *Tetrasphaera* enrichment did not occur, as microalgae did not grow (Table 2). It is possible that microalgae could not utilise the amino acids that were fed to *Tetrasphaera* and instead preferred the acetate and propionate fed to the *Accumulibacter* reactor. The absence of microalgae limited oxygen production in the test; therefore, aeration was turned on during the light phase to promote P uptake and prevent the washout of *Tetrasphaera* and understand if an acclimation period were necessary to induce phototrophy. Minimal P release and uptake were observed under dark/light cycling compared to anaerobic/aerobic cycling, even with the addition of air in the final light phase. Additionally, phototrophic and autotrophic genes/proteins could not be found in the UNIPROT genome database for *Tetrasphaera*. These results support the conclusion that phototrophic P uptake and the selection of photo-EBPR should not be expected for this group of PAOs.

The extended light test results indicated that *Accumulibacter* is capable of switching between two different metabolisms. When oxygen is available, *Accumulibacter* consumes the oxygen to perform P uptake with PHA consumption and glycogen replenishment (Fig. 2D and Figure S1). When oxygen is restricted, but light is available, *Accumulibacter* can obtain ATP phototrophically (Fig. 1a) after a period of adaptation. The rapid adaptability of *Accumulibacter* to both oxygenic and anoxygenic illuminated conditions for energy generation is of great interest in the development of phototrophic EBPR systems. Moreover, targeting *Accumulibacter* for such systems appears advantageous over *Tetrasphaera* type PAOs, as the former possess the capability for phototrophic P uptake. However, the observed decrease in TSS and VSS (Table S2), along with the decreased *Accumulibacter* abundance from FISH quantification (Table 1) throughout the extended light test, suggests a potential washout of *Accumulibacter*, likely attributed to microalgae proliferation. Further investigation into conditions that can mitigate microalgae proliferation while promoting *Accumulibacter*'s phototrophic metabolism is warranted.

3.3. Metabolic model assessment of the phototrophic behaviour of *Accumulibacter*

To assess if *Accumulibacter* within the enriched culture were indeed responsible for the phototrophic behaviour found experimentally, mass, redox and energy balances were performed based on the known and hypothesised metabolic reactions of *Accumulibacter* (See Supplemental Information Section 1 for more descriptions). The proposed metabolic reactions during the light are summarised in Table 3 and involve a combination of anaerobic, aerobic from *Accumulibacter* and known reactions in microalgae and PPB.

Reaction 1 (R1) is typically observed under anaerobic conditions for *Accumulibacter*, but was incorporated into the proposed reaction set due to the glycogen consumption observed during the light phase. Reactions 2–6 are typically employed for PHA consumption (R2), biomass synthesis (R3), P uptake (R4), maintenance (R5) and oxidative phosphorylation (R6) by *Accumulibacter*, as described in literature (Oehmen et al., 2007b; Zeng et al., 2003b), where R2, R4 and R6 were observed typically under aerobic conditions. While PHA consumption (R2) was adapted from aerobic models (or anoxic models, where nitrate is the electron acceptor) of PAOs, it can also occur under anaerobic conditions where PHA gets oxidised by losing electrons (H atom) to reduce NAD, as indicated by (Ferreira and Åkesson, 2020; Lee, 1996). In the context of this study, it is proposed that PHA consumption occurs independently of

Table 2
Phosphorus release and uptake and pigment concentration during the operational period in dark/light cycles with Tetrasphaera enriched biomass.

	$P_{release}$ mg/L	P_{uptake} total mg/L	P_{uptake} Light mg/L	P_{uptake} Light and air mg/L	Chlorophyll mgChl/gVSS	Bacteriochlorophyll mgChl/gVSS
Tetrasphaera SBR	43	70	–	–	0.64±0.04	0.29±0.04
Day 1	36	21	6	15	1.2 ± 0.25	1.0 ± 0.35
Day 20	17	29	12	17	0.47±0.04	0.16±0.10
Day 147	1	10.2	9.2	1	11±0.05	0

Table 3
Metabolic reactions during the light phase of the experiment.*.

N°	Equation	Reference
R1 - Glycogen hydrolysis	$-\ CH_{\frac{10}{6}}\ O_{\frac{5}{6}} - \frac{1}{6}H_2O + \frac{2}{3}CH_{1.5}O_{0.5} + \frac{1}{3}\ CO_2 + \frac{1}{2}\ NADH_2 + \frac{1}{3}\ ATP = 0$	(Oehmen et al., 2005)
R2 - PHA Catabolism	$-\ PHA - \left(1.5\ \lambda + \frac{5\beta}{3}\right)H_2O + (\lambda + \beta)\ CO_2 + \left(0.5\ \lambda + \frac{2\beta}{3}\right)ATP + (2.25\ \lambda + 2.5\beta)NADH_2 = 0$	(Oehmen et al., 2007b ; Zeng et al., 2003b)
R3 - Biomass synthesis from PHA	$-\ PHA - \left(\frac{0.19\lambda}{1.27} + \frac{0.19\beta}{1.06}\right)NH_3 - \left(\frac{1.7\lambda}{1.27} + \frac{1.38\beta}{1.06}\right)ATP - \left(\frac{0.41\lambda}{1.27} + \frac{0.27\beta}{1.06}\right)H_2O + \left(\frac{\lambda}{1.27} + \frac{\beta}{1.06}\right)CH_{1.84}O_{0.5}N_{0.19} + \left(\frac{0.72\lambda}{1.27} + \frac{0.52\beta}{1.06}\right)NADH_2 + \left(\frac{0.27\lambda}{1.27} + \frac{0.06\beta}{1.06}\right)CO_2 = 0$	(Oehmen et al., 2007b ; Zeng et al., 2003b)
R4 - P uptake[1]	$-\ H_3PO_4 - \left(\frac{\delta}{\varepsilon} + 1\right)\ ATP + HPO_3 + H_2O = 0$	(Oehmen et al., 2007b; Smolders et al., 1994b)
R5 - Maintenance[2]	$-\ ATP = 0$	(Oehmen et al., 2007b; Zeng et al., 2003b)
R6 - Oxidative phosphorylation	$-\ NADH_2 - 0.5\ O_2 + H_2O + \delta\ ATP = 0$	(Oehmen et al., 2007b)
R7- Oxygenic photosynthesis[3]	$-\ CO_2 - H_2O + O_2 + CH_2O = 0$	(Witt, 1996)
R8- Photoheterotrophy[4]	$-\ 1.5\nu + ATP = 0$	(Golomysova et al., 2010; Klamt et al., 2008)

[1] The equation was reformed by substituting R6 into the P uptake reaction (Smolders et al., 1994b) to represent the only ATP consumption associated with P uptake; ε=7 (Zeng et al., 2003b), δ= 1.85 (Oehmen et al., 2007b; Smolders et al., 1994b);.

[2] ATP required for cell maintenance (m^{ATP}) is determined through assuming aerobic maintenance coefficients 0.019 mol ATP/C-mol biomass·h (Smolders et al., 1994b).

[3] Oxygenic photosynthesis can occur in microalgae, with the rate of this reaction (SOPR) assumed to be at the higher end, 400 mol O2/mole chl.hr, within the range of 50–400 mol O2/mol chl.h proposed by Myers and Graham (1971).

[4] R8 is a sum up of reactions in photoheterotrophy, see (Golomysova et al., 2010) for more details, ν – photon.

* The stoichiometry are consistent with previously developed metabolic models of PAOs (Oehmen et al., 2005; Smolders et al., 1994a, 1994b), note that $NADH_2$ is shorthand for the full reaction of $NAD^+ + 2H^+ + 2e^- \rightarrow NADH + H^+$ (i.e. $NADH_2$) and that ATP is shorthand for the full reaction of ADP + H_3PO_4 → ATP + H_2O.

the presence of oxygen.

The oxygen demand in R6 is proposed to be supplied by photosynthetic organisms such as algae through oxygenic photosynthesis (R7), involving the conversion of CO_2 and H_2O to carbohydrates and oxygen (Witt, 1996). Although the specific oxygen production rate (SOPR) of algae is influenced by various factors, including algal species, culture environment, light intensity, and nutrient availability (Ye et al., 2018), each mole of chlorophyll has been reported to produce 50–400 mol of oxygen per hour (Myers and Graham, 1971). Based on this range, the maximum and minimum of oxygen production by algae can be estimated (See Table 4).

Regardless of the rate of oxygen production, the limited chlorophyll content (0.22 mgChl/gVSS) in the *Accumulibacter*-enriched culture resulted in a small amount of oxygen production. This led to a low level of ATP production through R6 and could not fulfill the total oxygen demand by *Accumulibacter* aerobic metabolism (See Table 4). After

Table 4
The stoichiometry and ATP balance in the light phase until the completion of P uptake.

		1st Cycle	2nd Cycle
Parameters	λ	0.75	0.77
	β	0.25	0.23
Glycogen consumption (mmol C / L)[1]		1.46	0.55
PHA consumption (mmol C / L)[1]		7.64	3.87
Biomass formation (mmol C / L)[2]		3.27	1.49
CO_2 formation (mmol C / L)[3]		4.84	2.56
Carbon balance (%)[4]		−12 %	−9 %
Oxygen production$_{Max\ SOPR}$ ($molO_2$/L)[5]		1.47	1.14
Oxygen production$_{Min\ SOPR}$ ($molO_2$/L)[5]		0.18	0.14
Oxygen requirement by *Accumulibacter* ($molO_2$/L)[6]		8.01	4.12
ATP production (mmol/L)			
ATP_{R1}		0.49	0.18
ATP_{R2}		1.98	1.10
ATP_{R6}[7]		5.44	4.22
Total ATP production		7.91	5.50
ATP consumption (mmol/L)			
ATP_{R3}		5.26	2.41
ATP_{R4}		3.45	2.35
ATP_{R5}		23.39	11.77
Total ATP consumption		32.10	16.53
ATP balance		−24.19	−11.03
ATPlight/Total ATP production (%)$_{Max\ SOPR}$[8]		75	67

ATP_{light}/Total ATP production (%): The percentage of ATP produced from light.

[1] Glycogen and PHA consumption measured in the test (mmol C/ L),.

[2] Biomass formation calculated through R3;.

[3] CO_2 formation calculated from R1, R2 and R3 (mmolC/L);.

[4] Carbon balance was calculated by subtracting the consumption of Glycogen and PHA (R1, R2, R3) from CO_2 production (R1, R2, R3), biomass produced (R3), and dividing by the production. This calculation does not include CO_2 consumption by microalgae;.

[5] The DO production by microalgae, using the SOPR Max: 400 and Min: 50 $molO_2$/mole chl.h as suggested in Myers and Graham (1971).

[6] DO requirement estimated for *Accumulibacter's* aerobic metabolism, using R6;.

[7] ATP production from R6 was calculated using the chlorophyll content of 0.22 mgChl/gVSS and the max SOPR of 400 molO2/mole chl.h;.

[8] Percentage of ATP produced from light, assuming a maximum SOPR;.

considering all potential ATP sources within *Accumulibacter*, including R1, R2, and R6, it became evident that they were insufficient to meet the ATP requirements for observed growth (R3), P uptake (R4) and maintenance (R5), as detailed in Table 4. This highlights the necessity of an additional pathway for ATP production to balance energy consumption, with phototrophic ATP production serving as the proposed source, based on the genomic potential of *Accumulibacter*.

In the case of assuming the maximum oxygen production previously observed by microalgae (SOPR of 400 molO2/mol chl.h), ATP production from light would still be needed to account for more than 67 % of the ATP demand in the two light cycles (See Table 4). This suggested that the phototrophic pathway in *Accumulibacter* must be present to fulfill the ATP requirement regardless of the oxygen availability. Similar results were observed in the long-term light test, where the algae's oxygen production was insufficient to meet the oxygen requirements of *Accumulibacter's* aerobic metabolism on day 1 (See Table 1). Thus, these results suggested that *Accumulibacter* not only possess phototrophic genes (Table S4 and S5) but can express these genes under oxygen stress and illuminated conditions.

The analysis of the experimental results in Sections 3.1 and 3.2 through mass and energy balances suggests that *Accumulibacter* may perform photoheterotrophy, using light as energy source and PHA as the organic carbon source. The consolidated light reaction given in R8 in Table 3, constitutes cytoplasmic membrane associated reactions, like the electron transport chain, and photophosphorylation that produces ATP. Under photoheterotrophic conditions, where PHA serves as the carbon source and light as the energy source, the electron transport chain operates cyclically, obviating the requirement for an external electron

donor (Klamt et al., 2008). As there are no additional electron sinks other than oxidative phosphorylation (R6) in R1-R6, excess $NADH_2$ would be diverted to other pathways (See Table S3), potentially including the CBB cycle, H_2 production, or release of soluble microbial products (SMP).

To maintain redox balance, carbon dioxide fixation via CBB (R9) and H_2 production (R10) are recognised electron sink pathways in PPB and are found as potential mechanisms for maintaining redox balance (Golomysova et al., 2010; McKinlay and Harwood, 2010). These reactions are deemed to be present in *Accumulibacter* based on the identification of corresponding genes within its genome. As shown in Table S5, *Accumulibacter* possess the genes needed for the CBB cycle (Skennerton et al., 2015) (Skennerton et al., 2015). Various types of nitrogenases, including both Mo- and Fe-nitrogenases, are known to be involved in hydrogen production in PPB and these gene have been found in *Accumulibacter* genomes UNIPROT.

$$-CO_2 - 2NADH_2 - 3ATP - \frac{5}{3}H_2O + CH_{\frac{7}{3}}O_2P = 0\ (R9)$$

$$-0.5proton - NADH_2 - 4ATP + H_2 = 0\ (R10)$$

On the other hand, cells could release soluble microbial products (SMPs) into the extracellular environment, acting as a sink for excess electrons within the cell (Yilmaz et al., 2010). Those secreted SMPs may get consumed by microalgae and they might also play a role in facilitating microalgal growth. This could be a reason that no microalgal enrichment was observed with *Tetrasphera* under light phase. It is hypothesised that one or a combination of these electron sink pathways could contribute towards balancing the excess electrons in *Accumulibacter's* photoheterotrophic growth. Indeed, the identification of electron sinks ($NADH_2$) was beyond the scope of this study, and it is not necessary to resolve the $NADH_2$ balance to indicate the ATP deficit without light reactions. Therefore, based on the genomic potential of *Accumulibacter* and the results from illuminated experiments, we propose the metabolic pathways in *Accumulibacter* under illuminated conditions, as shown in Fig. 3.

The above results suggest the potential for *Accumulibacter* to exhibit photoheterotrophic behaviour. The potential of *Accumulibacter* to contribute to photoautotrophic metabolism is worthy of further investigation. Photoautotrophs rely on light for anabolism and utilise CO_2 as their carbon source, converting it into intracellular carbohydrates, through the CBB cycle (Madigan and Jung, 2009). The CBB cycle is a metabolic pathway that can potentially enable *Accumulibacter* to replenish metabolic intermediates like glyceraldehyde-3-phosphate that act as precursors for biomass synthesis, from CO_2, ATP and $NADH_2$. However, from the data available in the present study, it was not possible to conclude if *Accumulibacter* employed the CBB cycle and photosynthetic metabolism in the experiments performed. Further research is necessary to gain insight into the potential photoautotrophic growth capabilities of *Accumulibacter* and the precise role of the CBB genes in its metabolic processes.

3.4. Impacts of the study

The observation of phototrophic metabolism in *Accumulibacter* could potentially present advantages for treating P in wastewater. Typically, *Accumulibacter* consume oxygen under aerobic conditions for P uptake, however, if oxygen is absent then light can be used as an energy source for P uptake. This potentially provides an alternative to aerated EBPR systems or phototrophic systems relying on oxygen supplied by algae, like photo-EBPR. This would be especially advantageous in situations where algae growth is constrained by insufficient inorganic carbon or limited light availability, as has been found in previous studies (Carvalho et al., 2023). Another potential application could involve oxygenation at night, while using a visible light filter to constrain microalgae growth and utilise phototrophic metabolism during the day. This way the aeration energy can be reduced during the day by utilising light metabolism in *Accumulibacter* for P uptake, as *Accumulibacter* is

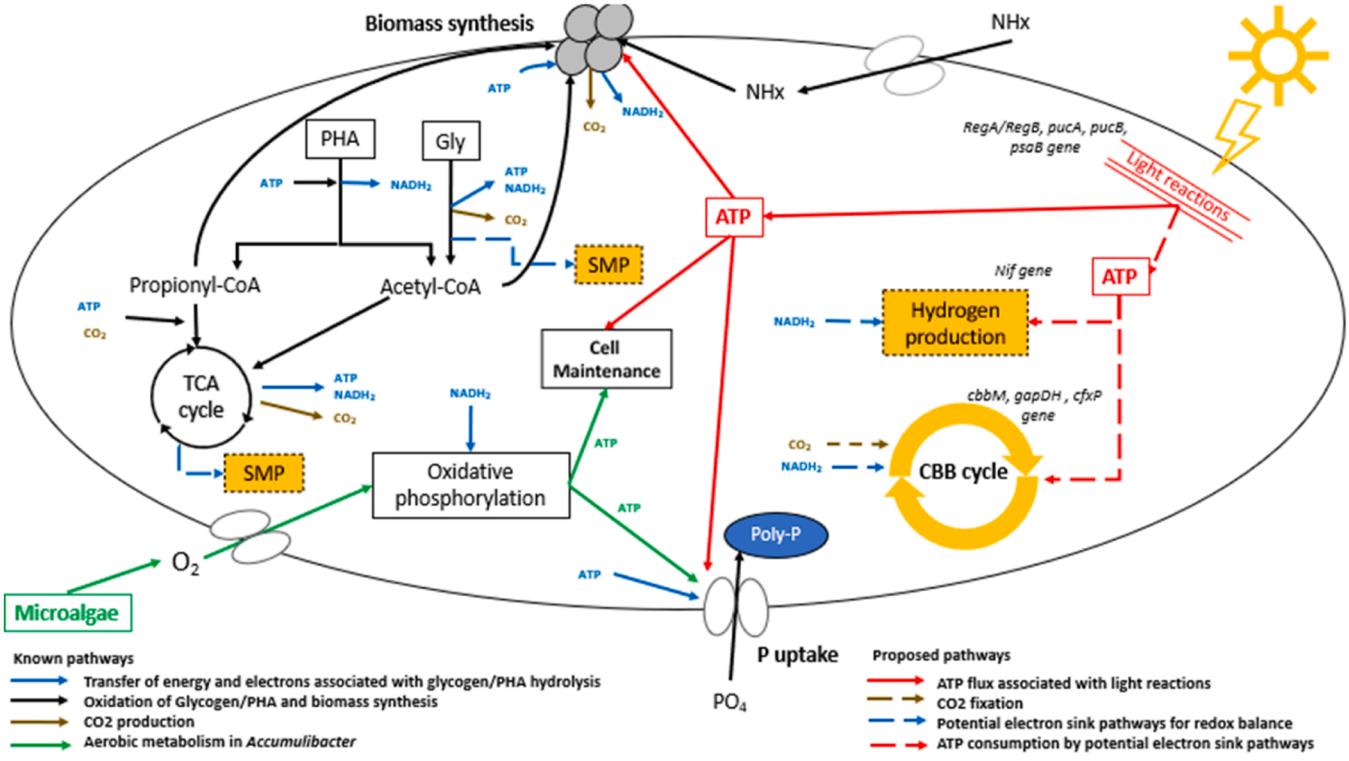

Fig. 3. Proposed biochemical pathway in Accumulibacter when exposed to light. The Calvin-Benson-Bassham (CBB) cycle, hydrogen production, and soluble microbial products (SMP) production are presented as potential electron sink pathways in Accumulibacter. Related genes are described on the diagram and a full list of these genes can be found in Tables S4 and S5 in the supplemental material.

more efficient than microalgae for P removal.

Both short and long light cycles indicate that *Accumulibacter* is likely to deplete its internal polymer, glycogen, during the light phase to provide sufficient carbon for growth and regenerate intermediates (metabolites) of central carbon metabolism along with $NADH_2$ and ATP. In the long-term test, glycogen levels declined from 12.0 C-mmol/L on day 1 to 1.88 C-mmol/L on day 4. Thus, glycogen limitation could be a potential bottleneck if trying to exploit the capacity of *Accumulibacter* to remove P phototrophically. Future research should focus on developing conditions that can address glycogen replenishment for practical applications. Since it has been proposed that Accumulibacter directly stores glucose as glycogen anaerobically (Ziliani et al., 2023), the strategy could involve either adding glucose to the feed or using sugar-rich wastewaters.

One explanation for the continuous glycogen consumption over time is that *Accumulibacter* might not be able to perform phototrophy to its full capacity due to suboptimal conditions provided. The presence of visible (VIS) light can enrich microalgae, leading to oxygen production. Previous studies have shown that oxygen suppresses phototrophic growth in PPB and causes complete loss of pigments like bacteriochlorophylls (BChls) and carotenoids eventually (Capson-Tojo et al., 2021). PPB utilise near infra-red (NIR) light (Capson-Tojo et al., 2022; Sepúlveda-Muñoz et al., 2022), *Accumulibacter* could also be able to utilise NIR due to its phylogenetic relationship with PPB. Therefore, to investigate *Accumulibacter's* phototrophic potential further, experiments using a VIS filter to restrict VIS light wavelengths and emit near-infrared (NIR) light could be conducted. This approach could eliminate microalgae and subsequent oxygen production, potentially enriching BChls and/or other light-harvesting pigments. Additionally, these tests with different wavelength could reveal the underlying phototrophic mechanisms in *Accumulibacter*, determining whether phototrophy is induced by NIR through light-harvesting pigments or by light-induced processes via VIS light. Conducting these light tests outdoors with natural sunlight would be crucial for demonstrating the system's practicality.

In theory, light harvesting could also be facilitated by rhodopsin-like proteins, as found in literature (Bryant and Frigaard, 2006; Lanyi, 2004). These membrane-bound enzymes have the capacity to absorb light energy, subsequently transporting protons across the membrane, thereby preserving the light energy in the form of a transmembrane electrochemical proton gradient (Gómez-Consarnau et al., 2010). In PPB, these proteins are often found to function as light sensors, aiding in the light harvesting process (Kyndt et al., 2004). Previous study found that the simultaneous activity of both bacteriochlorophyll-based and rhodopsin-based phototrophy works together to cover cellular energy needs during the light harvesting process (Kopejtka et al., 2020). As rhodopsin is likely stimulated under blue light (wavelength of 465–470 nm) in the visible light region (Gomelsky and Hoff, 2011), it is plausible that rhodopsin-based light harvesting plays a role in *Accumulibacter's* photoheterotrophic growth in this study, due to the illumination that was provided covered the wavelength range used by rhodopsin. To confirm the complete set of all ribonucleic acid (RNA) molecules and their corresponding light-harvesting protein expression during phototrophic growth in *Accumulibacter*, further proteomic and transcriptomic analysis is required.

In EBPR systems, the presence of GAOs can be undesirable, as they can compete with PAOs and disrupt the phosphorus removal process. By enriching PAOs phototrophically, this could potentially favour their growth in the competition with certain GAOs, (e.g., *Competibacter* and *Defluviicoccus*). The phototrophic genes identified in *Accumulibacter* (as shown in Table S4 and S5) were not found in *Competibacter* or *Defluviicoccus*, based on genomic data from UNIPROT. This suggests that the ability of PAOs to grow in illuminated systems and perform photoheterotrophic phosphorus removal could provide them with a competitive advantage over these GAOs. However, further experimentation involving light tests with these GAOs would be valuable for the development of a phototrophic-PAO system.

The integration of phototrophic *Accumulibacter* growth and metabolism into biological nutrient removal processes holds the potential to yield significant advantages for wastewater treatment. This includes the reduction of energy consumption and the facilitation of novel phosphorus recovery methods. Nevertheless, further investigation into the underlying light mechanisms within *Accumulibacter* is essential to adequately design optimal operating conditions for harnessing their phototrophic capabilities.

4. Conclusion

Illuminated tests with a highly enriched *Accumulibacter* culture, coupled with genome database and metabolic reaction analyses, supports the phototrophic capability of *Accumulibacter* for P uptake and growth. *Accumulibacter* exhibited an initial lag time of approximately 5 h before taking up phosphorus. Extended tests demonstrated that continuous light exposure stimulated the growth of microalgae (chlorophyll) and subsequent oxygen production, supporting *Accumulibacter's* conventional aerobic metabolism. In contrast, extended light tests suggested that the occurrence of phototrophic phosphorus uptake in *Tetrasphaera*-PAO is unlikely to occur. To the best of our knowledge, this study proposes the first metabolic model for phototrophic metabolism by *Accumulibacter*, predicting that phototrophic ATP production could fulfill over 67 % of the ATP demand. The phototrophic nature of *Accumulibacter* holds the potential to advance photo-EBPR technology development.

CRediT authorship contribution statement

V.C.F. Carvalho: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **A.Z.M. Gan:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **A. Shon:** Writing – review & editing, Investigation, Formal analysis. **S. Kolakovic:** Methodology, Investigation, Formal analysis. **E.B. Freitas:** Methodology, Investigation, Formal analysis. **M.A.M. Reis:** Writing – review & editing, Supervision, Resources, Funding acquisition. **J.C. Fradinho:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **A. Oehmen:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization.

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.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.121865.

References

Alberti, M., Burke, D.H., Hearst, J.E., 1995. Structure and sequence of the photosynthesis gene cluster. In: Blankenship, R.E., Madigan, M.T., Bauer, C.E. (Eds.), Anoxygenic Photosynthetic Bacteria, Advances in Photosynthesis and Respiration. Springer Netherlands, Dordrecht, pp. 1083–1106. https://doi.org/10.1007/0-306-47954-0_50.

Amann, R.I., 1995. In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Akkermans, A.D.L., Van Elsas, J.D., De Bruijn, F.J. (Eds.), Molecular Microbial Ecology Manual. Springer Netherlands, Dordrecht, pp. 331–345. https://doi.org/10.1007/978-94-011-0351-0_23.

APHA, 1998. Standard methods [WWW Document]. standard methods. URL https://www.standardmethods.org/(accessed 11.8.20).

Bélanger, G., Gingras, G., 1988. Structure and expression of the puf operon messenger RNA in rhodospirillum rubrum. J. Biol. Chem 263, 7639–7645.

Bryant, D.A., Frigaard, N.-U., 2006. Prokaryotic photosynthesis and phototrophy illuminated. Trends Microbiol. 14, 488–496. https://doi.org/10.1016/j.tim.2006.09.001.

Camejo, P.Y., Owen, B.R., Martirano, J., Ma, J., Kapoor, V., Santo Domingo, J., McMahon, K.D., Noguera, D.R., 2016. Candidatus Accumulibacter phosphatis clades enriched under cyclic anaerobic and microaerobic conditions simultaneously use different electron acceptors. Water Res. 102, 125–137. https://doi.org/10.1016/j.watres.2016.06.033.

Capson-Tojo, G., Lin, S., Batstone, D.J., Hülsen, T., 2021. Purple phototrophic bacteria are outcompeted by aerobic heterotrophs in the presence of oxygen. Water Res. 116941 https://doi.org/10.1016/j.watres.2021.116941.

Capson-Tojo, G., Batstone, D.J., Grassino, M., Hülsen, T., 2022. Light attenuation in enriched purple phototrophic bacteria cultures: implications for modelling and reactor design. Water Res. 219, 118572 https://doi.org/10.1016/j.watres.2022.118572.

Carvalho, V.C.F., Fradinho, J.C., Oehmen, A., Reis, M.A.M., 2023. Long term operation of a phototrophic biological nutrient removal system: impact of CO2 concentration and light exposure on process performance. J. Environ. Manage. 334, 117490 https://doi.org/10.1016/j.jenvman.2023.117490.

Carvalho, V.C.F., Freitas, E.B., Fradinho, J.C., Reis, M.A.M., Oehmen, A., 2019. The effect of seed sludge on the selection of a photo-EBPR system. N. Biotechnol 49, 112–119. https://doi.org/10.1016/j.nbt.2018.10.003.

Carvalho, V.C.F., Freitas, E.B., Silva, P.J., Fradinho, J.C., Reis, M.A.M., Oehmen, A., 2018. The impact of operational strategies on the performance of a photo-EBPR system. Water Res. 129, 190–198. https://doi.org/10.1016/j.watres.2017.11.010.

Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D., Blackall, L. L., 2000. Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. Appl. Environ. Microbiol 66, 1175–1182.

Ferreira, J.A., Åkesson, D., 2020. Aerobic and anaerobic degradation pathways of PHA. The Handbook of Polyhydroxyalkanoates. CRC Press.

Fernando, E.Y., McIlroy, S.J., Nierychlo, M., Herbst, F.-A., Petriglieri, F., Schmid, M.C., Wagner, M., Nielsen, J.L., Nielsen, P.H., 2019. Resolving the individual contribution of key microbial populations to enhanced biological phosphorus removal with Raman–FISH. ISME J 13, 1933–1946. https://doi.org/10.1038/s41396-019-0399-7.

García Martín, H., Ivanova, N., Kunin, V., Warnecke, F., Barry, K.W., McHardy, A.C., Yeates, C., He, S., Salamov, A.A., Szeto, E., Dalin, E., Putnam, N.H., Shapiro, H.J., Pangilinan, J.L., Rigoutsos, I., Kyrpides, N.C., Blackall, L.L., McMahon, K.D., Hugenholtz, P., 2006. Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities. Nat. Biotechnol 24, 1263–1269. https://doi.org/10.1038/nbt1247.

Golomysova, A., Gomelsky, M., Ivanov, P.S., 2010. Flux balance analysis of photoheterotrophic growth of purple nonsulfur bacteria relevant to biohydrogen production. Int. J. Hydrogen Energy, Asian Hydrogen Energy Confer. 2009 35, 12751–12760. https://doi.org/10.1016/j.ijhydene.2010.08.133.

Gomelsky, M., Hoff, W.D., 2011. Light helps bacteria make important lifestyle decisions. Trends Microbiol. 19 (9), 441–448. https://doi.org/10.1016/j.tim.2011.05.002.

Gómez-Consarnau, L., Akram, N., Lindell, K., Pedersen, A., Neutze, R., Milton, D.L., González, J.M., Pinhassi, J., 2010. Proteorhodopsin phototrophy promotes survival of marine bacteria during starvation. PLoS Biol. 8, e1000358 https://doi.org/10.1371/journal.pbio.1000358.

Hanada, S., Liu, W., Shintani, T., Kamagata, Y., 2002. Polyphosphate-accumulating bacterium isolated from activated sludge. Int. J. Syst. Evol. Microbiol. 52, 883–887.

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

Igarashi, N., Harada, J., Nagashima, S., Matsuura, K., Shimada, K., Nagashima, K.V.P., 2001. Horizontal transfer of the photosynthesis gene cluster and operon rearrangement in purple bacteria. J. Mol. Evol 52, 333–341. https://doi.org/10.1007/s002390010163.

Klamt, S., Grammel, H., Straube, R., Ghosh, R., Gilles, E.D., 2008. Modeling the electron transport chain of purple non-sulfur bacteria. Mol. Syst. Biol 4, 156. https://doi.org/10.1038/msb4100191.

Kolakovic, S., Freitas, E.B., Reis, M.A.M., Carvalho, G., Oehmen, A., 2021. Accumulibacter diversity at the sub-clade level impacts enhanced biological phosphorus removal performance. Water Res. 199, 117210 https://doi.org/10.1016/j.watres.2021.117210.

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

Kopejtka, K., Tomasch, J., Zeng, Y., Selyanin, V., Dachev, M., Piwosz, K., Tichý, M., Bína, D., Gardian, Z., Bunk, B., Brinkmann, H., Geffers, R., Sommaruga, R., Koblížek, M., 2020. Simultaneous Presence of Bacteriochlorophyll and Xanthorhodopsin genes in a freshwater bacterium. mSystems 5. https://doi.org/10.1128/msystems.01044-20.

Kyndt, J.A., Meyer, T.E., Cusanovich, M.A., 2004. Photoactive yellow protein, bacteriophytochrome, and sensory rhodopsin in purple phototrophic bacteria. Photochem. Photobiol. Sci 3, 519–530. https://doi.org/10.1039/b315731h.

Lanham, A., 2012. Full-scale biological phosphorus removal: quantification of storage polymers, microbial performance and metabolic modelling.

Lanham, A.B., Oehmen, A., Saunders, A.M., Carvalho, G., Nielsen, P.H., Reis, M.A.M., 2013a. Metabolic versatility in full-scale wastewater treatment plants performing enhanced biological phosphorus removal. Water Res., Microb. Ecol. Drink. Water Wastewater Treatment 47, 7032–7041. https://doi.org/10.1016/j.watres.2013.08.042.

Lanham, A.B., Ricardo, A.R., Albuquerque, M.G.E., Pardelha, F., Carvalheira, M., Coma, M., Fradinho, J., Carvalho, G., Oehmen, A., Reis, M.A.M., 2013b. Determination of the extraction kinetics for the quantification of polyhydroxyalkanoate monomers in mixed microbial systems. Process Biochem 48, 1626–1634. https://doi.org/10.1016/j.procbio.2013.07.023.

Lanyi, J.K., 2004. Bacteriorhodopsin. Annu. Rev. Physiol. 66, 665–688. https://doi.org/10.1146/annurev.physiol.66.032102.150049.

Lee, S.Y., 1996. Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng. 49, 1–14. https://doi.org/10.1002/(SICI)1097-0290(19960105)49:1<1::AID−BIT1>3.0.CO;2-P.

Lopez, C., Pons, M.N., Morgenroth, E., 2006. Endogenous processes during long-term starvation in activated sludge performing enhanced biological phosphorus removal. Water Res. 40, 1519–1530. https://doi.org/10.1016/j.watres.2006.01.040.

Lopez-Vazquez, C.M., Oehmen, A., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J., Yuan, Z., van Loosdrecht, M.C.M., 2009. Modeling the PAO–GAO competition: effects of carbon source, pH and temperature. Water Res. 43, 450–462. https://doi.org/10.1016/j.watres.2008.10.032.

Lu, H., Keller, J., Yuan, Z., 2007. Endogenous metabolism of Candidatus Accumulibacter phosphatis under various starvation conditions. Water Res. 41, 4646–4656. https://doi.org/10.1016/j.watres.2007.06.046.

Madigan, M.T., Jung, D.O., 2009. An overview of purple bacteria: systematics, physiology, and habitats. In: Hunter, C.N., Daldal, F., Thurnauer, M.C., Beatty, J.T. (Eds.), The Purple Phototrophic Bacteria, Advances in Photosynthesis and Respiration. Springer Netherlands, Dordrecht, pp. 1–15. https://doi.org/10.1007/978-1-4020-8815-5_1.

McKinlay, J.B., Harwood, C.S., 2010. Carbon dioxide fixation as a central redox cofactor recycling mechanism in bacteria. PNAS 107, 11669–11675. https://doi.org/10.1073/pnas.1006175107.

Myers, J., Graham, J.-R., 1971. The photosynthetic unit in chlorella measured by repetitive short flashes. Plant Physiol 48, 282–286. https://doi.org/10.1104/pp.48.3.282.

Nielsen, P.H., 2009. FISH handbook for biological wastewater treatment. wio 8. https://doi.org/10.2166/9781780401775.

Nguyen, P.Y., Marques, R., Wang, H., Reis, M.A., Carvalho, G., Oehmen, A., 2023. The impact of pH on the anaerobic and aerobic metabolism of Tetrasphaera-enriched polyphosphate accumulating organisms. Water Research X 19, 100177. https://doi.org/10.1016/j.wroa.2023.100177.

Oehmen, A., Zeng, R.J., Yuan, Z., Keller, J., 2005. Anaerobic metabolism of propionate by polyphosphate-accumulating organisms in enhanced biological phosphorus removal systems. Biotechnol. Bioeng 91, 43–53. https://doi.org/10.1002/bit.20480.

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

Oehmen, A., Zeng, R.J., Keller, J., Yuan, Z., 2007b. Modeling the aerobic metabolism of polyphosphate-accumulating organisms enriched with propionate as a carbon source. Water Environ. Res 79, 2477–2486. https://doi.org/10.1002/j.1554-7531.2007.tb00347.x.

Powell, N., Shilton, A., Pratt, S., Chisti, Y., 2011. Luxury uptake of phosphorus by microalgae in full-scale waste stabilisation ponds. Water Sci. Technol 63, 704–709. https://doi.org/10.2166/wst.2011.116.

Ritchie, R.J., 2018. Measurement of chlorophylls a and b and bacteriochlorophyll a in organisms from hypereutrophic auxinic waters. J. Appl. Phycol 30, 3075–3087. https://doi.org/10.1007/s10811-018-1431-4.

Singleton, C.M., Petriglieri, F., Wasmund, K., Nierychlo, M., Kondrotaite, Z., Petersen, J. F., Peces, M., Dueholm, M.S., Wagner, M., Nielsen, P.H., 2022. The novel genus,'Candidatus Phosphoribacter', previously identified as Tetrasphaera, is the dominant polyphosphate accumulating lineage in EBPR wastewater treatment plants worldwide. ISME J 16 (6), 1605–1616.

Skennerton, C.T., Barr, J.J., Slater, F.R., Bond, P.L., Tyson, G.W., 2015. Expanding our view of genomic diversity in Candidatus Accumulibacter clades. Environ. Microbiol. 17, 1574–1585. https://doi.org/10.1111/1462-2920.12582.

Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C., Heijnen, J.J., 1994a. Model of the anaerobic metabolism of the biological phosphorus removal process:

stoichiometry and pH influence. Biotechnol. Bioeng 43, 461–470. https://doi.org/10.1002/bit.260430605.
Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1994b. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnol. Bioeng. 44, 837–848. https://doi.org/10.1002/bit.260440709.
Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1995. A structured metabolic model for the anaerobic and aerobic stoichiometry of the biological phosphorus removal process. Biotechnol. Bioengineer. Biotechnol Bioengineer 47, 277–287.
Sepúlveda-Muñoz, C.A., Torres-Franco, A.F., de Godos, I., Muñoz, R., 2022. Exploring the metabolic capabilities of purple phototrophic bacteria during piggery wastewater treatment. J. Water Process Engineer 50, 103317. https://doi.org/10.1016/j.jwpe.2022.103317.
Stackebrandt, E., Scheuner, C., Göker, M., Schumann, P., 2014. The family intrasporangiaceae. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes: Actinobacteria. Springer, Berlin, Heidelberg, pp. 397–424. https://doi.org/10.1007/978-3-642-30138-4_176.
Stokholm-Bjerregaard, M., McIlroy, S.J., Nierychlo, M., Karst, S.M., Albertsen, M., Nielsen, P.H., 2017. A critical assessment of the microorganisms proposed to be important to enhanced biological phosphorus removal in full-scale wastewater treatment systems. Front. Microbiol 8. https://doi.org/10.3389/fmicb.2017.00718.
Tichy, H.V., Oberlé, B., Stiehle, H., Schiltz, E., Drews, G., 1989. Genes downstream from pucB and pucA are essential for formation of the B800-850 complex of Rhodobacter capsulatus. J. Bacteriol. 171, 4914–4922. https://doi.org/10.1128/jb.171.9.4914-4922.1989.
Tu, Y., Schuler, A.J., 2013. Low acetate concentrations favor polyphosphate-accumulating organisms over glycogen-accumulating organisms in enhanced biological phosphorus removal from wastewater. Environ. Sci. Technol. 47, 3816–3824. https://doi.org/10.1021/es304846s.
Vargas, M., Yuan, Z., Pijuan, M., 2013. Effect of long-term starvation conditions on polyphosphate- and glycogen-accumulating organisms. Bioresour. Technol. 127, 126–131. https://doi.org/10.1016/j.biortech.2012.09.117.
Wang, Z., Li, W., Li, H., Zheng, W., Guo, F., 2020. Phylogenomics of Rhodocyclales and its distribution in wastewater treatment systems. Sci Rep 10, 1–12. https://doi.org/10.1038/s41598-020-60723-x.
Witt, H.T., 1996. Primary reactions of oxygenic photosynthesis. Berichte der Bunsengesellschaft für physikalische Chemie 100, 1923–1942. https://doi.org/10.1002/bbpc.19961001202.
Ye, J., Liang, J., Wang, L., Markou, G., 2018. The mechanism of enhanced wastewater nitrogen removal by photo-sequencing batch reactors based on comprehensive analysis of system dynamics within a cycle. Bioresour. Technol. 260, 256–263. https://doi.org/10.1016/j.biortech.2018.03.132.
Yilmaz, L.S., Kontur, W.S., Sanders, A.P., Sohmen, U., Donohue, T.J., Noguera, D.R., 2010. Electron partitioning during light- and nutrient-powered hydrogen production by Rhodobacter sphaeroides. Bioenerg. Res. 3, 55–66. https://doi.org/10.1007/s12155-009-9072-8.
Yuan, Z., Pratt, S., Batstone, D.J., 2012. Phosphorus recovery from wastewater through microbial processes. Curr. Opin. Biotechnol., Phosphor. Biotechnol. • Pharmaceut. Biotechnol 23, 878–883. https://doi.org/10.1016/j.copbio.2012.08.001.
Zeng, R.J., Saunders, A.M., Yuan, Z., Blackall, L.L., Keller, J., 2003a. Identification and comparison of aerobic and denitrifying polyphosphate-accumulating organisms. Biotechnol. Bioeng. 83, 140–148. https://doi.org/10.1002/bit.10652.
Zeng, R.J., van Loosdrecht, M.C.M., Yuan, Z., Keller, J., 2003b. Metabolic model for glycogen-accumulating organisms in anaerobic/aerobic activated sludge systems. Biotechnol. Bioeng. 81, 92–105. https://doi.org/10.1002/bit.10455.
Ziliani, A., Bovio-Winkler, P., Cabezas, A., Etchebehere, C., Garcia, H.A., López-Vázquez, C.M., Brdjanovic, D., van Loosdrecht, M.C.M., Rubio-Rincón, F.J., 2023. Putative metabolism of Ca. Accumulibacter via the utilization of glucose. Water Res. 229, 119446 https://doi.org/10.1016/j.watres.2022.119446.