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

Weerasekara, A. W., Jenkins, S., Abbott, L. K., Waite, I., McGrath, J. W., Larma, I., Eroglu, E., O'Donnell, A., & Whiteley, A. S. (2016). Microbial phylogenetic and functional responses within acidified wastewater communities exhibiting enhanced phosphate uptake. *Bioresource Technology*, *220*, 55-61. https://doi.org/10.1016/j.biortech.2016.08.037

Published in:
Bioresource Technology

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

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

Anjani W. Weerasekara[a,b], Sasha Jenkins[a,b], Lynette K. Abbott[a,b], Ian Waite[a,b], John W. McGrath[d], Irma Larma[e], Ela Eroglu[f], Anthony O'Donnell[a,b] and Andrew S. Whiteley[a*]

Affiliations:

[a]School of Earth and Environment, Faculty of Science, The University of Western Australia, 35 Stirling Highway, Crawley, 6009, Western Australia, Australia

[b]Institute of Agriculture, The University of Western Australia, 35 Stirling Highway, Crawley 6009. Western Australia, Australia

[d]School of Biological Sciences and the Institute for Global Food Security, The Queens University of Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland

[e]Centre for Microscopy, Characterisation and Analysis (CMCA), The University of Western Australia, Crawley, 35 Stirling Highway, 6009, Western Australia, Australia

[f]Department of Chemical Engineering, Curtin University, Perth, 6845, Western Australia, Australia

[*]**Author for Correspondence:**

Prof. Andrew Whiteley (andy.whiteley@uwa.edu.au)

School of Earth and Environment, Faculty of Science, The University of Western Australia, 35 Stirling Highway, Crawley, 6009, Western Australia, Australia

ABSTRACT

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

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

1. Introduction

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

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

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

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

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

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

2. Methods

2.1. Sampling site description and sampling

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

Samples for laboratory phosphorus removal experiments were collected from the aerobic pond by suction using a 12V marine grade bilge pump connected to a PVC hosepipe. The hosepipe was placed into the aerobic pond and run for 5 mins to flush the sampling line, and samples from each point were collected into several autoclaved containers with corresponding samples mixed together to make a composite sample for laboratory incubation experiments. The chemical composition of the composite wastewater was assessed as follows: pH 8.5; EC 5.2 mS/cm; chemical oxygen demand 483 mg/L; total solids 0.1 %; volatile solids 25.8 %; total nitrogen 6.8 %; total C 45.9 %, 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 KH_2PO_4 (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^{o}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^{o}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).

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

Flow cytometric analyses were performed using a BD Influx cell sorter (Becton Dickinson, USA) at the Centre for Microscopy, Characterisation and Analyses at The University of Western Australia. DAPI was excited with a 355 nm (UV) laser with standard DAPI emission collected with a 460/50 nm band pass filter; the yellow shifted DAPI-PolyP bound emission was collected with a 585/29 bandpass filter. Measurements for DAPI and polyP were acquired on a logarithmic scale and post-acquisition analysis performed using Flow Jo software version 7.6.5. Single cells were gated on forward scatter area (FSC-A) vs forward scatter height (FSC-H) to exclude any doublets and DAPI-DNA and DAPI-polyP were gated to determine proportions of bacteria accumulating polyphosphate. Briefly, DAPI-bound polyP cell populations formed a distinct cluster in the polyP 570-600nm range whilst non-accumulators exhibited only DAPI-DNA fluorescence in the DAPI fluorescence 435-485 nm range.

2.4. DNA extraction and 16S rRNA Tag sequencing

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

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

2.5 Whole genome shotgun sequencing

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

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

2.6. Statistical analysis.

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

3. Results and Discussion

3.1. PolyP accumulation in a laboratory incubation experiment

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

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

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

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

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

3.3. Metabolic reconstruction of enhanced polyP accumulation by shotgun metagenomics

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

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

For polyP conversion, the bacterial *ppk1* gene encoding for the enzyme polyphosphate kinase is responsible for polyP synthesis in many bacteria (Mielczarek et al., 2013). There was an increase in the relative gene abundance of *ppk* genes at pH 5.5 that were characteristic of homologs from *Aeromonas hydrophila, Aeromonas salmonicida, Enterobacter* sp., *Pseudomonas aeruginosa, Klebsiella variicola, 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

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

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

4. Conclusions

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

Acknowledgements

This work was funded by grants from Australia Pork Ltd and the Australian Grains Research and Development Corporation (GRDC). The authors would also like to acknowledge the facilities, and the scientific and technical assistance of the Australian Microscopy and Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis (CMCA), The University of Western Australia (UWA), which is funded by the University and by State and Commonwealth Governments. The authors would like to acknowledge Dr. Kathy Heel for her assistance in Flow cytometry, Dr Richard Allcock for sequence generation and Dr.Falko Mathes for his input during the sequencing analysis. ASW is supported by the 2012 West Australian Fellowship Program award, funded jointly by the Department of Premier and Cabinet, WA and The University of Western Australia.

References

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

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

15. Lamarche, M.G., Wanner, B.L., Crépin, S., Harel, J., 2008. The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. FEMS Microbiol. Rev. 32, 461-473.
16. Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Schumann, P., Burghardt, J., Tokiwa, Y., Stratton, H.M., 2000. Three isolates of novel polyphosphate-accumulating gram-positive cocci, obtained from activated sludge, belong to a new genus, Tetrasphaera gen. nov., and description of two new species, *Tetrasphaera japonica* sp. nov. and *Tetrasphaera australiensis* sp. nov. Int. J. Syst. Evol. Microbiol. 50, 593-603.
17. McGrath, J.W., Cleary, S., Mullan, A., Quinn, J.P., 2001. Acid-stimulated phosphate uptake by activated sludge microorganisms under aerobic laboratory conditions. Water Res. 35, 4317-4322.
18. McGrath, J.W., Quinn, J.P., 2000. Intracellular accumulation of polyphosphate by the yeast *Candida humicola* G-1 in response to acid pH. Appl. Environ. Microbiol. 66, 4068-4073.
19. McGrath, J.W., Quinn, J.P., 2003. Microbial phosphate removal and polyphosphate production from wastewaters. Adv. Appl. Microbiol. 52, 75-100.
20. McMahon, K.D., Read, E.K., 2013. Microbial contributions to phosphorus cycling in eutrophic lakes and wastewater. Annu. Rev. Microbiol. 67, 199-219.
21. Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E.M., Kubal, M., Paczian, T., Rodriguez, A., Stevens, R., Wilke, A., 2008. The metagenomics RAST server–a public resource for the automatic phylogenetic and functional 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-Garcıa, M.R., Kornberg, A., 2009. Inorganic polyphosphate:

essential for growth and survival. Annu. Rev. Biochem. 78, 605-47.

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

Figure & Table Captions

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

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

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

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

Supplementary Figure captions

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

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

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