# Revealing metabolic flexibility of *Candidatus* Accumulibacter phosphatis through redox cofactor analysis and metabolic network modeling

**Running title: Metabolic flexibility of Accumulibacter**

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## ABSTRACT

*Candidatus* Accumulibacter phosphatis, a polyphosphate accumulating organism (PAO) growing under alternating anaerobic/aerobic conditions in enhanced biological phosphorus removal (EBPR) systems, displays an intricate metabolic strategy. The organism relies on combining features from either anaerobic- or aerobic-only metabolisms and connecting phases using different reserve polymers. Analysis of the metabolic functions is not trivial, specially without isolates of this microorganism available and without employing advanced metabolic modelling techniques. So far, there is no consensus regarding the type of glycolysis nor the operating mode of the tricarboxylic acid cycle anaerobically. Here, we simulated different conditions using flux balance analyses and a reduced central carbon metabolic network based on previously published meta-omics data and validated with enzyme activity assays. Interestingly, we observed that the polyhydroxyalkanoates (PHA) production was NADH preferring instead of the commonly reported NADPH dependency. This feature allows for higher metabolic flexibility while maintaining a near-optimal carbon conservation and without extra energetic costs, which explains PAOs' competitiveness under anaerobic/aerobic shifting environments.

## KEYWORDS

*Candidatus* Accumulibacter phosphatis, Polyphosphate Accumulating Organisms (PAO), Enhanced Biological Phosphate Removal (EBPR), Anaerobic, Central carbon metabolism, Redox cofactors, Flux Balance Analysis, Enzymatic assays

51 **INTRODUCTION**

52 Enhanced biological phosphate removal (EBPR) from wastewater is enabled by
53 circulating activated sludge through alternating anaerobic/aerobic and substrate
54 feast/famine conditions. Under these dynamic conditions, organisms are selected that can
55 accumulate growth substrates when these are abundant to compensate for moments
56 when these are exhausted (1). *Candidatus* Accumulibacter phosphatis (hereafter referred
57 to as Accumulibacter) makes use of such strategy for multiple types of nutrients;
58 particularly, by aerobic uptake of phosphate (removal from the wastewater) and
59 intracellular accumulation as polyphosphate that can be later used as anaerobic energy
60 source. For this feature Accumulibacter is known as polyphosphate accumulating
61 organism (PAO) as it allows for fast and competitive harvesting of organic matter in the
62 absence of an external electron acceptor (i.e. anaerobic conditions).

63 In the past decades, a number of researchers have derived hypotheses about
64 Accumulibacter's physiology (2–5). One of the most significant inconclusive discussions
65 is the source of reducing power for the anaerobic accumulation of polyhydroxyalkanoates
66 (PHA) from volatile fatty acids (e.g. acetate, propionate), as reviewed by (6). While most
67 experimental approaches were adequate to study the population's physiology, the
68 inherent heterogeneity of the system has not yet sufficiently been addressed; for example,
69 in the last decade, researchers have reported different Accumulibacter clades can each
70 have different kinetic characteristics (7). Furthermore, novel meta-omics approaches
71 were used, which allowed for Accumulibacter-targeted analysis without the interference
72 of side-populations. One of the metatranscriptomic studies, (8) highlighted the need of
73 validating assumptions often made in metabolic models of Accumulibacter. In particular,
74 they refined the discussion on reducing power sources and requirements by making a
75 distinction between the different redox cofactors, NADH and NADPH. This distinction
76 imposes a constraint between sources and sinks for each redox cofactor, which can only
77 be alleviated by energy-consuming transhydrogenase(-like) mechanisms or using

78 electrons for hydrogen production. However, in their analysis, the specificity of the
79 involved oxidoreductases was derived from analogy with other organisms.

80 In Accumulibacter, the reducing power is required for the conversion of acetate to 3-
81 hydroxybutyrate (3HB), the monomer of the reserve polymer poly-3-hydroxybutyrate
82 (PHB). The intermediate reduction step is catalyzed by the enzyme acetoacetyl-CoA
83 reductase (AAR). In common PHA accumulating bacteria such as *Cupriavidus necator* and
84 *Zoogloea ramigera*, the preferred electron donor cofactor of the acetoacetyl-CoA
85 reductase is NADPH (9), which was then assumed by analogy for Accumulibacter.
86 However, dual preference (accepting both NADH and NADPH) has been reported for
87 *Azotobacter beijerinckii* (10,11) and *Allochromatium vinosum* (12).

88 So far and to our knowledge, the nature of the cofactor accepted by Accumulibacter's
89 acetoacetyl-CoA reductase has not yet been experimentally established. In this study, we
90 addressed this knowledge gap by measuring the NADH- and NADPH-dependent
91 acetoacetyl-CoA reductase activities in a cell free extract from a highly enriched culture
92 of Accumulibacter. We further extended the analysis to the other key oxidoreductases
93 involved in the anaerobic metabolism of Accumulibacter. These results were then used to
94 update Accumulibacter's biochemical model and as constraints in a flux balance analysis
95 (FBA) framework. Our simulations demonstrate how flexible Accumulibacter's
96 metabolism can be by making use of different pathways as reducing power sources to
97 balance their redox state under unpredictable anaerobic environments.

98 # MATERIALS AND METHODS

99 **Reactor operation**

100 Two independent Accumulibacter enrichments were obtained in sequencing batch
101 reactors (SBR). The main operational conditions are described in Table 1 and were
102 adapted from the SBR-S as described in (7).

103 **Cell free extracts preparation**

104 Broth samples (10 mL) collected from the bioreactor during both anaerobic and
105 aerobic phases were centrifuged (2500 g, 10 min, 4ºC) and the pellet was washed using
106 10 mL buffer (hereafter named Buffer 1X) containing 50 mM Tris at pH 8, 5 mM $MgCl_2$, 5
107 mM NaCl and 5% (v/v) glycerol, and the obtained suspension was centrifuged again
108 (2500 g, 10 min, 4ºC). After centrifugation, pellets were kept at -20ºC for no longer than
109 four days until further analysis. For the enzymatic assay, cellular pellets were suspended
110 in 10 mL of Buffer 1X supplemented with 2 mM (L+D) 1,4-Dithiothreitol (DTT) and
111 protease inhibitor cocktail (Roche). The cells were disrupted using a sonicator (Sonifier
112 250, Branson) setting the output power to the level 3, 30% duty cycle, during 3 min. The
113 cellular suspension was kept cold during the disruption using ice. The resulting
114 suspension was centrifuged (4ºC, 15000 g for at least 45 min). The obtained supernatant,
115 a cell free extract (CFE), was used for the enzymatic assays.

116 **Enzymatic assays**

117 The reaction mixtures used for each enzymatic assay are described in

118 Table 1. Activities were calculated using the initial rate of reduction of $NAD(P)^+$ or
119 oxidation of NAD(P)H, which was obtained by following the changes in the absorbance at
120 340 nm, at 30ºC. To control the contribution of putative background reactions, reaction
121 mixtures were monitored that contained all the components except one substrate or
122 without addition of cell free extract (CFE, see "Controls" in Supplementary Document 4).

123 The measured rates were normalized to the total protein concentration in the
124 respective CFE. The protein concentration was measured using the Bradford dye-binding
125 protein assay (Bio-Rad), using known concentrations of bovine serum albumin as
126 external standards.

127 Table 1 – Origin of CFE, buffer, substrates, controls and references (26,53–60) used for each
128 enzymatic assay. Controls used can be found in Supplementary Document 4.

| CFE | Tested enzyme | Buffer | Substrates | Reference |
|---|---|---|---|---|
| SBR-1 | acetoacetyl-CoA reductase | 1X, supplemented | 200 µM acetoacetyl-CoA, 200 µM NADH or 200 µM NADPH | (53) |
| SBR-2 | acetoacetyl-CoA reductase | 1X, supplemented | 40 µM acetoacetyl-CoA, 100 µM NADH or 200 µM NADPH | (54) |
| SBR-2 | 3-hydroxybutyryl-CoA dehydrogenase | 1X, supplemented | 100 µM 3-hydroxybutyryl-CoA 1000 µM $NAD(P)^+$ | (54) |
| SBR-1 | glucose-6-phosphate dehydrogenase | 1X, supplemented | 2 mM glucose-6-phosphate; 2000 µM $NAD^+$ or 200 µM $NADP^+$ | (55) |
| SBR-2 | glucose-6-phosphate dehydrogenase | 1X, supplemented | 4 mM glucose-6-phosphate; 400 µM $NAD(P)^+$ | |
| SBR-2 | isocitrate dehydrogenase | 1X, supplemented | 400 µM $NAD(P)^+$ 400 µM isocitrate | (56) |
| | malate dehydrogenase | 1X, supplemented | 200 µM NAD(P)H 200 µM oxaloacetate | (26) |
| | malic enzyme | 1X, supplemented | 200 µM NADP 200 µM L-malate | (57) |
| | isocitrate lyase | 40 mM HEPES, pH 7, 6 mM $MgCl_2$ | 4 mM isocitrate, 280 µM NADH 45 units lactate dehydrogenase from rabbit muscle (Roche) | (58) |
| | fumarate reductase | 50 mM HEPES, pH 7 | 15 mM fumarate 250 µM NADH | (59) |
| | α-ketoglutarate dehydrogenase | 50 mM HEPES, pH 7, 1mM $MgCl_2$, 1mM Thiamine pyrophosphate, 2.5 mM DTT | 2 mM α-ketoglutaric acid 100 µM Coenzyme A 2.5 mM $NAD(P)^+$ | (60) |

129

130 **Microbial community characterization**

131 **FISH**

132 Fluorescence *in situ* hybridization (FISH) was used to qualitatively assess the presence
133 of Accumulibacter in our enrichments. All bacteria were targeted by the EUB338 mix
134 (general bacteria probe) (13–15). Accumulibacter clade I and II were targeted by the
135 probes Acc-1-444 and Acc-2-444 (16), respectively. Hybridized samples were examined
136 with a Zeiss Axioplan-2 epifluorescence microscope.

137 **16S-rRNA amplicon sequencing**

138 To identify typical side-populations present in our enrichments, 16S-rRNA amplicon
139 sequencing was applied. DNA samples from cell pellets of our enrichments were extracted
140 using the DNeasy UltraClean Microbial Kit (Qiagen, The Netherlands). Approximately 250
141 mg wet biomass was treated according to the standard protocol except an alternative lysis
142 was implemented. This included a combination of 5 min of heat (65$^{o}$C) followed by 5 min
143 of bead-beating for cell disruption on a Mini-Beadbeater-24 (Biospec, U.S.A.). After
144 extraction the DNA was checked for quality by gel electrophoresis and quantified using a
145 Qubit 4 (Thermo Fisher Scientific, U.S.A.).

146 After quality control, samples were sent to Novogene Ltd. (Hongkong, China) for
147 amplicon sequencing of the V3-4 region of the 16S-rRNA gene (position 341-806) on a
148 Illumina paired-end platform. After sequencing, the raw reads were quality filtered,
149 chimeric sequences were removed and OTUs were generated on the base of ≥ 97%
150 identity. Subsequently microbial community analysis was performed by Novogene using
151 Mothur & Qiime software(V1.7.0). For phylogenetical determination, the most recent
152 SSURef database from SILVA (http://www.arb-silva.de/) was used. The microbial
153 communities were compared by generating a top 10 genera graph. The 16S-rRNA gene
154 amplicon libraries have been deposited under project PRJNA490689.

155 **PPK analysis**

156 To further confirm the specific PAO clade, the presence of the gene encoding for the
157 polyphosphate kinase I (*ppk1*) present in Accumulibacter was tested as described in

158 (17,18) and using the primers ACCppk1-254F and ACCppk1-1376R targeting the *ppk1*
159 gene from Accumulibacter sp. like bacteria (19). The *ppk1* gene sequences obtained in this
160 study have been deposited in the GenBank database under accession numbers
161 MH899084-MH899086.

162 **Flux balance analysis (stoichiometric modelling)**

163 The metabolic network shown in Figure 1 is based on the ancestral genome
164 reconstruction done by (20). Despite not being annotated in the genome, the ED pathway
165 has been suggested as the route for glycogen degradation (21,22). A more extensive
166 overview of evidence for each pathway can be found in Supplementary Document 1. The
167 stoichiometric model (see Table 2) used in the simulations was built based on the
168 metabolic network shown in Figure 1, including the experimental observations obtained
169 in this present study.

170 To estimate the possible metabolic flux distributions through this underdetermined
171 system, Flux Balance Analysis (FBA) can be applied (23). Inequality constraints were
172 introduced for physiologically irreversible fluxes (i.e. these should always be positive). All
173 fluxes were normalized to the sum of acetate and glycogen consumption rates (hereafter,
174 C consumed). To simulate the different ratios of glycogen over C consumed, glycogen
175 dissimilation ratio was varied between 0 to 1 Cmol glucose per Cmol consumed
176 (inversely, acetate varied from 1 to 0). This simulation assumes that none of the balanced
177 intermediates is accumulated inside the cell (steady-state assumption).

178 Commonly, maximization of biomass synthesis is used in FBA as optimization
179 objective. However, Accumulibacter only grow later when oxygen is available and from
180 the intracellular carbon reserves (PHAs) accumulated during anaerobiosis. Therefore, we
181 assume maximal carbon conservation, resp. minimal $CO_2$ production, as the cellular
182 objective to simulate Accumulibacter's metabolism anaerobically.

183 $$v_{opt} = \arg\min v_{CO2_{prod}} \quad \text{subject to} \begin{cases} Nv = 0 \\ v_{Ac_{upt}} = 1 : 0\ Cmol/Cmol\ C_{consumed} \\ v_{Glyc_{deg}} = 0 : 1\ Cmol/Cmol\ C_{consumed} \\ v_{irr} \geq 0 \end{cases}$$

184 For this optimization problem, MATLAB's function *linprog* was used.

185 To prevent stoichiometric constraints from specific assumption on the polymer
186 composition (PHB, PHV, PH2MV, etc), we introduce the polymer amount as reduced
187 precursors Ac-CoA* and Pr-CoA* such that:

188 2Ac-CoA* → 3HB;

189 Ac-CoA* + Pr-CoA* → 3HV (or 3H2MB);

190 2Pr-CoA* → 3H2MV;

191 **Experimental data reconciliation**

192 The results of the aforementioned simulations were compared to experimental data
193 taken from literature. Anaerobic-feast yields were used when available, otherwise the
194 yields of the whole anaerobic phase were used. In case of missing compound rates
195 (usually $CO_2$, $PH_2MV$), these were estimated using electron and carbon balancing (see
196 Supplementary Document 5). For datasets with redundant measurements, data
197 reconciliation was applied (24). For all calculations, we assumed acetate and glycogen
198 monomers were the only substrates and $CO_2$, PHB, PHV and $PH_2MV$ were the only
199 products. For yields reported without the respective error, we estimated their error by
200 propagating the assumed relative errors of 5% for acetate and PHB measurements and
201 10% for PHV, $PH_2MV$ and glycogen measurements. From the (reconciled) rates for PHB,
202 PHV and $PH_2MV$, the respective Ac-CoA* and Pr-CoA* rates were determined and
203 normalized to the amount of consumed substrates, in C-mol.

204 # RESULTS

205 ## Characterization of Accumulibacter enrichments

206 This study was carried out using two independent Accumulibacter enrichment
207 cultures. Performance data indicated a high level of PAO activity and microbial
208 characterization confirmed by FISH, 16S-rRNA and *ppk1* gene analysis that the biomass
209 was highly dominated by Accumulibacter clade I (see Table 1 and Supplementary
210 Document 3).

211 Table 1 – Process parameters and key performance indicators of the two independent
212 enrichments of Accumulibacter.

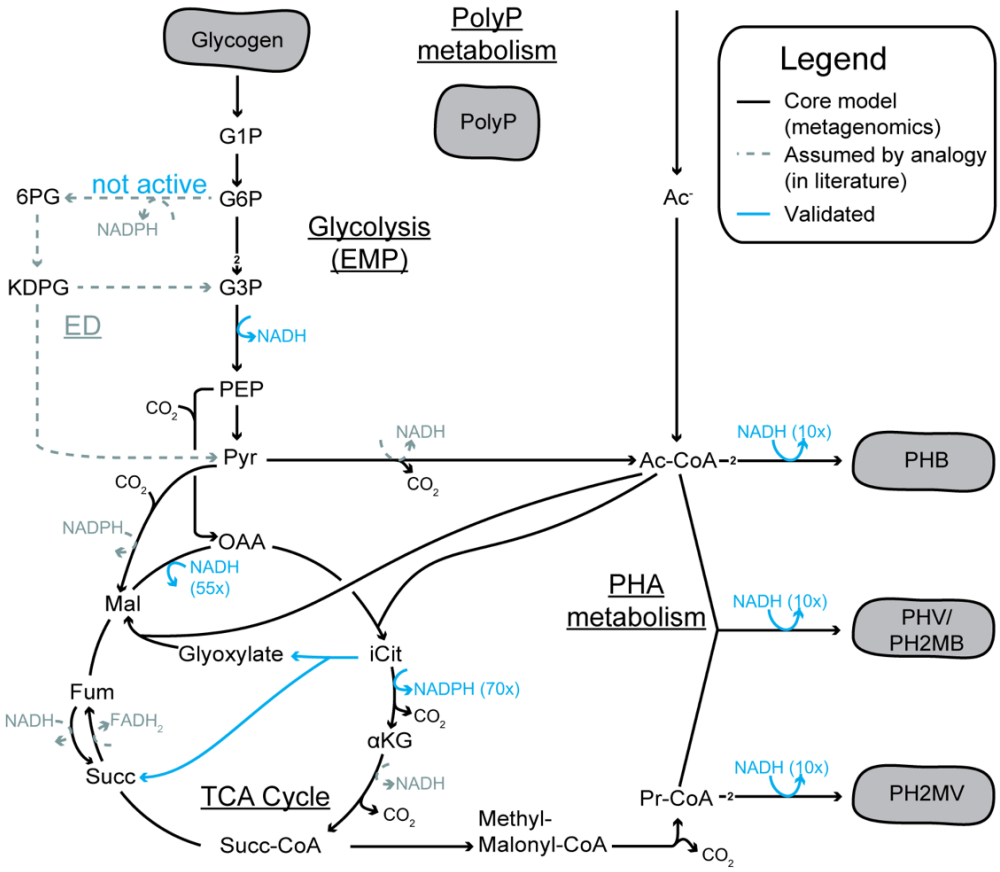
| | **SBR-1** | **SBR-2** | |
|---|---|---|---|
| pH | 7.6 | 7.6 | |
| T | 20°C | 20°C | |
| Carbon source | 210 mg COD/L (63:37 Acetate:Propionate) | 400 mg COD/L (75:25 Acetate:Propionate) | |
| Phosphate load per C | 0.1 Pmol/Cmol | 0.1 Pmol/Cmol | |
| | | | |
| Cycle time | 6 h<br>Settling – 76 min;<br>Anaerobic – 135 min;<br>Aerobic – 135 min. | 6 h<br>Settling – 30 min;<br>Anaerobic – 112 min;<br>Aerobic – 200 min. | |
| HRT | 12 h | 12 h | |
| SRT | 4 days | 5.6 days | |
| Aerobic SRT | 1.5 days | 3.1 days | |
| | | | |
| Anaerobic P-release per C-fed | 0.58 Pmol/Cmol | ~0.75 Pmol/Cmol | |
| Anaerobic-feast length | ~41 min | ~42 min | |
| | | | |
| FISH | PAO Acc I | PAO Acc I | |
| 16S amplicon sequencing (dominant OTUs) | Ca_Accumulibacter<br>Rhodopseudomonas<br>Blastochloris | Start:<br>Dechloromonas<br>Ca_Accumulibacter<br>genus from<br>f__Hyphomonadaceae | End:<br>Ca_Accumulibacter<br>Chryseobacterium<br>genus from<br>o__Sphingobacteriales |
| PPK analysis | Dominant: clade IC | Dominant: clade IA | |

213

214 ## Redox cofactor preferences of key oxidoreductases

215 Enzymatic assays can be a useful tool to investigate the presence and potential
216 extension of an enzymatically catalyzed conversion in a microbial community. However,

217 employed at that level, these assays will not specify "who" in the community is performing
218 the assessed conversion. Considering this limitation, we can still say 1) Accumulibacter
219 seems to not have a given function when such activity is not detected during the assay for
220 the whole community, 2) an enzyme from Accumulibacter is NAD(H) or NADP(H)
221 preferring when the community also shows the same clear preference. To further
222 characterize Accumulibacter's oxidoreductases (including their exact relative cofactor
223 preference), specific assays with purified enzymes can be performed as described in (25),
224 however this goes beyond the scope of our current analysis. The enzymatic activity assay
225 results are shown in blue in the metabolic network in Figure 1 and further details can be
226 found in Supplementary Document 4.



227

228 Figure 1 – Central carbon metabolic network of Accumulibacter including redox cofactor
229 preference of oxidoreductases assayed in this study. The value shown corresponds to the factor
230 difference between the preferred cofactor and the alternative one. This metabolic network is based
231 on the ancestral genome reconstruction done by (20). The arrows show the expected flux direction
232 under anaerobic conditions. The remaining cofactor preferences (in gray) remain as assumed by
233 analogy with other microorganisms. Despite not being annotated in the genome, the ED pathway
234 has been suggested as the route for glycogen degradation (21,22). A more extensive overview of
235 evidence for each pathway can be found in Supplementary Document 1.

236 The first question addressed was whether PHA accumulation is NADPH or NADH
237 dependent; it is clear from these results that the microbial community under study has a
238 NADH preferring accumulation of PHA with, at least, 10x more activity with NADH than
239 with NADPH. To further confirm this cofactor preference, the acetoacetyl-CoA reductase
240 activity was assayed using cell-free extracts from a second enrichment and both
241 directions of the reaction were monitored (i.e. NAD(P)H + acetoacetyl-CoA ↔ $NAD(P)^+$ +
242 3-hydroxybutyryl-CoA). In addition, a purified acetoacetyl-CoA reductase, encoded by a
243 gene highly similar to Accumulibacter's *phaB* (see Supplementary Document 4), was
244 employed as positive control as it is NAD(H)-preferring.

245 The second question tackled was related to the long-standing debate on glycolysis
246 proceeding via EMP or ED. To clarify this, we looked for the presence of the glucose-6-
247 phosphate dehydrogenase, the enzyme that catalyzes the first step of ED, and which is
248 also common to the oxidative branch of the pentose phosphate pathway (oxPP). No
249 activity was found in the cell-free extracts obtained from our enrichments, while the
250 biological positive control used, a cell-free extract from *P. putida* KT2440, showed clear
251 activity both with $NAD^+$ and $NADP^+$. Therefore, Accumulibacter is likely to use EMP as
252 glycolytic route and to produce the NADH required for PHA synthesis.

253 Since for many organisms oxPP is an important source of NADPH for growth (26) and
254 it is not used by Accumulibacter, the next question was if NADPH could be provided by
255 the isocitrate dehydrogenase (a TCA cycle enzyme). The oxidation of isocitrate was tested
256 using either $NAD^+$ or $NADP^+$ and the activity with $NADP^+$ was, at least, 70x higher, thus
257 isocitrate dehydrogenase is indeed a candidate supplier of NADPH in Accumulibacter.

258 Additionally, we tested the activity of other TCA oxidoreductases and anaplerotic
259 routes: 1) the reduction of oxaloacetate to malate (catalyzed by malate dehydrogenase)
260 had, at least, 55x higher activity when using NADH than NADPH. Unfortunately, the small
261 activity when using NADPH impeded the study of the cofactor preference of the malic
262 enzyme (oxidation of malate to pyruvate) using our assay; 2) the glyoxylate shunt was

263 also found active and with an activity comparable to the positive biological control *E. coli*
264 grown in acetate; and lastly, 3) fumarate reductase and α-ketoglutarate dehydrogenase
265 were also tested but their activity was very low compared to all other enzymes tested in
266 our study.

267 Next to assaying the cofactor preference of the aforementioned TCA oxidoreductases,
268 we also evaluated the differences in enzymatic activity between the anaerobic and aerobic
269 phases. The enzyme assays gave similar activities for anaerobic and aerobically harvested
270 biomass and thus do not allow for concluding on the anaerobic operation mode of the
271 TCA. These results are in line with the previous observation of proteomics studies where
272 relative protein abundances do not change notably across an anaerobic/aerobic cycle
273 (27,28). Nevertheless, the collected information on cofactor preferences and glycolysis
274 type (summarized in Figure 1) allowed for setting up an intracellular carbon flux balance
275 analysis (FBA) simulator to test different hypotheses for the anaerobic metabolism of
276 Accumulibacter against published experimental data (Figure 2).

277 **Lumped stoichiometric model of Accumulibacter for Flux Balance Analysis**

278 The goal of the flux balance analysis was to assess the balance of reducing equivalents
279 by Accumulibacter during anaerobic acetate conversion to PHAs. To this end, we used the
280 stoichiometric model of Accumulibacter's anaerobic metabolism presented in Table 2,
281 which is a lumped version of the central carbon metabolic network shown in Figure 1.
282 Given the presented evidence that PHA accumulation in Accumulibacter is NADH-
283 consuming (instead of the previously assumed NADPH preference), it follows that the
284 previously-made assumption in many Accumulibacter models that the type of redox
285 cofactor used does not affect the stoichiometry is valid: If the reducing reaction is feasible
286 using NADH, which is usually a worse electron donor than reduced ferredoxin or NADPH,
287 then the latter two can always be converted to NADH using transhydrogenases and
288 without any energetic cost for the cell (26). The same does not hold for FADH, which
289 would require an input of energy (ATP) to be re-oxidized using $NAD^+$. Moreover, there is

290 still no experimentally validated mechanism on how Accumulibacter could re-oxidize
291 FADH in the absence of oxygen or nitrate. Therefore, for this simulation, we blocked this
292 FADH producing step in the TCA cycle (i.e. Succ-CoA to Fum) and for the remaining
293 reactions we neglected the different types of redox cofactors by simply balancing
294 "electrons" (note that each redox cofactor carries 2 electrons and in some publications
295 these are referred to as [H]). Since the FADH producing step in the TCA cycle is blocked,
296 only the oxidative branch (OAA to Succ-CoA via ICDH and AKGDH) and/or the reductive
297 branch (OAA to Succ-CoA via MDH and FR) are possible.

298 Table 2 – Stoichiometry used for flux balance analysis (in mol basis) to simulate
299 Accumulibacter's anaerobic metabolism. Reversible reaction: <->, irreversible reaction: ->. Note
300 that these are lumped reactions and each may involve several enzymatic steps.

| Pathway | Stoichiometry | Enzyme assayed in this study |
|---|---|---|
| **Glycolysis (EMP)** | $(Glycogen)_1$ -> 2Pyr + 4electrons | G6PDH<br>6PGDH<br>GAPDH |
| **Pyruvate to Acetyl-CoA** | Pyr -> Ac-CoA + 2electrons + $CO_2$ | |
| **Pyruvate to Oxaloacetate** | Pyr + $CO_2$ <-> OAA | |
| **Oxidative TCA branch** | OAA + Ac-CoA -> Succ-CoA + $2CO_2$ + 4electrons | ICDH<br>AKGDH |
| **Reductive TCA branch (reverse TCA)** | OAA + 4electrons -> Succ-CoA | MDH<br>FR |
| **Glyoxylate shunt** | 2Ac-CoA -> Succ-CoA + 2electrons | ICL |
| **Succinate-propionate shunt** | Succ-CoA <-> Pr-CoA + $CO_2$ | |
| **Ac-CoA* production (precursor for 3HB, 3HV and 3HMB)** | Ac-CoA + electron -> Ac-CoA*<br>(* means "+1electron") | AcAc-CoA red |
| **Pr-CoA* production (precursor for 3H2MV, 3HV and 3HMB)** | Pr-CoA + electron -> Pr-CoA*<br>(* means "+1electron") | AcAc-CoA red |

301 Regarding the ATP generation, there are still, unfortunately, too many unknowns: 1)
302 how much ATP can be generated from efflux of ions (potassium, magnesium and
303 phosphate) associated with polyphosphate hydrolysis; 2) how much is required for

acetate uptake; 3) how much is needed to upgrade redox cofactors; 4) and how much ATP is actually used for cellular maintenance. Therefore, we will not balance ATP in this simulation. Still, it is important to note that the different pathways used for redox balancing lead to different levels of ATP generation via direct substrate-level phosphorylation, and that, in cases of polyphosphate limitation, more ATP-generating pathways will be preferred over less generating ones.

Experiments show that the ratio between glycogen and acetate consumption by PAO's is variable, e.g. between high/low temperature (29) or polyphosphate/glycogen availability (30–32). Therefore, in our simulations, we varied this ratio between the glycogen and acetate fluxes and we compared the results with experimental data reported in literature.

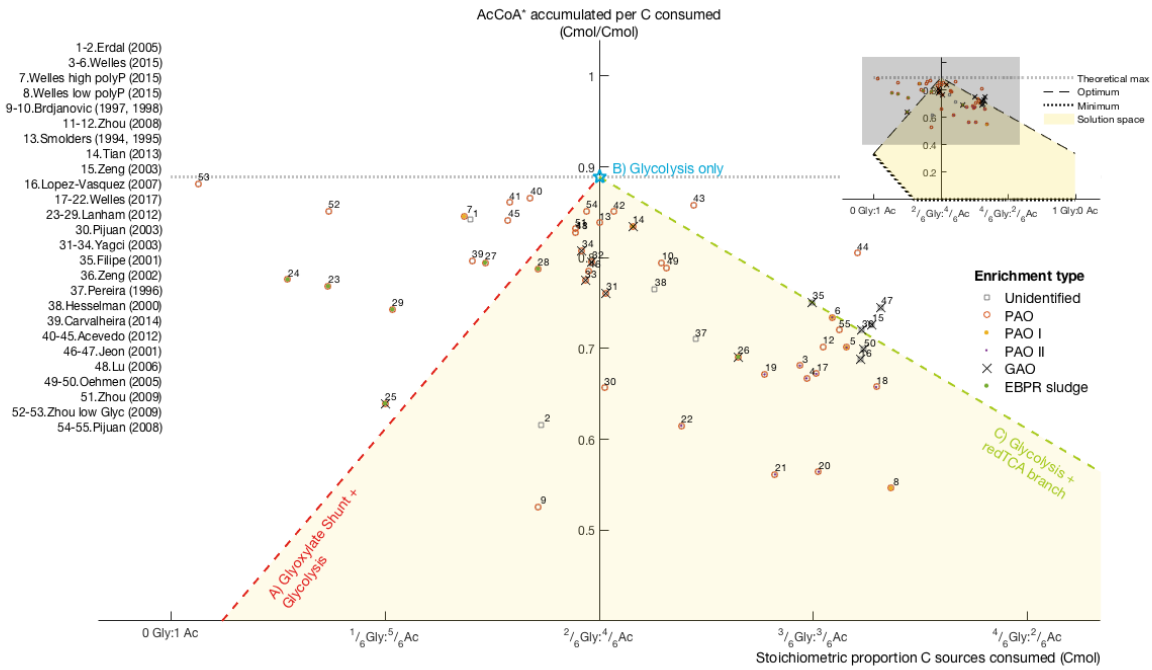



Figure 2 – The amount of Ac-CoA* accumulated (PHB and PHV precursor) depends on the different proportion of glycogen to acetate consumed and on the available pathways. The three optimum redox balancing strategies are shown in Figure 3 ABC. From 0 to approx. 2/6 glycogen Cmol, it can be considered that bacteria are performing a polyphosphate-based metabolism (PAM) and from approx. 3/6 to 1 glycogen Cmol, a Glycogen-based metabolism (GAM) is used. The "optimum" and "minimum" lines were obtained by minimizing and maximizing $CO_2$ production, respectively. The feasible solution space (in yellow) lies in between these two cellular objectives, in which a mixture of different redox balancing strategies is used. The minimum carbon conserving strategy is that which only uses the oxidative TCA branch. Experimental data was retrieved from (7,22,29,31,32,35,47,50,61–72) and reconciled to respect carbon and energy conservation principles. Similar plots for Pr-CoA*, $CO_2$, alternative simulations and including error bars on the experimental data points can be found in Supplementary Document 6.

328 For the anaerobic sequestration of acetate as PHA, acetate needs to be reduced to form
329 PHA. The Ac-CoA* optimum in Figure 2 corresponds to the situation in which most carbon
330 is kept inside the cell, most likely in the form of PHB. The source of reducing power can
331 come from either glycolysis or partial TCA cycle operation and the corners of the optimum
332 line in Figure 2 represent the extreme cases, which are presented in Figure 3. If the
333 amount of glycogen degraded can supply exactly the amount of electrons needed for the
334 uptake and conversion of acetate, then only Ac-CoA* (as PHB) is expected and there is no
335 need to use any part of the TCA cycle (Figure 3B). However, in a typical GAO-like
336 metabolism, an excess of glycogen is degraded compared to the acetate consumed to
337 supply all ATP needed. This leads to the generation of an excess of reducing power, which
338 is used to produce Pr-CoA* leading to PHAs more reduced than PHB (e.g. PHV or even
339 $PH_2MV$), using the reductive TCA branch (Figure 3C). On the other hand, in a PAO-like
340 metabolism when most ATP is provided by polyphosphate metabolism, the amount of
341 reducing equivalents generated by glycolysis is usually limited when compared to the
342 amount of acetate consumed. In this situation, part of the acetate also needs to be oxidized
343 to produce reducing equivalents. With the enzymatic activity observation of an active
344 glyoxylate shunt, our simulations show this as the optimal pathway to produce PHAs
345 when glycogen is limiting (Figure 3A); it allows for higher carbon conservation (i.e. higher
346 PHA yield) than the "horseshoe" TCA operation and also avoids the problem of
347 regenerating FADH produced in a full TCA cycle run.

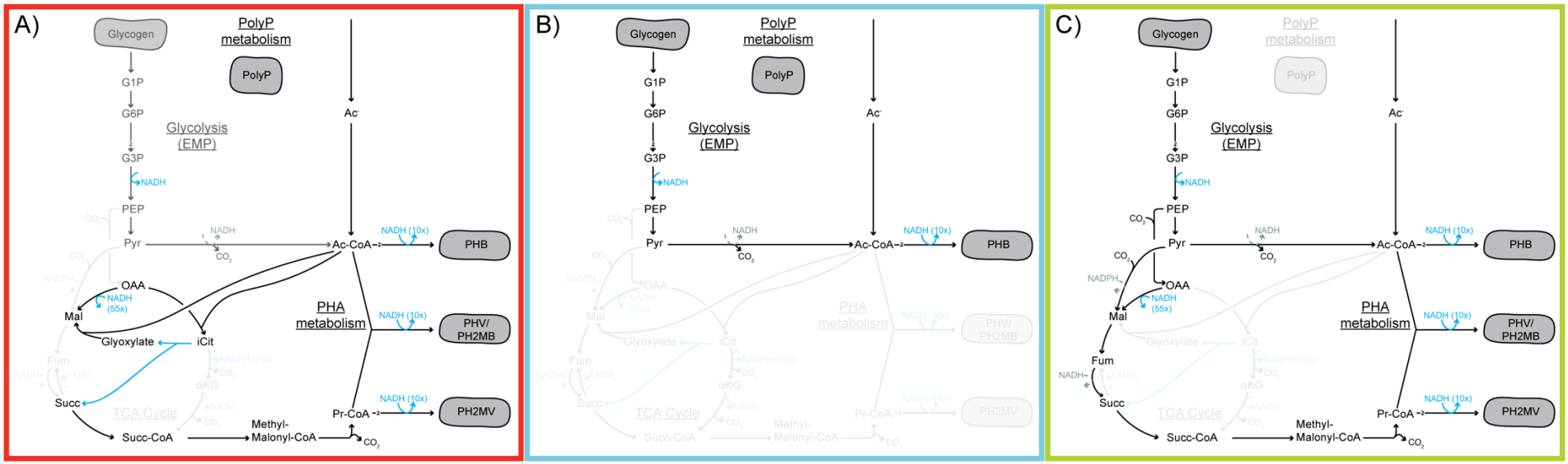



Figure 3 – Different optimum redox balancing strategies for Accumulibacter under anaerobic conditions. A) When little glycogen is degraded and is not enough to reduce all acetate to PHB, the glyoxylate shunt as recommended by (47) is the most optimal way to provide these electrons. B) When the stoichiometric amount of glycogen is degraded for acetate reduction to PHB, there is no real need to operate any part of the TCA cycle to balance redox and only PHB is then produced, as proposed in (48). C) When more glycogen than needed for acetate reduction is degraded, the reductive TCA branch can be used as proposed by (47) for GAOs to sink electrons in more reduced PHAs (e.g. PHV and PH2MV).

354 # DISCUSSION

355 ## Characterization of Accumulibacter enrichments

356 Firstly, it is important to note that all presented results and following interpretations
357 hold for the microbial community present in our PAO enrichments (mainly *Ca.*
358 Accumulibacter phosphatis clade I). Any extrapolation of these results to Accumulibacter
359 clade II or GAOs must be considered an assumption until further validation. This is due to
360 the well-known fact that, depending on their environment, different types of
361 Accumulibacter clades can be found, and each may have a slightly different function
362 (7,28,33–36).

363 ## Updated biochemical (stoichiometric) model

364 Accumulibacter and their flexible metabolism have shown to defy many of the
365 traditional characteristics of anaerobic metabolism due to their energy and reducing
366 power storage, for example: anaerobic PHB synthesis decoupled from growth or the fast
367 anaerobic acetate uptake are thanks to ATP provided by their polyphosphate storage.
368 Consequently, general biochemical knowledge may only help guiding thoughts and
369 analyses on Accumulibacter's unique metabolism, and the actual characteristics of their
370 biochemistry should still be validated. Here in this study, enzymatic assays were
371 performed to define what are the redox cofactor preferences of the main oxidoreductases
372 in the central metabolic pathways of Accumulibacter. The key finding in this study is the
373 NADH-preferring PHA accumulation in Accumulibacter. Unlike for NAPDH-PHA
374 producers, this NADH-preference allows for a direct consumption of the NADH produced
375 in most of Accumulibacter's reducing power sources. This prevents the need for an
376 additional conversion of NADH into NADPH at the cost of proton motive force via the
377 membrane-bound transhydrogenase (PntAB) as suggested by (8). Although previous
378 reports of the use of NADH to drive PHB accumulation are found in literature (10,37–40),
379 the level of NADH preference of the acetoacetyl-CoA reductase from Accumulibacter is the

380 largest so far observed with virtually no NADPH-activity (see Supplementary Document
381 S4). This observation allows to re-think the role of PHAs: we hypothesize that depending
382 on the environment where microorganisms thrive, PHA accumulation will play a role as
383 carbon reservoir during metabolic over-flow (NADPH-driven accumulation) or as an
384 electron reservoir during scarcity of external electron acceptors (NADH-driven
385 accumulation). Thus, for Accumulibacter, PHA is essentially a fermentation product.

386 Another important finding was the absence of activity of the first step of the
387 sometimes-implicated ED glycolytic pathway. These results match those observed by (29)
388 and in (41) for GAOs, where no glucose-6-phosphate dehydrogenase activity was found,
389 using $NADP^+$ only. The experimental findings are in line with the absence of key ED genes
390 in the genome annotations of Accumulibacter and closest relatives *Dechloromonas*
391 *aromatica* and *Azoarcus sp. EbN1* (36,42). Furthermore, we also verified the absence of
392 the ED pathway with $NAD^+$ as electron acceptor since there are microorganisms known
393 to use both cofactors (25). On the other hand, these findings are in contradiction with
394 earlier $^{13}C$ NMR studies (21,22), which indicated that the ED pathway was more likely the
395 route for glycolysis than EMP. Nevertheless, it has to be noted that these early studies
396 comprise interpretations of $^{13}C$ patterns using simplified metabolic models, with limited
397 information on the reversibility of each reaction and potentially with reactions missing;
398 these are common pitfalls of the $^{13}C$-labelling method (43). Furthermore, these $^{13}C$ NMR
399 studies were performed before the first draft genome of Accumulibacter was available
400 (42), and only the $^{13}C$ pattern in the different PHAs was measured and not in the metabolic
401 intermediates of each pathway used.

402 While NADPH is not required anaerobically, this reducing cofactor still has to be
403 produced aerobically for growth. Given the observation that NADPH is not produced by
404 the oxPP pathway, the next logical candidate is the isocitrate dehydrogenase in the TCA
405 cycle. This is indeed the case, which is not unexpected as most acetate consumers will use
406 this conversion to produce NADPH for their anabolism (44) and confirms the protein

407 annotation done by (36). Since PHA accumulation is now known to be NADH-preferring
408 and without another sink of NADPH, the latter might accumulate anaerobically and
409 thereby inhibit the isocitrate dehydrogenase reaction. Alternatively, a soluble
410 transhydrogenase can convert this NADPH into NADH and allow the oxidative branch of
411 the TCA to be operational under anaerobic conditions.

412 Regarding the activity of other TCA oxidoreductases and anaplerotic routes: 1) the
413 oxidation of malate using $NAD^+$ was also found in the studies of (29); 2) the glyoxylate
414 shunt was also found active as observed by (22,29,45), which is expected as this is the
415 anaplerotic route that allows for microorganisms to convert C2 sources like acetate into
416 C4 building blocks for anabolism (44); and 3) fumarate reductase and α-ketoglutarate
417 dehydrogenase activities were very low compared to all other enzymes tested in our
418 study and alike in the studies of (29). Since we did not include biological controls, we
419 cannot interpret these low activities. We recommend that these enzymes are assayed
420 again next to biological controls to at least show that the conditions used in the assay were
421 appropriate. We also propose to assay the oxidation of α-ketoglutarate using ferredoxin
422 as electron acceptor as the α-ketoglutarate:ferredoxin oxidoreductase has been identified
423 in Accumulibacter's genome and proteome (36).

424 All the aforementioned redox cofactor specificities were used to update
425 Accumulibacter's central carbon metabolic network. With this biochemical model, we
426 performed a Flux Balance Analysis to study Accumulibacter's main activity under
427 anaerobic conditions. Since growth is negligible, the main anaerobic metabolic activity is
428 restricted to the central pathways, hence why we consider a reduced metabolic network
429 sufficient for this analysis. Furthermore, the inherent FBA assumption of no intermediate
430 accumulation is acceptable while the flux going through each intermediate is much larger
431 than its own concentration. This is the case during acetate uptake when it is present at
432 high concentrations (i.e. uptake rate is maximal and steady). Inversely, it might not be
433 valid during the anaerobic famine phase when no acetate is present since it will depend

434 on how large the glycogen degradation flux is compared to the intracellular concentration
435 of intermediates.

436 **Flexible anaerobic metabolism – Adjustments depending on available compounds**
437 **accumulated and on the environment**

438 As (6) suggested in their review, the flexibility of Accumulibacter's metabolism is likely
439 the major reason why there is controversy in literature regarding how reducing power is
440 balanced under anaerobic conditions. Long-term exposure to set conditions (e.g. pH,
441 temperature, oxidation level of the substrate, nutrient availability, counter-ions, SRT,
442 settling time) will select for the best strategy for those conditions, but short-term
443 perturbations of those set conditions have shown that Accumulibacter seems to still be
444 able to solve the redox balancing problem even if sub-optimally regarding carbon
445 conservation (7,30,31,46). Thus, based on our analysis, we believe that there is not one
446 fixed stoichiometry, but a range of possible stoichiometries that in the end are defined by
447 the relative proportions of each of the intervening substrates, supplied to the system or
448 produced by a side-population (e.g. acetate, glycogen, polyphosphate, other VFAs, oxygen,
449 nitrate, hydrogen).

450 The simulations showed Accumulibacter can face three distinct situations: A) when
451 reducing equivalents from glycolysis are limiting compared to the acetate imported
452 (Yagci's model (47)); B) when glycolysis supplies exactly enough reducing equivalents to
453 convert all imported acetate into PHB (Mino's model (48)); and lastly C) when glycolysis
454 is producing an excess of reducing equivalents (alike Yagci's model for GAOs (47)).

455 The anaerobic use of the glyoxylate shunt in a PAO-like metabolism to provide for the
456 reducing equivalents needed for PHA accumulation is supported by the studies of (45,47);
457 However, as seen in Figure 2, it does not explain the higher levels of Ac-CoA* found (points
458 above the optimum line) when more reduced PHAs are expected. This indicates that there
459 might be an alternative process that allows the cell to conserve extra carbon, in other
460 words, more Ac-CoA* and less Pr-CoA*. It could be that 1) fully anaerobic conditions were

461 not attained when performing the experiments, or that 2) another side population is
462 providing electrons in the form of a more reduced organic substrate or even hydrogen gas
463 (49) or 3) Accumulibacter has yet another, alternative way of balancing redox which has
464 yet to be described/demonstrated.

465 Also for the scenario where glycolysis produces an excess of reduced cofactors as in a
466 GAO-like metabolism, a few experimental data points fall outside of the feasible solution
467 space of our simulations (points above the optimum line in Figure 2); these could be
468 explained in the case that Accumulibacter produces $H_2$ to solve an excess of NADH as
469 described by (8).

470 **The multilayered complexity of a physiological analysis of Accumulibacter**

471 The experimental data gathered are likely also noisy due to the intrinsic heterogeneity
472 of Accumulibacter enrichments; clade differences, population heterogeneity or even
473 different environment (e.g. depending on the position of the cell in a floc/granule).
474 Therefore, experimental data points under the optimum line in Figure 2 likely represent
475 mixtures of cells, each with a different mode of operation (Figure 3 A, B or C).
476 Furthermore, any kinetic limitation (i.e. pathway capacity) may also explain a sub-
477 optimal, mixed phenotype. And from this analysis, when all enzymatic machinery is
478 available anyway, it seems possible that the same cell might be able to go from A to B to C
479 depending on the availability of acetate (e.g. transition feast/famine) or if polyphosphate
480 becomes limiting, or even from C to A when glycogen becomes limiting.

481 Nevertheless, despite the noise, the experimental data outside the solution space
482 suggests this stoichiometric model is still incomplete. For example, when allowing for
483 hydrogen production in excess glycogen-to-acetate conditions or the oxidation of acetate
484 in a fully operational TCA cycle in a glycolysis-limiting scenario, the observed data can be
485 explained (see Supplementary Document 6). The first is supported by the detection of
486 hydrogen gas produced by an Accumulibacter enrichment (8), while the latter has been
487 previously suggested based on $^{13}C$ NMR observations (50) and stoichiometric analyses

488 such as done by (30). Furthermore, a novel protein has been proposed based on
489 metagenomic analysis to allow for full TCA operation under anaerobic conditions (42);
490 however, there is still no experimental evidence that indeed validates this peculiar
491 anaerobic TCA operation. Moreover, to confirm full TCA cycle activity anaerobically, we
492 suggest the use of $^{13}C$ labelled acetate together with MS techniques, which allow for
493 tracking $^{13}C$ patterns in the different pathway intermediates. In (51), labelled propionate
494 has been used and $^{13}C$ was found back in the PHV fragments that come from acetyl-CoA,
495 thus supporting the full TCA should be active although alternative metabolic routes could
496 also explain this observation. Furthermore, it may also be considered that Accumulibacter
497 can also make use of energy conservation features employed by many anaerobes such as
498 electron bifurcation (52), which employs ferredoxin oxidoreductases to allow for
499 alternative pathways in the central carbon metabolism.

500 ## CONCLUSION

501 In this study an interdisciplinary approach was used, which integrated findings from
502 genomics, transcriptomics and different physiological datasets as well as biochemical
503 assays, to unravel the metabolic flexibility of Accumulibacter under dynamic conditions.

504 This flexible metabolic function can explain previous controversy in PAO literature.
505 Depending on the exact history and cultivation conditions, Accumulibacter can exhibit
506 different metabolic phenotypes. The NADH dependent PHA synthesis generates a direct
507 link to central pathways used by Accumulibacter without the previously assumed
508 requirement to generate NADPH for PHA synthesis through an oxidative PPP or ED
509 pathways, or an energy-consuming transhydrogenase. Thus, under anaerobic conditions,
510 Accumulibacter can make combinations of glycolysis (EMP), different branches of the TCA
511 cycle (incl. glyoxylate shunt) and potentially other pathways not yet considered. This
512 poses a challenge to predictive EBPR modelling, as it implies that stoichiometry is not
513 fixed, but variable, spanning continuously from polyphosphate to glycogen-based
514 phenotypes.

515 This metabolic versatility is likely what allows Accumulibacter to be, in most
516 situations, very close to a carbon conservation optimum, which is key to ensure their
517 competitiveness in the dynamic environments of EBPR systems.

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## COMPETING INTERESTS

The authors declare no conflict of interest.

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