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Microbial phylogenetic and functional responses within acidified wastewater communities exhibiting enhanced phosphate uptake

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1 **Microbial Phylogenetic and Functional Responses within Acidified Wastewater**
2 **Communities Exhibiting Enhanced Phosphate Uptake**

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25 **ABSTRACT**

26 Acid stimulated accumulation of insoluble phosphorus within microbial cells is highly
27 beneficial to wastewater treatment but remains largely unexplored. Using single cell
28 analyses and next generation sequencing, the response of active polyphosphate
29 accumulating microbial communities under conditions of enhanced phosphorus uptake
30 under both acidic and aerobic conditions was characterised. Phosphorus accumulation
31 activities were highest under acidic conditions (pH 5.5 > 8.5), where a significant
32 positive effect on bioaccumulation was observed at pH 5.5 when compared to pH 8.5.
33 In contrast to the *Betaproteobacteria* and *Actinobacteria* dominated enhanced biological
34 phosphorus removal process, the functionally active polyP accumulators at pH 5.5
35 belonged to the *Gammaproteobacteria*, with key accumulators identified as members of
36 the families *Aeromonadaceae* and *Enterobacteriaceae*. This study demonstrated a
37 significant enrichment of key polyphosphate kinase and exopolyphosphatase genes
38 within the community metagenome after acidification, concomitant with an increase in
39 P accumulation kinetics.

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41 **Key words:** Polyphosphate accumulating organisms / pH/ acid-stimulated biological
42 phosphorus removal / Polyphosphate kinase

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49 **1. Introduction**

50 Phosphorus is present at high loading in wastewater streams and is a key agent of
51 environmental eutrophication. However, the impact of soluble phosphorus contained
52 within release streams can be mitigated using technologies that induce enhanced
53 phosphate uptake by the microbial community and its intracellular accumulation as
54 insoluble polyphosphate (polyP) (McGrath and Quinn, 2003; Nielsen et al., 2010).
55 Enhanced Biological Phosphorus Removal (EBPR) is an effective, low cost
56 biotechnological process for P removal from wastewater which is achieved by
57 modifying the conventional activated sludge system to include alternating anaerobic and
58 aerobic phases (McGrath and Quinn, 2003; Yuan et al., 2012).

59 Although EBPR has been widely studied from the engineering and chemical
60 perspective, an understanding of the microorganisms performing the C and P
61 transformation involved in EBPR, together with the environmental factors affecting
62 enhanced P (and polyP) accumulation efficiency, have yet to be fully resolved. Such
63 knowledge is clearly fundamental to the design of effective P removal systems (Nielsen
64 et al., 2010). Indeed, EBPR processes can show variation in the efficiency and
65 effectiveness of P removal (Kawaharasaki et al., 1999; Oehmen et al., 2007). Functional
66 EBPR systems depend on the interaction of complex microbial communities with the
67 prevailing environment within a specific compartment of the treatment process.
68 Consequently, process optimisation is maintained through the control of the operational
69 environment within the process compartments, but this can be difficult to do without a
70 fundamental understanding of the types of microorganisms involved and their pathways
71 under different environmental conditions, such as anaerobic and aerobic transitions.

72 Therefore, the desire for simplified systems has promoted the analyses of simpler,
73 alternative environmental conditions which can induce enhanced phosphorus uptake.

74 PolyP biosynthesis is not just confined to those microorganisms exposed to the
75 alternating anaerobic/aerobic environment of the EBPR treatment regime. Given the
76 prevalence of polyP throughout the microbial world, it is clear that polyP accumulation
77 can be triggered by many environmental factors (Powell et al., 2008; Zheng et al., 2014)
78 and extensive microbial polyP accumulation has been measured in response to nitrogen,
79 amino acid or P limitation as well as to osmotic or oxidative stress (Mullan et al., 2006;
80 Rao et al., 2009; Temperton et al., 2011). Other evidence has shown that enhanced
81 polyP accumulation can be induced in fully aerobic cultures, under acidic conditions,
82 without the need for prior anaerobiosis (McGrath et al., 2001; Moriarty et al., 2006;
83 Mullan et al., 2002). McGrath and Quinn (2000) reported a 10.5-fold increase in
84 intracellular polyP accumulation in *Candida humicola* G-1, grown at pH 5.5 versus pH
85 7.5. In operational mixed, aerobic wastewater communities of activated sludge,
86 phosphate uptake increased between 50% and 143% when the pH was adjusted down to
87 5.5 as compared to normal operating levels around pH 7.5 (McGrath et al., 2001). In
88 these acidic systems, 34% of the activated sludge microflora proved to be capable of
89 increased phosphate uptake following acidification. These findings showed that
90 phosphate removal could be substantially enhanced by acidification under strictly
91 aerobic conditions (McGrath et al., 2001; Moriarty et al., 2006; Mullan et al., 2002) and
92 that the process was economically feasible (Mullan et al., 2006). Duguid et al. (1954)
93 have previously reported that intracellular polyP production in *Klebsiella aerogenes*
94 occurred only during growth at pH 4.0 to 5.0 and not at neutral pH values. Similar
95 observations have been made in soil fungi where Gerlitz (1996; 1997) demonstrated

96 that maximal polyP accumulation in the ectomycorrhizal fungus *Suillus bovinus* was
97 35% greater at pH 5.5 than at pH 7.5. However, information on how acidic conditions
98 influence microbial community dynamics and the bioaccumulation of polyP during
99 wastewater treatment acidic remains largely unexplored.

100 Using previous observations of acid stimulated P removal and polyP accumulation,
101 we sought to understand the mechanism and taxa involved in engineered biological
102 systems which could accumulate phosphorus under these conditions. It was
103 hypothesised that there would be a significant microbial polyphosphate accumulation
104 under acid conditions in engineered wastewater treatment communities and that this
105 would be accompanied by an increase in functionally active polyP accumulators and P
106 accumulation kinetics. To understand the microbial communities involved, a combined
107 phylogenetic and metagenome analysis was used to investigate changes in the bacterial
108 community structure (16S rRNA genes) and functional diversity (metagenomics) of
109 waste treatment systems run under acidic and near-neutral conditions. These changes in
110 structural and functional diversity were evaluated relative to increases in P uptake. To
111 quantify the organisms responsible for poly P accumulation, flow cytometry and
112 coincident single cell analyses was used to enumerate functional polyphosphate
113 accumulators coupled with next generation sequencing to determine the community
114 structure and diversity of the organisms involved. The approach used provides a 'cell to
115 population' understanding of the polyP accumulation process that can then be used as
116 the basis of an optimisation strategy for acid stimulated P removal from wastewater
117 streams.

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120 2. Methods

121 2.1. Sampling site description and sampling

122 A covered anaerobic pond (CAP) fitted with an impermeable cover was constructed
123 at Medina Research Station, Western Australia (GPS geocoder: Latitude -32.223000,
124 Longitude 115.805801) to treat piggery effluent waste and capture bioenergy. The waste
125 treatment process can be separated into 5 stages: collection pits in the pig shed; solid
126 separation screens, holding tank, the CAP and finally a secondary aerobic pond.
127 Effluent from the pig pens was collected into the pits followed by release into a 100,000
128 L underground tank from where it was pumped over a static run-down screen (solid
129 separator) that removed 10-15% of the total solids. The remaining wastewater was
130 transferred to a holding tank prior to being pumped into the CAP digester (ca. 25m
131 x25m x5m) on a weekly basis (75,000 L/wk). The biogas produced from the CAP was
132 removed through a perforated pipe system placed around the perimeter of the pond.
133 Treated effluent was finally transferred to the secondary aerobic pond (ca. 50m x50m
134 x5m) for evaporation (Fig. S1).

135 Samples for laboratory phosphorus removal experiments were collected from the
136 aerobic pond by suction using a 12V marine grade bilge pump connected to a PVC
137 hosepipe. The hosepipe was placed into the aerobic pond and run for 5 mins to flush the
138 sampling line, and samples from each point were collected into several autoclaved
139 containers with corresponding samples mixed together to make a composite sample for
140 laboratory incubation experiments. The chemical composition of the composite
141 wastewater was assessed as follows: pH 8.5; EC 5.2 mS/cm; chemical oxygen demand
142 483 mg/L; total solids 0.1 %; volatile solids 25.8 %; total nitrogen 6.8 %; total C 45.9
143 %, total P 13.6 mg/ L; orthophosphate 12.2 mg/L; Ca 21 mg/L; Mg 148 mg/L; K 681

144 mg/L; Fe 0.1 mg/L using standard methods for the analysis of water and wastewater
145 (Eaton et al., 2005).

146 *2.2. Lab-scale incubation experiment*

147 The initial pH and inorganic orthophosphate concentration (Pi) of the wastewater at the
148 sampling was pH 8.5 and 12.2±0.3 mg/L respectively. The Pi concentration was
149 adjusted to 25 mg/L using KH₂PO₄ (25mM) to simulate a moderately low P loaded
150 wastewater system for lab-scale evaluation. The experimental design comprised of five
151 different pH treatments (5.5, 6.0, 6.5, 7.0 and 8.5 [control]) run in triplicate to determine
152 the best pH level for polyP accumulation. The control represented the natural pH level
153 of the wastewater at the time of sampling (pH 8.5). For each treatment, effluents (300
154 mL) were placed into autoclaved jars (500 mL) and kept under aerobic conditions
155 (regular oxygen bubbling) at room temperature (25°C) for 48 h under a natural
156 light/dark illumination cycle. Orthophosphate concentration at the beginning and after
157 48h of incubation was determined using published methods (Eaton et al., 2005).
158 Microcosm samples from pH 5.5 and 8.5 were chosen, after initial determination of
159 optimum acidification conditions (Fig. S2), for downstream epi-fluorescence
160 microscopy, flow cytometry and molecular analyses.

161 *2.3. Sample preparation for epi-fluorescence microscopy and flow cytometry*

162 At the end of the incubation experiment (48 h), aliquots (1 mL) from each
163 microcosm were taken and centrifuged at 5000 x g for 5 min. The cell pellet was
164 washed with phosphate buffered saline (PBS) then resuspended in PBS, fixed with 4%
165 (w/v) paraformaldehyde fixative solution (PFA) and incubated overnight at 4°C.

166 For samples analysed by flow cytometry, cells were stained with 15 ug/mL DAPI
167 (4',6-diamidino-2-phenylindole) for 20 mins, according to Kawaharasaki et al. (1999).

168 For epi-fluorescence microscopy, the PFA fixed cells were washed with PBS and
169 distilled water before mounting fifty microliters (50 μ L) of cell suspension in the middle
170 of a microscope slide and air drying. Slides were subsequently stained with DAPI (15
171 μ g/mL for 20 mins), rinsed with distilled water and airdried. Cells were visualised under
172 x100 objective using a Zeiss Axioplan epifluorescence microscope under UV excitation
173 (DAPI filter block).

174 Flow cytometric analyses were performed using a BD Influx cell sorter (Becton
175 Dickinson, USA) at the Centre for Microscopy, Characterisation and Analyses at The
176 University of Western Australia. DAPI was excited with a 355 nm (UV) laser with
177 standard DAPI emission collected with a 460/50 nm band pass filter; the yellow shifted
178 DAPI-PolyP bound emission was collected with a 585/29 bandpass filter.

179 Measurements for DAPI and polyP were acquired on a logarithmic scale and post-
180 acquisition analysis performed using Flow Jo software version 7.6.5. Single cells were
181 gated on forward scatter area (FSC-A) vs forward scatter height (FSC-H) to exclude any
182 doublets and DAPI-DNA and DAPI-polyP were gated to determine proportions of
183 bacteria accumulating polyphosphate. Briefly, DAPI-bound polyP cell populations
184 formed a distinct cluster in the polyP 570-600nm range whilst non-accumulators
185 exhibited only DAPI-DNA fluorescence in the DAPI fluorescence 435-485 nm range.

186 *2.4. DNA extraction and 16S rRNA Tag sequencing*

187 DNA from microcosm treatments (pH 5.5 and pH 8.5) was extracted using the
188 MoBio Powersoil DNA isolation kit (Geneworks, Australia) with bead beating and
189 column purification, as described by the manufacturer. Extracted DNA was quantified
190 and checked for purity at A260/280 nm (Nanodrop, Thermo Fisher Scientific, USA)
191 prior to storage at -20°C. Fragments of the 16S ribosomal RNA gene were amplified by

192 polymerase chain reaction (PCR) from the DNA samples using Golay barcodes fused to
193 Ion Torrent adapter modified core primers 341F and 518R (Whiteley et al., 2012), using
194 amplification conditions described previously (Jenkins et al., 2010). All PCR products
195 were checked for size and specificity by electrophoresis on 1.5% w/v agarose, gel
196 purified and adjusted to 10 ng/ μ L using molecular grade water and then pooled equally
197 for subsequent sequencing. Sequencing was performed using an Ion Torrent Personal
198 Genome Machine (PGM) (Life technologies, USA) using 200 base-pair chemistry as
199 described previously by Whiteley et al. (2012). All the PGM quality filtered data were
200 exported as FastQ files and split into *.fasta and *.qual files and analysed using the
201 QIIME pipeline (Caporaso et al., 2010). Assigning the multiplexed reads to samples
202 was performed using standard parameters (minimum quality score = 25;
203 minimum/maximum length = 130/220; no ambiguous base calls; removal of reverse
204 primers; and no mismatches allowed in the forward and reverse primer sequences).
205 Chimera checking was done using USEARCH61 (Edgar et al., 2011) and only non-
206 chimeric sequences were assigned operational taxonomic units (OTUs) using the
207 Greengenes (GG) reference database with clustering at 97% identity using the UCLUST
208 algorithm. Singletons were removed and taxonomy was assigned to the representative
209 sequence of each OTU. Alpha rarefaction was performed using the phylogenetic
210 diversity, Chao1, the Shannon Index and Observed Species metrics.

211 *2.5 Whole genome shotgun sequencing*

212 DNA was extracted from the laboratory microcosm experiments (pH 5.5 and pH 8.5)
213 using the MoBio Powersoil DNA isolation kit (Geneworks, Australia), as described
214 above. Genomic DNA sequencing of these samples was performed using whole genome
215 shotgun sequencing where 150 ng of DNA was used to generate a whole genome

216 shotgun library using a NEBnext Ultra library preparation kit (New England
217 Biosciences). Fragments of 320-330bp were selected from the final library by gel-
218 excision and sequenced for 520 flows on a Proton sequencer (Life Technologies),
219 yielding reads of 230-240bp modal length. Quality filtering and trimming were
220 performed 'on instrument' using TorrentSuite 4.0. The sequencing data for each of the
221 sampling points were rarefied to the same sequencing depth (300000 reads) and
222 uploaded to MG-RAST (Metagenome Rapid Annotation using Subsystem Technology
223 (MG-RAST) server (<http://metagenomics.nmpdr.org/>). Metagenomic data sets are
224 publicly available in the MG-RAST system under project identifiers 4553566.3 (pH 8.5;
225 control) and 4553567.3 (pH 5.5). Assignment of metabolic function and phylogenetic
226 identification were performed as described previously (Meyer et al., 2008).

227 *2.6. Statistical analysis.*

228 ANOVA was performed using the Statistical Analysis System (SAS) version 9.2
229 software package (SAS Institute, Inc. Cary, NC, USA). Means were separated using
230 least significant difference (LSD) at 5% significance level.

231

232 **3. Results and Discussion**

233 *3.1. PolyP accumulation in a laboratory incubation experiment*

234 Soluble orthophosphate concentrations within aerobic microcosms indicated the
235 absolute removal of P was 45% higher at pH 5.5 than at pH 8.5 after 48 hours (Fig. S2).
236 Subsequently, the observed increases in polyP accumulation were matched by an
237 increase in the number of microorganisms able to accumulate polyP, as determined by
238 direct epi-fluorescence microscopy and flow cytometry. Epifluorescence microscopy
239 (Fig. S3) showed that qualitatively, the number of DAPI-polyP stained cells increased at

240 pH 5.5 (Fig. S3b) when compared to pH 8.5 (Fig. S3a). Flow cytometric analyses for
241 quantification of polyP accumulators (Fig. 1a and 1b) indicated increased P
242 accumulation was mediated by a doubling of the cells actively accumulating polyP at
243 pH 5.5 when compared to pH 8.5 (70% versus 36% respectively). This was consistent
244 with previous findings in other treatment systems, where growth of polyphosphate
245 accumulators was enhanced and the aerobic uptake of phosphate reached a maximum at
246 pH 5.5 (McGrath et al., 2001; Mullan et al., 2002; Moriarty et al., 2006).

247 *3.2. Phylogenetic community structure of polyphosphate accumulating microorganisms* 248 *under enhanced accumulation conditions*

249 DNA extracted from incubated microcosms was used to assess the phylogenetic
250 affiliations and relative abundances of the bacteria in microcosms incubated at pH 5.5
251 and pH 8.5 by next generation sequencing. After normalising all samples to 5000
252 sequence reads, alpha diversity metrics indicated that bacterial diversity was higher in
253 samples incubated at pH 8.5 (246 OTUs) when compared to pH 5.5 (173 OTUs),
254 indicating that acidification selected a sub-set of the diversity present within the control
255 community. Further, 16S rRNA sequencing indicated a different phylogenetic
256 community structure at pH 5.5 when compared to pH 8.5 (Fig. 2). The bacterial
257 community composition under 'normal' pH 8.5 operating conditions was dominated by
258 *Actinobacteria* (50.7%), followed by *Betaproteobacteria* (19.9%), *Erysipelotrichi* (8.2
259 %), TM7 (5.1%) and *Gammaproteobacteria* (2.9%). In comparison, members of the
260 *Gammaproteobacteria* accounted for 90% of the sequences recovered at pH 5.5. This
261 indicated a 30 fold increase in *Gammaproteobacteria* taxa, represented by
262 *Aeromonadaceae* (72% of sequences classified), *Enterobacteriaceae* (16%),
263 *Alteromonadales* (4%), and the genera *Citrobacter* (3%), *Pseudomonas* (3%), and

264 *Acinetobacter* (2%). Although previous work has shown that members of the
265 *Gammaproteobacteria* are effective polyP accumulating bacteria and have been
266 detected in other wastewater treatment systems (Nielsen et al., 2010) the most
267 commonly reported polyP accumulating organisms in both full scale EBPR systems and
268 laboratory EBPR bioreactors are from lineages of the *Betaproteobacteria* and
269 *Actinobacteria*, in particular *Candidatus accumulibacter* and *Tetrasphaera* respectively
270 (Maszenan et al., 2000; McMahon and Reid 2013). For cultured isolates, polyP
271 synthesis has been studied extensively in a wide range of microorganisms including
272 *Escherichia coli*, *Saccharomyces cerevisiae* and *Helicobacter pylori* (Rao et al., 2009)
273 and is well known within the *Actinobacteria*, *Bacteroidetes*, and *Alpha- Beta-* and
274 *Gammaproteobacteria* (McMahon and Read, 2013). This emerging evidence suggests
275 that microbial polyP accumulation is likely ubiquitous, and enrichment for r-selected
276 polyP accumulators such as the fast growing *Gammaproteobacteria* can occur under
277 relatively simple selection pressure, such as acidification. This contrasts with the much
278 more complex engineering requirements needed to impose anaerobic-aerobic cycling in
279 EBPR systems and indicates that acidification clearly represents a much more
280 simplified method to stimulate phosphate uptake.

281 3.3. *Metabolic reconstruction of enhanced polyP accumulation by shotgun*

282 *metagenomics*

283 Shotgun metagenomics was used to assess the presumptive functional genes involved
284 in aerobic acid stimulated polyP accumulation. The genetic potential for phosphorus
285 metabolism by the community, in terms of the richness and changes in the abundance of
286 genes involved in polyP metabolism, was compared by assigning functional annotations
287 to metagenomic sequences with subsequent sequence assignment to subsystems. The

288 genetic potential for phosphorus metabolism was demonstrated by comparing changes
289 (elevation) in the polyphosphate kinase1 (*ppk1*: EC 2.7.4.1) and exopolyphosphatase
290 (*ppx*; EC 3.6.1.11) genes at pH 5.5 and 8.5; both genes are considered essential in polyP
291 synthesis and hydrolysis, respectively (Rao et al., 2009; Fig. 3). Under acidified
292 conditions, *ppk1* was the most abundant gene sequence when compared to all other
293 genes involved in phosphorus metabolism. Reid et al. (2008) also found that *ppk1*
294 dominated when *Campylobacter jejuni* was subject to acid shock. Furthermore, prior to
295 polyP synthesis, phosphate is taken up and transported across the cytoplasmic
296 membrane, a process governed by a number of enzymes and regulons (Lamarche et al.,
297 2008). For these transport systems, metagenomic analyses indicated that the low affinity
298 *Pit* system (phosphate inorganic transport) and high affinity *Pst* (phosphate specific
299 transport) systems (*PstA*, *PstB*, and *PstC*), were higher at pH 5.5 than at pH 8.5 (Fig. 3).
300 These data indicate that the selective enrichment for organisms that contained both
301 phosphate uptake and polyP synthesis systems are more pronounced under acid
302 conditions. This phenomenon occurs at multiple regulatory levels and increases with
303 acidification, the overall increase being underpinned by increases in the P accumulating
304 cell phenotype, driven by selection for taxa capable of increased P transport and polyP
305 conversion.

306 For polyP conversion, the bacterial *ppk1* gene encoding for the enzyme
307 polyphosphate kinase is responsible for polyP synthesis in many bacteria (Mielczarek et
308 al., 2013). There was an increase in the relative gene abundance of *ppk* genes at pH 5.5
309 that were characteristic of homologs from *Aeromonas hydrophila*, *Aeromonas*
310 *salmonicida*, *Enterobacter* sp., *Pseudomonas aeruginosa*, *Klebsiella variicola*,
311 *Citrobacter koseri*, and *Salmonella enterica* (Table 1). In contrast, at pH 8.5, homologs

312 related to *Bordetella avium*, *Burkholderia mallei*, *Kribbella flavida*, *Kineococcus*
313 *radiotolerans*, *Cellulomonas flavigena* and *Chromobacterium* were most abundant
314 (Table 1). Thus, based upon this evidence coupled with the 16S rRNA studies, it would
315 appear that acidification is selecting for the *Gammaproteobacteria* and their pathways
316 as key agents in the enhanced P uptake and polyP accumulation. This is in contrast to
317 EBPR processes that appear to be driven by representatives of the *Betaproteobacteria*
318 and *Actinobacteria* (McMahon and Read, 2013). These community differences may
319 explain the results of Filipe et al. (2001) who report a 53% decrease in phosphate uptake
320 during EBPR at pH 6.5. This contrast leads to the hypothesis that a drop in P uptake in
321 normal EBPR systems at low pH may be due to the absence of *Gammaproteobacteria*
322 capable of low pH accumulation, whereas these taxa are abundant in our study and
323 correspond with species known to possess sequences homologs to *ppk* (Rao et al.,
324 2009).

325 The exact reason for polyP accumulation under acidic conditions and its subsequent
326 physiological role is still unclear. One hypothesis which deserves future attention is
327 that enhanced polyP accumulation may help regulate intracellular phosphate and pH
328 levels (Harold, 1966). For example, acid pH optimums for cellular P transport have
329 been shown in *Saccharomyces cerevisiae* (pH 5.5: Borst-Pauwels and Peters, 1977),
330 *Yarrowia lipolytica* (pH 4.5: Zvyagilskaya et al., 2000) and *Burkholderia cepacia*
331 (Moriarty et al., 2006) which cause both an increase in cellular P and a decrease in
332 cellular pH. Conversion of excess phosphorus entering into the cell under optimum
333 transport levels may allow a mechanism of homeostasis whereby any excess P can be
334 effectively stored and the resulting polyP further acts as a buffer for cellular pH by
335 acting as an intracellular cation trap, sequestering H⁺ ions. Circumstantial evidence that

336 this strategy may be in operation and deserves further attention is that polyP has
337 previously been linked to cellular pH homeostasis in both the unicellular alga
338 *Dunaliella salina* (Bental et al., 1991) and in *Saccharomyces cerevisiae* (Castro et al.,
339 1995) whilst *Lactococcus* ppk- mutants show diminished growth at acid pH (Alcántra et
340 al., 2014). However, irrespective of the physiological reasons for PolyP accumulation in
341 response to acid pH, it is demonstrated that this phenomenon may have significant
342 implications for overall ecological P-cycling and the applied aspects of developing
343 alternative technologies for P removal from waste streams.

344 Application of next-generation sequencing of metagenomes, in tandem with cellular
345 physiological assays and biogeochemical P speciation using ^{31}P NMR, will provide a
346 comprehensive understanding of functional polyP accumulators in both engineered and
347 natural ecosystems, such as those microbial communities responsible for P cycling
348 kinetics in freshwater and marine sediments (McMahon and Read, 2013). Ultimately,
349 resolution of the genetic basis and physiological ability of microbial populations to
350 accumulate P as polyP, and its subsequent release as P_i , under a range of environmental
351 conditions (e.g. redox, temperature, pH etc) are key components still to be resolved
352 within the natural microbial P cycle and its exploitation within engineered wastewater
353 treatment systems. For the first time in an applied context, these data generated the
354 fundamental understanding of how acid stimulation modulates P transformation. This
355 knowledge base is applicable to other engineered systems and allows targeted process
356 monitoring by genetic analyses, leads to design of optimum physical process conditions
357 as well as the potential for bioengineering the wastewater community for optimal
358 activity and P recovery.

359

360 **4. Conclusions**

361 This study demonstrated the microorganisms capable of mediating enhanced
362 phosphorus removal from wastewater systems under acid stimulation and the genetic
363 basis for this enhanced P accumulation. Both the phylogenetic and metagenomic
364 analyses indicate increases in polyP accumulation are the result of substantial
365 population increases in *Gammaproteobacteria* taxa, in tandem with an enhanced
366 community genetic capacity for both cellular P transport and polyP production. These
367 community shifts result in a substantial quantitative population increase in P removal
368 capacity which can be visualised within the accumulator cells as insoluble P deposition
369 as acid stimulation modifies the nascent community structure and function.

370

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383

384 **References**

- 385 1. Alcántara, C., Blasco, A., Zúñiga, M., Monedero, V., 2014. Accumulation of
386 Polyphosphate in *Lactobacillus* spp. and its involvement in stress resistance.
387 Appl. Environ. Microbiol. 80, 1650-1659.
- 388 2. Bental, M., Pick, U., Avron, M., Degani, H., 1991. Polyphosphate metabolism in
389 the alga *Dunaliella salina* studied by P31-NMR. Biochim. Biophys. Acta. 1092,
390 21-28.
- 391 3. Borst-Pauwels, G.W.F.H., Peters, P.H.J., 1977. Effect of the medium pH and the
392 cell pH upon the kinetical parameters of phosphate uptake by yeast. Biochim.
393 Biophys. Acta. 466, 488-495.
- 394 4. Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D.,
395 Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., 2010.
396 QIIME allows analysis of high-throughput community sequencing data. Nat.
397 Methods. 7, 335-336.
- 398 5. Castro, C.D., Meehan, A.J., Koretsky, A. P., Domach, M. M., 1995. In situ ³¹P
399 nuclear magnetic resonance for observation of polyphosphate and catabolite
400 responses of chemostat-cultivated *Saccharomyces cerevisiae* after alkalization.
401 Appl. Environ. Microbiol. 61, 4448-4453.
- 402 6. Duguid, J. P., Smith, I.W., Wilkinson, J.F., 1954. Volutin production in
403 *Bacterium aerogenes* due to development of an acid reaction. J. Path. Bact.
404 67.289-300.
- 405 7. Eaton, A.D., Clesceri, L.S., Greenberg, A.E., 2005. Standard methods for the
406 examination of water and wastewater American Public Health Association.
407 Washington, DC, 20001-23710.

- 408 8. Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME
409 improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–
410 2200.
- 411 9. Filipe, C.D.M., Daigger, G.T., Grady, C.P., 2001. Effects of pH on the rates of
412 aerobic metabolism of phosphate-accumulating and glycogen-accumulating
413 organisms. *Water Environ. Res.* 73, 213-222.
- 414 10. Gerlitz, T.G.M., 1996. Effects of aluminium on polyphosphate mobilization of
415 the ectomycorrhizal fungus *Suillus bovinus*. *Plant Soil* 178, 133-140.
- 416 11. Gerlitz, T.G.M., Gerlitz, A., 1997. Phosphate uptake and polyphosphate
417 metabolism of mycorrhizal and nonmycorrhizal roots of pine and of *Suillus*
418 *bovinus* at varying external pH measured by in vivo P-31-NMR. *Mycorrhiza* 7,
419 101-106.
- 420 12. Harold, F.M., 1966. Inorganic polyphosphate in biology: structure, metabolism,
421 and function. *Bacteriol. Rev.* 30, 772-794.
- 422 13. Jenkins, S.N., Rushton, S.P., Lanyon, C.V., Whiteley, A.S., Waite, I.S.,
423 Brookes, P.C., Kemmitt, S., Evershed, R.P., O'Donnell, A.G., 2010. Taxon-
424 specific responses of soil bacteria to the addition of low level C inputs. *Soil*
425 *Biol. Biochem.* 42, 1624-1631.
- 426 14. Kawaharasaki, M., Tanaka, H., Kanagawa, T., Nakamura, K., 1999. In situ
427 identification of polyphosphate-accumulating bacteria in activated sludge by
428 dual staining with rRNA-targeted oligonucleotide probes and 4', 6-diamidino-2-
429 phenylindol (DAPI) at a polyphosphate-probing concentration. *Water Res.* 33,
430 257-265.

- 431 15. Lamarche, M.G., Wanner, B.L., Crépin, S., Harel, J., 2008. The phosphate
432 regulon and bacterial virulence: a regulatory network connecting phosphate
433 homeostasis and pathogenesis. *FEMS Microbiol. Rev.* 32, 461-473.
- 434 16. Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Schumann, P., Burghardt, J.,
435 Tokiwa, Y., Stratton, H.M., 2000. Three isolates of novel polyphosphate-
436 accumulating gram-positive cocci, obtained from activated sludge, belong to a
437 new genus, *Tetrasphaera* gen. nov., and description of two new species,
438 *Tetrasphaera japonica* sp. nov. and *Tetrasphaera australiensis* sp. nov. *Int. J.*
439 *Syst. Evol. Microbiol.* 50, 593-603.
- 440 17. McGrath, J.W., Cleary, S., Mullan, A., Quinn, J.P., 2001. Acid-stimulated
441 phosphate uptake by activated sludge microorganisms under aerobic laboratory
442 conditions. *Water Res.* 35, 4317-4322.
- 443 18. McGrath, J.W., Quinn, J.P., 2000. Intracellular accumulation of polyphosphate
444 by the yeast *Candida humicola* G-1 in response to acid pH. *Appl. Environ.*
445 *Microbiol.* 66, 4068-4073.
- 446 19. McGrath, J.W., Quinn, J.P., 2003. Microbial phosphate removal and
447 polyphosphate production from wastewaters. *Adv. Appl. Microbiol.* 52, 75-100.
- 448 20. McMahon, K.D., Read, E.K., 2013. Microbial contributions to phosphorus
449 cycling in eutrophic lakes and wastewater. *Annu. Rev. Microbiol.* 67, 199-219.
- 450 21. Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E.M., Kubal, M.,
451 Paczian, T., Rodriguez, A., Stevens, R., Wilke, A., 2008. The metagenomics
452 RAST server—a public resource for the automatic phylogenetic and functional
453 analysis of metagenomes. *BMC Bioinformatics* 9, 386.

- 454 22. Mielczarek, A.T., Nguyen, H.T.T., Nielsen, J.L., Nielsen, P.H., 2013. Population
455 dynamics of bacteria involved in enhanced biological phosphorus removal in
456 Danish wastewater treatment plants. *Water Res.* 47, 1529-1544.
- 457 23. Moriarty, T., Mullan, A., McGrath, J., Quinn, J., Elborn, J., Tunney, M., 2006.
458 Effect of reduced pH on inorganic polyphosphate accumulation by *Burkholderia*
459 *cepacia* complex isolates. *Lett. Appl. Microbiol.* 42, 617-623.
- 460 24. Mullan, A., McGrath, J.W., Adamson, T., Irwin, S., Quinn, J.P., 2006. Pilot-
461 scale evaluation of the application of low pH-inducible polyphosphate
462 accumulation to the biological removal of phosphate from wastewaters. *Environ.*
463 *Sci. Technol.* 40, 296-301.
- 464 25. Mullan, A., Quinn, J. P., McGrath, J.W., 2002. Enhanced phosphate uptake and
465 polyphosphate accumulation in *Burkholderia cepacia* grown under low-pH
466 conditions. *Microbial ecol.* 44, 69-77.
- 467 26. Nielsen, P.H., Mielczarek, A.T., Kragelund, C., Nielsen, J.L., Saunders, A.M.,
468 Kong, Y., Hansen, A.A., Vollertsen, J., 2010. A conceptual ecosystem model of
469 microbial communities in enhanced biological phosphorus removal plants.
470 *Water Res.* 44, 5070-5088.
- 471 27. Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L.,
472 Reis, M.A., 2007. Advances in enhanced biological phosphorus removal: from
473 micro to macro scale. *Water Res.* 41, 2271-2300.
- 474 28. Powell, N., Shilton, A.N., Pratt, S. and Chisti, Y., 2008. Factors influencing
475 luxury uptake of phosphorus by microalgae in waste stabilization ponds.
476 *Environ. Sci. Technol.* 42, 5958-5962.
- 477 29. Rao, N.N., Gomez-Garcia, M.R., Kornberg, A., 2009. Inorganic polyphosphate:

- 478 essential for growth and survival. *Annu. Rev. Biochem.* 78, 605-47.
- 479 30. Reid, A.N., Pandey, R., Palyada, K., Whitworth, L., Doukhanine, E., Stintzi, A.,
480 2008. Identification of *Campylobacter jejuni* genes contributing to acid
481 adaptation by transcriptional profiling and genome-wide mutagenesis. *Appl.*
482 *Environ. Microbiol.* 74, 1598-1612.
- 483 31. Temperton, B., Gilbert, J.A., Quinn, J.P., McGrath, J.W., 2011. Novel analysis
484 of oceanic surface water metagenomes suggests importance of polyphosphate
485 metabolism in oligotrophic environments. *PLoS One* 6, e16499.
- 486 32. Whiteley, A.S., Jenkins, S., Waite, I., Kresoje, N., Payne, H., Mullan, B.,
487 Allcock, R., O'Donnell, A., 2012. Microbial 16S rRNA Ion Tag and community
488 metagenome sequencing using the Ion Torrent (PGM) Platform. *J. Microbiol.*
489 *Methods.* 91, 80-88.
- 490 33. Yuan, Z.G., Pratt, S., Batstone, D.J., 2012. Phosphorus recovery from
491 wastewater through microbial processes. *Curr. Opin. Biotechnol.* 23, 878-883.
- 492 34. Zheng, X., Sun, P., Han, J., Song, Y., Hu, Z., Fan, H., Lv, S., 2014. Inhibitory
493 factors affecting the process of enhanced biological phosphorus removal (EBPR)
494 - A mini-review. *Process Biochem.* 49, 2207-2213.
- 495 35. Zvyagilskaya, R., Allard, P., Persson, B.L., 2000. Two systems for phosphate
496 uptake in *Yarrowia lipolytica* cells grown at acidic conditions. *IUBMB Life* 49,
497 143-147.

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501 **Figure & Table Captions**

502 **Fig. 1.** Flow cytometric community analysis of polyP positive microbial populations
503 within wastewater communities at (a) pH 8.5 and (b) pH 5.5.

504 **Fig. 2.** Identities and relative abundance (%) of the bacteria in laboratory microcosms
505 incubated at pH 8.5 (natural pH of wastewater) and at pH 5.5 (acidified wastewater).
506 Inset chart shows the composition of the *Gammaproteobacteria* at pH 5.5.

507 **Fig. 3.** Abundance of genes involved in the uptake and transport of inorganic phosphate
508 across the cytoplasmic membrane (low affinity *Pit* system and high affinity *Pst*), polyP
509 synthesis (polyphosphate kinase), and hydrolysis (exopolyphosphatase) in laboratory
510 microcosms at pH 8.5 and pH 5.5. Polyphosphate kinase (*ppk*), phosphate transport
511 system permease protein (*pstC*), a probable low-affinity inorganic phosphate transporter
512 (LAT), a phosphate transport ATP-binding protein (*pstB*), alkaline phosphatase (*ALPL*),
513 phosphate transport system permease protein (*pstA*), exopolyphosphatase (*ppx*),
514 phosphate regulon sensor protein (*phoR*), and phosphate regulon transcriptional
515 regulatory protein (*phoB*).

516

517 **Table 1.** Functional affiliations of polyphosphate accumulating microorganisms and
518 their percentage identities to cultured strains based on the presence of the *ppk* genes in
519 microcosms incubated at pH 5.5 and pH 8.5.

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522 **Supplementary Figure captions**

523 **Fig. S1.** (a) Location of the study site. (b) The piggery waste treatment process at
524 Medina Research Station, Department of Agriculture and Food, Western Australia
525 (DAFWA) for treating piggery effluent waste and capture bio-energy.

526 **Fig. S2.** Overall phosphate removal efficiencies from laboratory microcosms incubated
527 at different pH treatments (pH 5.5, 6.0, 6.5, 7.0, 8.5). Error bars indicate the standard
528 deviation where points with different letters (A, B, C, D, and E) are significantly
529 different from each other ($P < 0.05$).

530 **Fig. S3.** Epifluorescence micrographs of DAPI stained cells from laboratory
531 microcosms incubated at (a) the natural pH of wastewater at the time of sampling (pH
532 8.5) and (b) pH 5.5.

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