An Interdisciplinary Review of Polyphosphate Accumulating Organism (PAO)-Mediated Phosphorus Cycling for Landscape-Scale Water Quality Management

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Abstract

Despite ongoing management efforts, phosphorus (P) loading from agricultural landscapes continues to impair water quality. Concurrent, established research in engineered systems (e.g., wastewater treatments plants) and natural systems (e.g., stream, lakes, sediments, and marine waters) has enhanced our understanding of microbial mechanisms influencing P cycling. However, there is limited application of this research to reducing P loading and improving water quality in agricultural systems (e.g., field soils and downstream waterbodies). Herein, we review microbial-mediated P cycling in engineered and natural systems—focusing on the role of polyphosphate accumulating organisms (PAOs). We discuss emerging technologies and frameworks to evaluate the diversity, structure, and function of PAO communities while highlighting opportunities to apply PAO-focused research in agricultural systems. The overall goal of this review is to motivate interdisciplinary collaborations that consider both biotic (here microbial) and abiotic P cycling mechanisms across the landscape. We argue that these collaborations and considerations are needed to reduce P loading and improve water quality.

Keywords: agriculture; best management practices; enhanced biological phosphorus removal; microbial processes; phosphorus; polyphosphate; polyphosphate accumulating organisms; water quality

1. Introduction

Sustained excess phosphorus (P) from anthropogenic sources (e.g., urban and agricultural runoff) leads to freshwater eutrophication and subsequent decreases in water transparency, potential growth of toxin producing cyanobacteria, hypoxic (i.e. low oxygen) or anoxic (i.e. no oxygen) conditions, and fish die-offs (Bennett, Carpenter, & Caraco, 2001; Carpenter et al., 1998; Carpenter, 2005; Dodds & Smith, 2016; Schindler, 2012; Seviour, Mino, & Onuki, 2003). Freshwater eutrophication is estimated to cost the United States $2.2 billion annually (Dodds et al., 2009) and can lead to issues such as increased spending on drinking water treatment, potential recreational space closures, loss of waterfront real estate, increased spending to manage threatened and endangered species, and decreased fish and wildlife production (Carpenter, 2005; Dodds et al., 2009; Sekaluvu, Zhang, & Gitau, 2018; Seviour et al., 2003).

Despite years of effort to reduce P loadings, excess P still impacts the water quality of many lakes, rivers, and streams (Carpenter et al., 1998; Carpenter, 2005; Dodds et al., 2009; Dubrovsky et al., 2010; Jarvie et al., 2013, 2017; Kleinman, Sharpley, Buda, Mcdowell, & Allen, 2011; Mekonnen & Hoekstra, 2018; Obersteiner, Peñuelas, Ciais, van der Velde, & Janssens, 2013; Sharpley et al., 2013). The use of phosphate-free soaps and improved wastewater treatment technologies have reduced urban point sources (e.g., wastewater treatment plant effluents) of P (Jarvie et al., 2013; Litke, 1999; Tong et al., 2017). However, non-point P sources such as agricultural landscapes still contribute a substantial fraction of diffuse P loading to water bodies due to the applications of chemical fertilizer and manure. Regional models of the United States estimate that croplands, pasturelands, and rangelands delivered over 80% of P loads to the Gulf of Mexico from 1992 to 2002 (Alexander et al., 2008) and global models estimate that agricultural land accounted for 34% of anthropogenic P loads to rivers (Mekonnen & Hoekstra, 2018). In addition to model estimates, long-term water sampling records also emphasize the impact of agriculture. The majority of stream samples taken near agricultural sites exceeded the United States Environmental Protection Agency (USEPA) recommendations for P (i.e. 10 to 1000 μg/L depending on ecoregion) over the 1992-2004 study period (Dubrovsky et al., 2010).

Land management strategies may explain sustained agricultural P additions to receiving waterbodies despite concerted management efforts to minimize diffuse sources of P loading (see review by Smith, King, & Williams, 2015b). Historically, long-term accumulation of amended P in agricultural soils—termed ‘legacy’ P—consistently contributes to P loading for > 10 years after P fertilizer application stops; to counteract this effect, more aggressive P management strategies are required (Cassidy, Doody, & Watson, 2017; Gregory et al., 2007; Haygarth et al., 2014; Jarvie et al., 2013; Kleinman et al., 2011; Kleinman, Smith, Bolster, & Easton, 2015; Powers et al., 2016; Sharpley et al., 2013; Sharpley, Kleinman, Flaten, & Buda, 2011; Sharpley & Rekolainen, 1997). Calls for reductions in sediment-bound, particulate P (PP) to Lake Erie in the early 2000s led to increased implementation of no-tillage and tile drains in the Midwestern United States (Jarvie et al., 2017; Sekaluvu et al., 2018; Smith et al., 2015a; 2015b). However, while no-tillage and tile drains decreased surface transport of PP, they led to unintended increases in the subsurface transport of dissolved P (DP; Christianson, Harmel, Smith, Williams, & King, 2016; Geohring et al., 2001; Jarvie et al., 2017; Smith et al., 2015a; 2015b). In addition, researchers suspect that the shift from PP to DP transport led to an increase in the number of harmful algal blooms along the western shore of Lake Erie (Jarvie et al., 2017; Sekaluvu et al., 2018; Smith et al., 2015a; 2015b). These examples emphasize the need for agricultural water quality management strategies that address a wide range of P forms and cycling mechanisms.

P forms in soil and water samples are distinguished by chemical and/or operational means. For a summary of P form terminology used in the literature, see Table 1 [Table 1 near here]. Chemical forms are typically categorized as either inorganic P (P_i) or organic P (P_o). P_i includes free phosphate (PO_4^{3-})—also known as orthophosphate or dissolved P_i. Additionally, P_i includes phosphate chains such as polyphosphate (polyP) and phosphate bound to metals such as Fe(III)-phosphate ($FePO_4$) and apatite ($Ca_{10}(PO_4)_6(OH)_2$). P_o includes phosphate bound to carbon molecules such as humic-metal-P complexes (Gerke, 2010) as well as P-containing biomolecules such as DNA, RNA, phospholipids, and intracellular polyP (Cade-Menun, 2015; 2017). We note that while polyP is not an organic molecule itself (Table 1), it is typically included alongside other P_o analyses because of its intracellular nature (Cade-Menun, 2017). Henceforth, we refer to intracellular polyP as polyP. Operational P forms are categorized as either dissolved P (DP) if they can pass through a 0.45 um filter or particulate P (PP) if the material is retained on the filter. Both DP and PP may have P_i or P_o fractions (River & Richardson, 2018; Ruttenberg & Dyhrman, 2012). For additional discussion of operationally and chemically defined P forms, see Hedley, Stewart, & Chauhan (1982), Tiessen and Moir (1993), Cross and Schlesinger (1995), Guppy, Menzies, Moody, & Blamey (2005), Gerke (2010), DeLaune, Reddy, Richardson, & Magonigal (2013), Zeckoski, Smolen, Moriasi, Frankenberger, & Feyereisen (2015), Cade-Menun, Benitez-Nelson, Pellechia, & Paytan (2005), and Cade-Menun (2015).

Table 1. A description of phosphorus (P) forms commonly used in the literature.

Form	Common Abbreviation	Description	References
dissolved phosphorus	DP	Operationally defined form of P that passes through 0.45um filter.	USEPA, 1974; Zeckoski et al., 2013
particulate phosphorus	PP	Operationally defined form of P that is obtained by subtracting DP from TP.	Zeckoski et al., 2013
total phosphorus	TP	Total amount of P in a soil or water sample. For water samples TP is measured on an unfiltered sample and for soil samples TP is determined after digestion using strong acids and bases like fluoric acid, hydrogen peroxide, hydrochloric and nitric acids, sodium hydroxide.	USEPA, 1974; Zeckoski et al., 2013
soluble reactive phosphorus, dissolved orthophosphate	SRP	Portion of the DP fraction (i.e., P passing through 0.45 um filter) that can be detected with the molybdenum blue assay. SRP consists of primarily Pi but may also include hydrolyzed Po due to the required acidity of the molybdenum blue assay.	USEPA, 1978; Benitez-Nelson, 2000; Zeckoski et al., 2013
inorganic phosphorus	Pi	P that is not associated with organic (carbon) molecules. Examples include phosphate, polyphosphate, and phosphate bound directly to metals (e.g., apatite - Ca10(PO4)6(OH)2).	Zeckoski et al., 2013
dissolved inorganic phosphorus	DIP	The Pi fraction of DP.	USEPA, 1974; Benitez-Nelson, 2000
phosphate, orthophosphate	PO43-	The most basic form of Pi in the environment.	Zeckoski et al., 2013
organic phosphorus	Po	P associated with organic (carbon containing) material/molecules. This may include phosphate bound to organic matter as well as phosphate inside plants, animals, and microorganisms (e.g., as DNA or polyP).	Zeckoski et al., 2013; Cade-Menun et al., 2005; Cade-Menun, 2015; 2017
polyphosphate	polyP	Two or more phosphate molecules bound together by a high energy phosphoanydride bond (i.e., tetrahedral phosphate groups are linked via O2 bonds). It is a Pi on its own, but because it is typically stored intracellularly, it is often considered a Po.	Harold, 1964; Kornberg, 1995; Cade-Menun et al., 2005; Cade-Menun, 2015; 2017
pyrophosphate	--	A polyP with only two phosphate molecules bound together by a phosphoanydride bond. It is a Pi on its own, but because it is typically stored intracellularly, it is often considered a Po.	Cade-Menun et al., 2005; Cade-Menun, 2015; 2017
dissolved organic phosphorus	DOP	The Po fraction of DP.	USEPA, 1974; Benitez-Nelson, 2000
orthophosphate monoesters	--	Extracted from organic P. Includes sugar phosphates (e.g., glucose 1-phosphate).	Cade-Menun et al., 2005; Cade-Menun 2015
orthophosphate diesters	--	Extracted from organic P and they include DNA, RNA, and phospholipids.	Cade-Menun et al., 2005; Cade-Menun, 2015
phosphonate	--	Extracted from organic P and they are most commonly found as free molecules or membrane phosphonolipids.	Cade-Menun et al., 2005; Cade-Menun, 2015
microbial phosphorus	--	Pi and Po stored intracellularly by microorganisms; therefore, it is considered a form of Po. Calculated based on the difference in P detected before and after fumigation of soil or sediment samples.	Hedley et al., 1982
bound phosphorus	--	A general term that refers to P (usually inorganic P) that is attached to soil or sediment via bonds or associations with organic matter or metals such as Ca, Fe, and Al.	--
labile phosphorus	--	P that is easy converted into other forms.	Zeckoski et al., 2013
biologically available phosphorus	--	P that can easily be taken up by plants, animals, microorganisms. Determined by summing SRP and what is extracted from PP using NaOH.	Sharpley et al., 1991; Zeckoski et al., 2013

Abiotic and biotic P cycling mechanisms play an important role in defining which P forms may be present (or abundant) at a given time and location in the landscape and how readily those forms are transported. Abiotic P cycling mechanisms include chemical processes such as dissolution and precipitation of P with metals (e.g., Fe, Al, Mg, and Ca) and physical processes such as perturbation (e.g., freezing/thawing or wetting/drying) or settling; both impact whether P forms are more or less available to living organisms or can be easily transported in overland or subsurface flows (Benitez-Nelson, 2000; Blackwell et al., 2010; Reddy, Kadlec, Flaig, & Gale, 1999; Richardson, 1985). Biotic P cycling mechanisms include processes initiated by plants or organisms (macroorganisms or microorganisms) associated with P uptake and release (Cross & Schlesinger, 1995; Dodds & Smith, 2016; Olander & Vitousek, 2000; Reddy et al., 1999; Richardson, 1985). Both abiotic and biotic P cycling mechanisms may be influenced by changes in environmental factors like pH, temperature, and redox status (Blackwell et al., 2010; Dodds, 2003; Hoffmann, Kjaergaard, Uusi-Kämppä, Hansen, & Kronvang, 2009; Hupfer & Lewandowski, 2008; Reddy et al., 1999; Sharpley et al., 2013; Tiessen & Moir, 1993).

Despite the acknowledgement of numerous P forms and cycling mechanisms, many research opportunities exist to explore how lesser studied P_o forms and biotic cycling mechanisms influence water quality P management strategies in agricultural systems. P_o may account for up to 95% of soil P and this percentage increases as soils become more weathered (Cross & Schlesinger, 1995; P. M. Haygarth, Harrison, & Turner, 2018). However, why and how soils support this large pool of P_o and how associated biotic P cycling mechanisms influence P availability to crops is largely unknown (Dodd & Sharpley, 2015; George et al., 2018; Haygarth et al., 2018; Haygarth, Hinsinger, &

Blackburn, 2018; McLaren et al., 2015). Furthermore, researchers hypothesize that a deeper exploration of mechanisms influencing soil P_o cycling may be key to (1) solving persistent water quality issues due to legacy P and (2) finding a balance between food security and clean water (Dodd & Sharpley, 2015; George et al., 2018; Haygarth et al., 2018).

In this review, we synthesize research on P_o stored by microbes as polyP for several reasons. First, microbes immobilize a large proportion (i.e., 20-50%) of P_o in P-limited soils (Bünemann, Smernik, Marschner, & McNeill, 2008; Cross & Schlesinger, 1995) and this proportion typically increases 30-240% under agricultural management (Dodd & Sharpley, 2015). In aquatic systems, 20% of the microbial P pool can consist of intracellular polyP (Bünemann et al., 2008). Second, microbially-sourced P_o (i.e., microbial P; Table 1) in soils is more easily converted to other P forms compared to plant-sourced P_o (Blackwell et al., 2010; Hoffmann et al., 2009) and there are many open questions regarding microbial P cycling as it relates to water quality (Blackwell et al., 2010; Dodd & Sharpley, 2015; George et al., 2018). Third, polyP storage and cycling by microbes has been studied extensively in engineered systems (e.g., wastewater treatment plants; WWTPs) and natural systems (e.g., lakes; see McMahon and Read, 2013) but has received limited attention in agricultural systems to manage water quality across the landscape (i.e., from soils to downstream waterbodies). Fourth, polyP is ubiquitous; it is found intracellularly in a wide range of living and degrading organisms including bacteria, archaea, fungi, plants, and animals (Brown & Kornberg, 2004; Harold, 1964; Kornberg, 1995; Rao, Gómez-García, & Kornberg, 2009; Seviour & Nielsen, 2010; Zhang, Ishige, & Kornberg, 2002). Last, polyP plays a role in many important metabolic functions. Besides serving as a universal energy source and P reservoir, polyP is needed for biofilm formation, is a strong ion chelator, buffers against alkali conditions, regulates gene expression in organisms under stress, and regulates virulence factors (Brown & Kornberg, 2004, 2008; Kornberg, 1995; Rao et al., 2009; Seviour & Nielsen, 2010).

Given the persistence of current P loading from agricultural systems, our limited understanding of microbial P cycling mechanisms, the ubiquity of polyP storage across the tree of life, and the potential cellular benefits of storing excess P in the environment as polyP, there is a need to assess the water quality benefits and impacts of microbial polyP storage and subsequent release of associated phosphate. Herein, we focus on the role of a group of microorganisms—known as polyphosphate accumulating organisms (PAOs)— credited with P removal in engineered systems such as certain WWTPs. We focus on PAOs because they are known to store polyP and there is also evidence for their activity outside of WWTPs. We will not synthesize research on inorganic (e.g., nanoparticles; River and Richardson, 2018) and organic (e.g., cable bacteria; Sulu-Gambari et al., 2016) Fe-P interactions as well as microbial Fe metabolism, which have been summarized previously (Gerke, 2010; Guppy et al., 2005; Pronk & Johnson, 1992; Roden, 2012; Straub, Benz, & Schink, 2000). Next, we review microbial controls on P in natural systems such as streams, lakes, estuaries, oceans, soils, and sediments. We synthesise knowledge gaps in engineered and natural systems with respect to PAOs and suggest tools that may propel each discipline forward while promoting constructive interaction between them. Last, we suggest research topics that may inform microbially-based P water quality management in agricultural systems and also discuss the capacity for knowledge sharing between engineered, and natural, and agricultural systems.

2. Biotic Controls on Phosphorus Cycling in Engineered Systems

Before the 1970s, operators designed WWTPs to remove urban point sources of carbon (C) and nitrogen (N). During the 1970s and in response to the growing eutrophication problem in lakes (e.g., Schindler 1977), WWTP designs employed enhanced biological P removal (EBPR) to simultaneously reduce operation costs and remove P from wastewater (Seviour et al., 2003). EBPR is more economical than conventional WWTPs with chemical P removal because it does not require Ca, Al, or Fe to precipitate out P (Oehmen et al., 2007) and does not generate metal-laden sludge. Instead, it relies on microbes that store P intracellularly to transfer P from solution to waste solids (Barnard, 1976; Seviour et al., 2003). More specifically, the EBPR process includes at least four operating components: (1) an anaerobic reactor with an organic (C-containing) energy source (e.g., acetate), (2) an anoxic zone, (3) an aerobic zone, and (4) a means to recycle settled biomass (Seviour et al., 2003). While optimal operating conditions were originally developed empirically rather than based on an understanding of microbial processes, it is now commonly accepted that the characteristic alternating anaerobic/aerobic conditions of EBPR selects for microbes capable of taking up phosphate in excess of normal cellular levels (Gebremariam, Beutel, Christian, & Hess, 2011; Seviour et al., 2003)—coined 'luxury uptake' (Yall, Boughton, Roinestad, & Sinclair, 1972). Typically, EBPR sludge is 5-7% P (dry weight) while the P content of conventional sludge ranges from 1-2% (Yuan, Pratt, & Batstone, 2012). PAOs—key microbes enriched by EBPR—play a large role in removing P from influent waters of EBPR WWTPs around the world (e.g., Mao *et al.*, 2015). The most frequently studied (model) PAO is known by the provisional scientific name *Candidatus* Accumulibacter phosphatis (CAP; Hesselmann *et al.*, 1999);

however, researchers have identified other non-CAP PAOs in engineered and natural systems (Table 2) [Table 2 near here].

Table 2. A summary of polyphosphate accumulating organisms (PAOs) identified in engineered and natural systems. Abbreviations: laboratory-scale sequencing batch reactor (SBR), full-scale wastewater treatment plant (WWTP), uncharacterized wastewater (Mixed).

Phylum/Class	Genus	Carbon Source	Reactor Type / Environment	Reference
Actinobacteria	--	Mixed	WWTP	Auling et al., 1991; Beer et al., 2006
Actinobacteria	--	Mixed	SBR	Bark et al., 1993
Actinobacteria	*Arthrobacter*	Mixed	Rhizosphere soil	Li et al., 2013
Actinobacteria	*Gordonia*	Mixed	WWTP	Beer et al., 2006
Actinobacteria	*Microlunatus*	Mixed	Pure culture	Kawakoshi et al., 2012
Actinobacteria	*Microlunatus*	Mixed	SBR	Nakamura et al., 1991; 1995a; 1995b; Kawaharasaki et al., 1998
Actinobacteria	*Microlunatus*	Mixed	WWTP	Beer et al., 2006
Actinobacteria	*Tetrasphaera*	Mixed	WWTP	Maszenan et al., 2000; Nguyen et al., 2011; Kong et al., 2005; Albertsen et al., 2012; Kristiansen et al., 2013
Actinobacteria	*Tetrasphaera*	Mixed	SBR	Günther et al., 2009
Alphaproteobacteria	--	Mixed	WWTP	Zilles et al., 2002a
Alphaproteobacteria	--	Mixed	SBR	Kawaharasaki et al., 1998
Alphaproteobacteria	*Defluviicoccus**	Acetate	SBR	Nobu et al., 2014
Bacilli	*Bacillus*	Mixed	Rhizosphere soil	DebRoy et al., 2013
Betaproteobacteria	--	Mixed	WWTP	Zilles et al., 2002a
Betaproteobacteria	--	Acetate	SBR	Stante et al., 1997
Betaproteobacteria	--	Mixed	SBR	Ge et al., 2015
Betaproteobacteria	Accumulibacter	Mixed	WWTP	Beer et al., 2006; Albertsen et al., 2012; Nguyen et al., 2012; Mao et al., 2015
Betaproteobacteria	Accumulibacter	Mixed	SBR	Liu et al., 2001; Günther et al., 2009; Mao et al., 2014; Skennerton et al., 2014
Betaproteobacteria	Accumulibacter	Acetate	SBR	Hesselmann et al., 1999; Crocetti et al., 2000; McMahon et al., 2002; Garcia-Martin et al., 2006; Kim et al., 2010
Betaproteobacteria	Accumulibacter	Propionate	SBR	Garcia-Martin et al., 2006
Betaproteobacteria	Accumulibacter	Mixed	Estuary	Kunin et al., 2008; Peterson et al., 2008
Betaproteobacteria	Accumulibacter	Mixed	Freshwater	Kunin et al., 2008; Peterson et al., 2008
Betaproteobacteria	Accumulibacter	Mixed	Soil	Kunin et al., 2008; Valdivia, 2009; Archibald, 2010
Betaproteobacteria	Accumulibacter	Mixed	Sediment	Kunin et al., 2008
Betaproteobacteria	*Dechloromonas*	Mixed	WWTP	Zilles et al., 2002b; Kong et al., 2007
Betaproteobacteria	*Propinoibacter*	Acetate	SBR	Crocetti et al., 2000
Betaproteobacteria	*Rhodocyclus*	Mixed	WWTP	Zilles et al., 2002a; 2002b; Kong et al., 2004; 2005
Betaproteobacteria	*Rhodocyclus*	Acetate	SBR	Hesselmann et al., 1999; Crocetti et al., 2000; McMahon et al., 2002
Cyanobacteria	*Leptolyngbya*	--	Marine, SBR	Zhang et al., 2015, Oyserman et al., 2017
Gammaproteobacteria	--	Mixed	SBR	Liu et al., 2001
Gammaproteobacteria	*Acinetobacter*	Mixed	WWTP	Wagner et al., 1994; Streichan et al., 1990
Gammaproteobacteria	*Halomonas*	Mixed	WWTP	Nguyen et al., 2012
Gammaproteobacteria	*Pseudomonas*	Mixed	WWTP	Nguyen et al., 2012
Gammaproteobacteria	*Pseudomonas*	Mixed	SBR	Günther et al., 2009
Gammaproteobacteria	*Thiomargarita*	Mixed	Sediment pore water	Schulz and Schulz, 2005
Gammaproteobacteria	*Beggiatoa*	Acetate	Pure culture	Brock and Schulz-Vogt, 2011
Gammaproteobacteria	*Thiothrix*	Acetate	SBR	Rubio-Ricón et al., 2017
Gemmatimonadetes	*Gemmatimonas*	Mixed	SBR	Zhang et al., 2003
Melainabacteria		Mixed	WWTP	Soo et al., 2014

*This organism was described as a competitor to PAOs, a glycogen accumulating organisms (GAO), but had genes coding for intracellular polyphosphate storage.

2.1 Polyphosphate Accumulating Organism Metabolism

Model PAOs like CAP are distinguished from non-PAOs because of their ability to make polyP, glycogen, and poly-β-hydroxyalkanoate (PHA) polymers under alternating anaerobic/aerobic conditions (Seviour & Nielsen, 2010). Their ability to synthesize large amounts of polyP under aerobic conditions and use it as an energy source under anaerobic conditions enables PAOs to outcompete other EBPR heterotrophs with more limited fermentative processes (Gebremariam et al., 2011). The metabolism of CAP in effective EBPR WWTPs is as follows. During anaerobic conditions (Figure 1a) [Figure 1 near here], PAOs like CAP take up short chain volatile fatty acids (VFAs) such as acetate and store them as PHAs like poly-β-hydroxybutyrate (PHB; Seviour et al. 2003; Seviour and Nielsen 2010). Intracellular polyP and glycogen concentrations decrease because they are used to convert VFA to PHA (Seviour et al., 2003; Seviour & Nielsen, 2010). Phosphate cleaved from the terminal end of a polyP chain during this process is exported from the cell, thereby contributing to an increase in the concentration of phosphate (i.e., P_i in Figure 1) in the bulk wastewater (Seviour et al., 2003; Seviour & Nielsen, 2010). CAP uses the energy released from the respiration of PHAs to replace polyP and glycogen (Seviour et al., 2003; Seviour & Nielsen, 2010) during aerobic periods (Figure 1b). As a result, CAP uptakes phosphate to build polyP chains, thereby drawing down bulk water phosphate concentrations in the wastewater prior to its discharge from the WWTP (Seviour et al., 2003; Seviour & Nielsen, 2010).

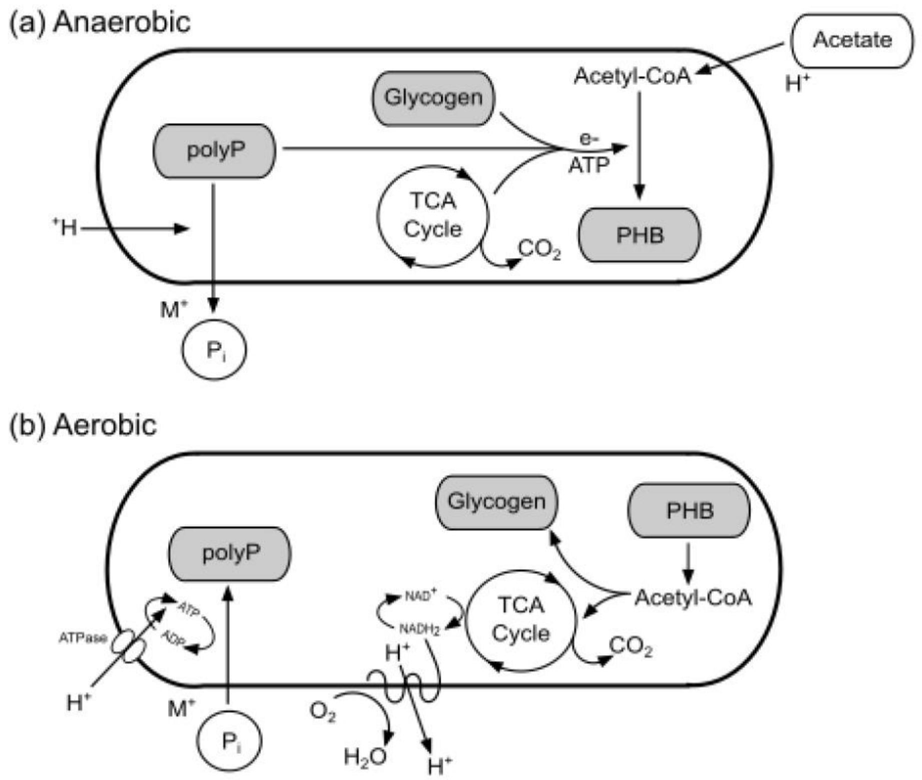

Figure 1. (a) Anaerobic and (b) aerobic metabolism of model polyphosphate accumulating organism (PAO), Candidatus Accumulibacter phosphatis (CAP). The poly-β-hydroxyalkanoate (PHA) known as poly-β-hydroxybutyrate (PHB) is specific to CAP. Abbreviations: metal cations (M+), phosphate (Pi). Adapted from Seviour et al. 2003, Seviour & Nielsen 2010, and Skennerton et al. 2014.

While we have a better understanding of PAO metabolism since EBPR was introduced, the metabolic mechanisms separating PAOs from non-PAOs are still debated and studied (e.g., Skennerton et al. 2014; Barnard et al. 2017). However, the genotypic and phenotypic diversity of CAP—and PAOs, in general—likely explains observed variation in metabolic processes under anaerobic conditions. Rather than a single metabolic model, many markedly different metabolic models may exist (Crocetti et al., 2000; Kawakoshi et al., 2012; Kristiansen et al., 2013; Mao, Yu, Xia, Chao, & Zhang, 2014; Mino, Van Loosdrecht, & Heijnen, 1998; Rubio-Rincón et al., 2017; Seviour &

McIlroy, 2008; Seviour et al., 2003; Skennerton et al., 2014). For example, *Tetrasphaera*-related PAOs, which can accumulate intracellular glycogen and polyP as well as denitrify and ferment, are still considered PAOs even though they do not accumulate PHAs under anaerobic conditions (Kristiansen et al., 2013). The PAO *Microlunatus phosphovorus* released phosphate under anaerobic conditions, stored polyP under aerobic conditions like CAP (Nakamura et al. 1995a). However, it was able to take up a wider range of C substrates under anaerobic conditions (Nakamura et al. 1995b). In a laboratory scale sequencing batch reactor (SBR) initially dominated by CAP, researchers demonstrated the emergence of the PAO *Thiothrix caldifontis* after exposing the SBR to sulfide (Rubio-Rincón et al., 2017). *T. caldifontis* took up P during aerobic conditions and released it during anaerobic conditions, removed P more effectively than CAP, and used both PHA and intracellular polysulfide as energy sources for growth (Rubio-Rincón et al., 2017). As we learn more about the diversity of PAOs, we may be motivated to revisit features that distinguish PAOs from non-PAOs as well as how EBPR WWTP microbial communities (including PAOs and non-PAOs) contribute to effective P removal in engineered systems.

2.2 Polyphosphate Accumulating Organism Phosphorus Cycling Functional Genes

There are several known functional genes associated with PAO-mediated P uptake and release in EBPR (Table 3) [Table 3 near here]. Polyphosphate kinases PPK1 and PPK2 (coded for by *ppk1* and *ppk2*) catalyse the reversible reaction of ATP to ADP to form intracellular polyP (Table 3). The nucleotide sequence for *ppk1* was first isolated from *Escherichia coli* (Akiyama et al. 1992) and since then other studies have identified PPKs in a wide range of bacterial, archaeal, and eukaryotic organisms (Kawakoshi et al., 2012; Rao et al., 2009; Trelstad, Purdhani, Geißdörfer, Hillen, & Keasling, 1999; H.

Zhang et al., 2002) as well as PAOs (He, Gall, & McMahon, 2007; McMahon, Dojka, Pace, Jenkins, & Keasling, 2002; Zhang et al., 2016). PPK1 is likely a membrane-bound protein with four domains concentrated in regions where the inner and outer cell membranes come together (Ahn & Kornberg, 1990). The nucleotide sequence of *ppk2* was first isolated from *Pseudomonas aeruginosa* (Zhang et al. 2002). PPK2 differs from PPK1 in its ability to catalyse the formation of polyP from both GTP and ATP as well as enzyme cofactors. Also, PPK2 has an affinity for Mn^{2+} while PPK1 has an affinity for Mg^{2+} (Rao et al., 2009; Zhang et al., 2002).

301 Table 3. Proteins associated with polyphosphate accumulating organism (PAO)-related P cycling functional genes.

Gene	Protein (Abbreviation)	Function and Key Traits	References
ppk1	polyphosphate kinase 1 (PPK1)	Catalyzes the de-phosphorylation of ATP to make polyP. Has an affinity for Mg^{2+}.	Ahn and Kornberg, 1990; Akiyama et al., 1992; Trelstad et al., 1999; Zhang et al., 2002; Zhu et al., 2005; Rao et al., 2009; Kawakoshi et al., 2012
ppk2	polyphosphate kinase 2 (PPK2)	Catalyzes the de-phosphorylation of ATP and GTP to make polyP. Has an affinity for Mn^{2+}.	Ishige et al., 1998; 2002; Zhang et al., 2002; Nocek et al., 2008; Rao et al. 2009; Kawakoshi et al. 2012
ppx1	exopolyphosphatase (PPX1)	Liberates the terminal phosphate molecule in longer (>500) polyP chain. Requires Mg^{2+} and KCl. Does not act on ATP and cannot be inhibited by ADP or ATP.	Reizer et al., 1992; Akiyama et al., 1993; Wurst and Kornberg, 1994; Kornberg, 1995; Zago et al., 1999; Ohtake et al., 2001; Rangarajan et al., 2006; Lichko et al., 2006; Rao et al., 2009; Kawakoshi et al., 2012
ppx2/gppA	exopolyphosphatase/ pentaphosphate phosphohydrolase (PPX/GPPA)	Liberates the terminal phosphate in a polyP chain and important in cellular stress response by hydrolyzing pppGpp to ppGpp.	Keasling et al., 1993; Reizer et al., 1993; Rao et al., 2009; Kawakoshi et al., 2012; Alcántara et al., 2014
ppn	endopolyphosphatase (PPN)	Cleaves phosphate from polyP chains (not selective to terminal phosphate). Has only been found in archaea, fungi, and mammals.	Kumble and Kornberg, 1996; Shi and Kornberg, 2005; Lichko et al., 2006; Rao et al., 2009
pap	AMP phosphotransferase (PAP)	Catalyzes reaction of AMP to ADP using energy from breakdown of polyP.	Rao et al., 2009; Mao et al., 2014; Skennerton et al., 2014
phoX, *phoD*, *phoA*	Alkaline phosphatase (APase)	Under P limited conditions, catalyzed the breakdown of Po to Pi.	Temperton et al., 2011; von Sperber et al., 2014; Morrison et al., 2016; Ragot et al., 2015; 2016; George et al., 2018; Margenot et al., 2018
pit	low-affinity phosphate transporter (PIT)	Binds phosphate and brings it into the cell.	Mao et al., 2014
pst	high-affinity phosphate transporter (PST)	Binds phosphate and brings it into the cell.	Mao et al., 2014

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ppk genes are an ideal marker for bacterial strain diversity because *ppk1* and *ppk2* are highly conserved (Rao et al., 2009; Zhang et al., 2002). Some microbes possess two *ppk* genes (*ppk1* and *ppk2)* while others only have one (Kawakoshi et al., 2012; Rao et al., 2009; H. Zhang et al., 2002). Specific to PAOs, the CAP genome has a single copy of *ppk1* and it evolves faster than CAP 16S rRNA genes (He & McMahon, 2011; Kunin et al., 2008). Researchers identified CAP *ppk1* in full-scale Canadian, United States, British, Danish, Chinese, Singaporean, Japanese, and Australian EBPR WWTPs (Albertsen, Hansen, Saunders, Nielsen, & Nielsen, 2012; Kunin et al., 2008; Mao et al., 2015)—indicating the ubiquity of this gene. Despite many studies focusing on CAP *ppk*, we found no studies quantifying the abundance and diversity of non-CAP PAO *ppk* in EBPR. Given the conserved nature of *ppk1* and *ppk2* as well as the ubiquity of polyP use, understanding the abundance and diversity of these genes in CAP and non-CAP PAOs may prove important to managing P cycling in engineered systems.

Exopolyphosphatase PPX1 and PPX2/GPPA (coded for by *ppx1* and *ppx2/gppA*) catalyse the breakdown of polyP. PPX1 breaks off the terminal phosphate molecules of a polyP chain when excess phosphate is present (Table 3). PPX1 preferentially acts on longer chains of polyP (i.e., 500 phosphate molecules or longer), does not act on ATP, and cannot be inhibited by ADP or ATP (Akiyama, Crooke, & Kornberg, 1993). PPX2/GPPA, also referred to as pentaphosphate phosphohydrolase, inhibits polyP accumulation at the enzymatic level by hydrolysing stress response nucleotides pppGpp to ppGpp or catalyses the release of phosphate by breaking polyP chains (Table 3). PPX2/GPPA is thought to be less active than PPX1, prefers longer polyP chains (i.e., 1000 residues or longer), and is inhibited by the presence of short- and medium-length polyP chains (Keasling, Bertsch, & Kornberg, 1993). Some organisms have both *ppx1* and *ppx2/gppA* (Alcántara, Blasco, Zúñiga, & Monedero, 2014; Keasling et al., 1993), but this trend is not well characterised for PAOs. We found only a few *ppx1* and *ppx2/gppA* studies (Table 3); none assessed their abundance and diversity in engineered systems. Due to their role in polyP breakdown under anaerobic conditions—an important defining metabolic characteristic of potential PAOs—further study of *ppx* genes are needed. Some studies found that P starvation induces transcription of *ppk* and *ppx* genes, but whether this can be applied to PAOs is unknown (Seviour et al., 2003).

Other P cycling genes of interest include *pap, phoX, phoD, phoA, pit, pst,* and *ppn*. Associated protein functionality and key traits are summarized in Table 3. As we mentioned previously for *ppk* and *ppx*, more work needs to be done to characterize the abundance, diversity, and role of these genes in P cycling with respect to engineered systems. While we only focus on P-related genes here, little is known about functional genes regulating other important PAO polymers: PHA and glycogen (Seviour et al., 2003).

3. Biotic Controls on Phosphorus Cycling in Natural Systems

Parallel research in natural systems, carried out at a similar time frame as developments in engineered systems, evaluated microbial controls on P cycling (e.g., Fleischer 1978). This work was motivated by an emerging understanding that anthropogenic P loadings lead to the eutrophication of marine and freshwater ecosystems world-wide and microbes may respond to this increase by storing excess P as polyP (Gächter, Meyer, & Mares, 1988; Kenney, Schelske, & Chapman, 2001; Uhlmann & Bauer, 1988). Cross-over research in engineering (e.g., Barnard 1976) and medical (e.g., Kornberg 1995) fields suggested intracellular polyP provided a biotic mechanism for observed P patterns in natural systems. Researchers in natural systems hypothesised alternating anaerobic/aerobic conditions in the upper layers of terrestrial soils and aquatic sediments led to polyP degradation/synthesis, respectively, by organisms that appeared to be similar to EBPR PAOs (Davelaar, 1993; Diaz et al., 2012; Gächter et al., 1988; Hupfer, Gloess, & Grossart, 2007; Hupfer, Glöss, Schmieder, & Grossart, 2008; Hupfer & Lewandowski, 2008; McMahon & Read, 2013; McParland et al., 2015; Peterson, Warnecke, Madejska, McMahon, & Hugenholtz, 2008; Pett-Ridge & Firestone, 2005; Reddy et al., 1999; Schulz & Schulz, 2005; Uhlmann & Bauer, 1988). The evidence synthesised below provides preliminary support for this hypothesis by highlighting the ubiquity of polyP use and EBPR PAO-like metabolism in the natural environment. This synthesis also sets a baseline for future studies exploring the potential role of PAOs in water quality management outside of traditional WWTPs.

3.1 Aquatic Systems

A number of studies in freshwater and marine systems documented intracellular presence of EBPR PAOs and intracellular polyP storage (Tables 2 and 4). Microscopy-based studies found intracellular polyP granules in freshwater and marine sediment bacteria (Diaz et al., 2008; Hupfer, Rube, & Schmieder, 2004; Schulz & Schulz, 2005), stream biofilms (Taylor 2016), and freshwater and marine microorganisms (Martin, Dyhrman, Lomas, Poulton, & Van Mooy, 2014; Sicko-Goad & Lazinsky, 1986; Stevenson & Stoermer, 1982). There is also evidence of polyP accumulation by diverse bacterial species in river sediments contaminated by heavy metals (Narancic et al., 2012). Bulk extraction hydrolysis techniques using hot water have been used to quantify polyP content in microbial stream biofilm assemblages (e.g., Price and Carrick 2011) and lake sediments (e.g., Kenney et al., 2001). More recently, enzymatic assays (e.g., Rier *et al.* 2016) and ^{31}P nuclear magnetic resonance (^{31}P-NMR) spectroscopy (e.g., Zhang et al., 2013; Read et al., 2014) have been used to estimate microbial polyP content. Researchers identified CAP genes in sediment and water samples across the United States (Kunin et al., 2008; Peterson et al., 2008) and in Portuguese lake sediments (Martins et al., 2011). Additionally, one study observed polyP accumulation by marine filamentous cyanobacterial symbionts within sponges and verified the presence of *ppk* genes using techniques established for EBPR PAOs (Zhang et al. 2015).

Consistent with EBPR PAO metabolism, there is evidence that O_2 concentrations influence microbial P cycling in freshwater environments. More specifically, microbes accumulated P as polyP under aerobic (i.e., O_2 rich conditions) and released P under anaerobic (i.e., O_2 poor conditions) in freshwater lake sediments (Amirbahman, Lake, & Norton, 2013; Martins et al., 2011), wetland sediments (Khoshmanesh, Hart, Duncan, & Beckett, 1999), and stream biofilms (Saia et al., 2017). Other researchers have observed coupled P and O_2 patterns that are generally consistent with EBPR PAO metabolism despite not directly measuring polyP concentrations or known EBPR PAO genes. As an example, in freshwater streams, diel water column P (as phosphate) cycling patterns were inversely related to O_2 availability—when dissolved O_2 was high during the day, phosphate was low and vice versa at night (Cohen et al., 2013; Ford, King, & Williams, 2018). In freshwater lakes, low O_2 levels have been linked to higher P concentrations (Weinke & Biddanda, 2018). Also, increasing/decreasing O_2 availability near the water column-sediment boundary or water column-periphyton boundary led to uptake/release of P, respectively (Carlton & Wetzel, 1988; Fleischer, 1978; Gächter et al., 1988; Read et al., 2014; Saia et al., 2017; Sherson, Van Horn, Gomez-Velez, Crossey, & Dahm, 2015). While abiotic processes (e.g., redox sensitive dissolution/precipitation of Fe and P; Richardson, 1985) cannot be completely ruled out in freshwater systems, there is consensus that biological processes play a role in coupled P and O_2 patterns, and in some cases, this role is large. For example, biotic processes accounted for 66% of stream water column P uptake and release—with the remaining being attributed to abiotic, Ca-P precipitation and dilution—in a diel P cycling study (Cohen et al., 2013). In freshwater wetlands, biotic mechanisms accounted for over 83% of short-term (12 hr) water column P removal (Scinto & Reddy, 2003).

Only a few researchers in marine systems have linked O_2 availability in the water column with P cycling, but these limited studies find support for PAO metabolism in marine environments. Namely, one study found that phosphate concentrations were ~3x greater in the redoxcline—a zone with a strong vertical redox gradient—of a coastal basin compared to the surface (McParland et al., 2015). Another found that polyP concentrations in water samples from a coastal inlet decreased as dissolved O_2 concentrations decreased (Diaz et al., 2012). One study observed that giant sulphur bacteria (*Thiomargarita namibiensis*) accumulated polyP under oxic sediment conditions and released phosphate under anoxic sediment conditions similar to EBPR PAOs (Schulz & Schulz, 2005).

In addition to O_2 concentrations, there is evidence that polyP storage in aquatic environments also depends on P availability. For instance, phytoplankton accumulated more polyP in P depleted regions of the Sargasso Sea compared to regions that were more P rich (Martin et al., 2014) and stream biofilm polyP storage was positively correlated with stream P load (Price & Carrick, 2016). Additionally, the abundance of P metabolism-associated genes in water column microorganisms increased along an urban to estuary transect (i.e., from P abundant inland to P limited open ocean); this same study also verified the presence of EBPR PAO bacterial classes in water column samples (Jeffries et al., 2016). A metagenomic study of marine environments found that *ppk1, ppk2*, *ppx, pstS,* and *phoX* gene abundances were inversely proportional to P availability (Temperton, Gilbert, Quinn, & McGrath, 2011). In freshwater algal cells, the development of intracellular polyP granules reflected the degree of nutrient additions to stream ecosystems and the surrounding landscape (Carrick and Price 2011). Inverse relationships between P availability and polyP accumulation as well as between P availability and P functional gene abundance support the hypothesis that polyP storage provides organisms with the ability to conserve energy and nutrients for future use.

3.2 Soils

Compared to aquatic systems, we found only a few studies documenting the presence of EBPR PAOs (Archibald, 2010; Kunin et al., 2008; Valdivia, 2009), relatives of EBPR PAOs (DeAngelis, Silver, Thompson, & Firestone, 2010; Pett-Ridge & Firestone, 2005), or unrepresented PAOs (DebRoy, Mukherjee, Roy, Thakur, & Raychaudhuri, 2013; Li, Yuan, Yang, & Li, 2013) in terrestrial environments such as soils (Table 2). These studies did not directly address the role of PAOs in soil P cycling. In terms of agricultural systems, we found one study that quantified polyP in overland flows (Bourke et al., 2009) and several studies characterizing soil P_o (B. J. Cade-Menun, 2017; Cade-Menun & Liu, 2014) but none directly addressed the role of PAOs. Despite the limited research on the role of PAOs in soils, we found several studies exploring the impacts of environmental perturbations such as wetting and drying events on soil P_o pools (i.e., microbial P). Namely, soil P_o mineralization showed a statistically significant, positive correlation between microbial biomass (Dinh, Schramm, Spohn, & Matzner, 2016) and microbial P (Grierson, Comerford, & Jokela, 1998; Turner & Haygarth, 2001) upon rewetting. Soils undergoing wetting/drying events showed larger increases in microbial P over time compared to soils that did not undergo these events (Grierson et al., 1998). One study estimated that 41% of added phosphate was stored as microbial P upon soil rewetting (Yevdokimov, Larionova, & Blagodatskaya, 2016). Increases in P release during saturated soil conditions have also been observed at larger landscape (Dupas et al., 2015) and watershed scales (Franklin, Steiner, Duke, Moriasi, & Starks, 2013) but whether and how much microbial P contributes to these patterns is still unknown (Blackwell et al., 2010).

We draw attention to research relating microbial P pools and soil wetting/drying events because we believe these findings provide additional evidence for PAO-mediated P cycling in soils. Wetting and drying events influence the diffusion of O_2 through soil pores; saturated soils tend to be anaerobic while unsaturated soils tend to be aerobic (Burgin & Groffman, 2012; Silver, Lugo, & Keller, 1999; M. S. Smith & Tiedje, 1979). If PAOs are present in soils, they may behave similarly to EBPR PAOs. In fact, researchers have hypothesized that soil PAOs may release P during wet periods and take up P during dry periods like EBPR PAOs (Davelaar, 1993; Peterson et al., 2008; Pett-Ridge & Firestone, 2005). The studies mentioned above provide support for this hypothesis; however, research opportunities exist to test this PAO-specific hypothesis in soils.

4. Research Approaches and Future Directions

Many research opportunities exist to explore the presence, metabolism, and ecological role of PAOs in engineered, natural, and agricultural systems. Additionally, by integrating research in these three fields, we may achieve reductions in P loading and improving water quality across the landscape—from soils to waterbodies. In general, key opportunities for these three systems include but are not limited to exploration of (1) non-CAP PAOs (including bacteria, eukaryotes, and archaea), (2) lesser studied functional genes (e.g., *ppx*), and (3) microbial responses to changing environmental conditions (e.g., O_2 and P availability). In this section, we highlight specific knowledge gaps and tools that may be used to address them.

Studies in engineered systems have used microscopy, molecular biology, and other tools to study the metabolism, functional genes, and communities (diversity, structure, and function) of EBPR PAOs (Table 4) [Table 4 near here], but these tools can be applied more broadly. Namely, we found only one *ppk* polymerase chain reaction (PCR) primer available for non-CAP PAOs (Table 5) [Table 5 near here]. We found many papers discussing the abundance and diversity of *ppk* in engineered systems (e.g., Table 5, Zilles *et al.*, 2002; Skennerton *et al.*, 2014; Mao *et al.*, 2015, 2016), one paper that assessed the abundance of *ppk* in natural systems (Temperton et al., 2011), a few that assessed *ppk* diversity in natural systems (Peterson et al., 2008), and no studies that assessed the abundance and diversity of *ppk* in agricultural systems. We found no studies that assessed the abundance and diversity of *ppx* in EBPR, one in natural systems that addressed *ppx* abundance (Temperton et al., 2011), and no studies in agricultural systems addressing either *ppx* abundance or diversity. As researchers seek out unrepresented non-CAP PAOs, a shift in focus from only P accumulation (i.e., via PPK) to both P accumulation and breakdown (i.e., via PPK and PPX) may be necessary to close the loop of the P cycle. Additionally, our limited understanding of P cycling functional genes may necessitate approaches that do not rely on complete *a priori* knowledge of DNA sequences for a gene of interest. Therefore, next generation sequencing (NGS) technologies such as amplicon and shotgun metagenomics (Table 4) may help identify relevant microorganisms, quantify their relative abundances, and compare previously unknown DNA sequences for PAO functional genes.

Table 4. Descriptions, references, and examples of tools used to study microbially-mediated P cycling in engineered and natural systems. Abbreviations: polyphosphate accumulating organism (PAO), transmission electron microscopy (TEM), 4',6-diamidino-2-phenylindole (DAPI), fluorescence in-situ hybridization (FISH), quantitative FISH (qFISH), polymerase chain reaction (PCR), quantitative PCR (qPCR), next generation sequencing (NGS), internal transcribed spacer (ITS), nanometer-scale second ion mass spectrometry (nanoSIMS).

Tool	Description/Purpose	Use Category	Use References	Example in Engineered Systems	Example in Natural Systems
Microscopy					
TEM	Used with energy dispersive x-ray analysis to identify intracellular polyP granules.	metabolism	Sick-Goad and Lazinsky, 1986	Günther et al., 2009	Uhlmann and Bauer, 1988
NanoSIMS	Microscopy based spectroscopy used to identify intracellular polyP granules as well as the 2D and 3D spatial distribution of P and various cations such as Fe, Al, Ca, and Mg.	metabolism	Herrmann et al., 2007; Hoppe et al., 2013; Mueller et al., 2013; Kruse et al., 2015; Gao et al., 2016	NA	Sulu-Gambari et al., 2016
Light microscopy					
Lead staining	Technique used to identify intracellular polyP granules	metabolism	Stevenson and Stoermer, 1982	NA	Stevenson and Stoermer, 1982
Neisser's (methylene) blue	Stain used to identify intracellular polyP granules.	metabolism	Crocetti et al., 2000	Crocetti et al., 2000	Schulz and Schultz, 2005
Toludine blue	Stain used to identify intracellular polyP granules.	metabolism	Schulz and Schulz, 2005	Streichan et al., 1990	Schulz and Schultz, 2005
Epifluorescence microscopy					
Tetracycline	Fluorescent stain used to identify intracellular polyP granules.	metabolism	Günther et al., 2009	Günther et al., 2009	NA
DAPI	Stain used to identify and quantify intracellular polyP granules.	metabolism	Hung et al., 2002; Aschar-Sobbi et al., 2008; Diaz and Ingall, 2010, Martin and Van Mooy, 2013	Hung et al., 2002	Rier et al., 2016
FISH/qFISH	Fluorescent oligonucleotide probe that hybridizes to 16S rRNA or 23S rRNA sequences. Used to quantify PAOs and study the diversity of PAO communities.	metabolism, microbial community	Amann et al., 1990; 2001; Seviour et al., 2003; Seviour and Nielsen, 2010	metabolism: Hesselman et al., 1999; diversity: Beer et al., 2006; quantification: Albertsen et al., 2012	Sulu-Gambari et al., 2016
Molecular Biology					
PCR/qPCR	Used to amplify and quantify PAO 16S rRNA and functional genes (e.g., *ppk*). Used to determine PAO strain diversity, PAO community functional gene diversity.	metabolism, functional genes, microbial community	He et al., 2010; Seviour and Nielsen, 2010	Zhang et al., 2016	Peterson et al., 2008
Shotgun metagenomics	Non-targeted NGS-based technique used to identify potential PAOs, study the metabolic potential of potential PAOs, and quantify the relative abundance of PAO functional genes.	metabolism, functional genes, microbial community	Riesenfeld et al., 2004; Howe et al., 2014; Zimmerman et al., 2014; Howe and Chain, 2015; Wang et al., 2015; Choi et al., 2016, Menzel and Krogh, 2016	Skennerton et al., 2014	Temperton et al., 2011
Amplicon metagenomics	Targeted (16S rRNA, 23S rRNA, ITS region) NGS-based technique used to quantify known PAOs and study PAO communities.	microbial community	Riesenfeld et al., 2004, Kozich et al., 2013, Zimmerman et al., 2014	Oyserman et al., 2017	Locke, 2015
Flow cytometry	Cell sorting technique often used in conjunction with DAPI to identify potential PAOs.	metabolism, microbial community	Zilles et al., 2002a; 2002b; Hung et al., 2002	Kim et al., 2010	Locke, 2015
Other					
^{31}P-NMR	Used to measure the concentration of various P-species (e.g., polyphosphate) in water and soil/sediments.	metabolism	Cade-Menun, 2015	NA	Read et al., 2014, Cade-Menun, 2017
Sensors	Used to measure environmental variables (e.g., P concentration, dissolved oxygen) along a range of time scales.	metabolism	Pellerin et al., 2016; Rode et al., 2016; Fares et al., 2016	Lanham et al., 2013	Cohen et al., 2013

Table 5. A summary of polyphosphate accumulating organism (PAO) polymerase chain reaction (PCR) primer sequences.

Gene Target	Primer Pair	Sequence (5'-3')	Reference
Most *ppk1*	NLDE-0199F	CGTATGAATTTTCTTGGTATTTATTGTACTAATCTngaygarttyt	McMahon et al., 2002; 2007
	TGNY-1435R	GTCGAGCAGTTTTTGCATGAwarttnccngt	
CAP *ppk1*	ACCppk1-254F	TCACCACCGACGGCAAGAC	McMahon et al., 2002; 2007; Kunin et al., 2008
	ACCppk1-1376R	ACGATCATCAGCATCTTGGC	
CAP *ppk1*	ppk274f	ACCGACGGCAAGACSG	Kunin et al., 2008
	ppk1156r	CGGTAGACGGTCATCTTGAT	
CAP *ppk1*	ppk734f	CTCGGCTGCTACCAGTTCCG	Kunin et al., 2008
	ppk1601r	GATSCCGGCGACGACGTT	
CAP Clade 1A *ppk1*	Acc-ppk1-763f	GACGAAGAAGCGGTCAAG	He et al., 2007; He and McMahon, 2011
	Acc-ppk1-1170r	AACGGTCATCTTGATGGC	
CAP Clade 1A *ppk1*	Acc-ppk1-974f	TGATGCGCGACAATCTCAAATTCAA	Zhang et al., 2016
	Acc-ppk1-1113r	AATGATCGGATTGAAGCTCTGGTAG	
CAP Clade 1B *ppk1*	Acc-ppk1-372f	TGAAGGCATTCGCTTCCT	Zhang et al., 2016
	Acc-ppk1-653r	AAGCAGTATTCGCTGTC	
CAP Clade 1C *ppk1*	Acc-ppk1-362f	AGCTGGCGAGTGAAGGCATTCG	Zhang et al., 2016
	Acc-ppk1-758r	AACAGGTTGCTGTTGCGCGTGA	
CAP Clade 1D *ppk1*	Acc-ppk1-634f	TGCGACAGCGAATACAG	Zhang et al., 2016
	Acc-ppk1-848r	ACTTCGAGGCGGACG	
CAP Clade 2A *ppk1*	Acc-ppk1-893f	AGTTCAATCTCACCGACAGC	He et al., 2007; He and McMahon, 2011
	Acc-ppk1-997r	GGAACTTCAGGTCGTTGC	
CAP Clade 2B *ppk1*	Acc-ppk1-870f	GATGACCCAGTTCCTGCTCG	He et al., 2007
	Acc-ppk1-1002r	CGGCACGAACTTCAGATCG	
CAP Clade 2C *ppk1*	Acc-ppk1-254f	TCACCACCGACGGCAAGAC	He et al., 2007
	Acc-ppk1-460r	CCGGCATGACTTCGCGGAAG	
CAP Clade 2D *ppk1*	Acc-ppk1-375f	GGGTATCCGTTTCCTCAAGCG	He et al., 2007
	Acc-ppk1-522r	GAGGCTCTTGTTGAGTACACGC	
CAP Clade 2E *ppk1*	Acc-ppk1-757f	TTCGTGGACGAGGAAGA	Zhang et al., 2016
	Acc-ppk1-1129r	ATTGTTCGAGCAACTCGATG	
CAP Clade 2G *ppk1*	Acc-ppk1-410f	CCGAGCAACGCGAATGG	Zhang et al., 2016
	Acc-ppk1-514r	TGTTGAGTACGCGCGGGA	
CAP Clade 2H *ppk1*	Acc-ppk1-701f	ACTCCTTCGTATTCCTCTCT	Zhang et al., 2016
	Acc-ppk1-928r	TCATCGCTTCGGAGCA	
CAP Clade 2I *ppk1*	Acc-ppk1-688f	AGTGATTATGCTTTCGTCTTTC	Zhang et al., 2016
	Acc-ppk1-946r	TGAACTGTCCGAGCAGGA	
CAP 16S	CAP438f	GGTTAATACCCTGWGTAGAT	Zhang et al., 2016
	CAP846r	GTTAGCTACGGCACTAAAAGG	
CAP 16S	PAO-518f	CCAGCAGCCGCGGTAAT	He et al., 2007; He and McMahon, 2011
	PAO-846r	GTTAGCTACGGCACTAAAAGG	
CAP Clade 1A 16S	16S-Acc-1Af	TTGCTTGGGTTAATACCCTGA	He et al., 2010
	16S-Acc-1Ar	CTGCCAAACTCCAGTCTTGC	
CAP Clade 2A 16S	16S-Acc-2Af	TTGCACGGGTTAATACCCTGT	He et al., 2010
	16S-Acc-2Ar	CTCTGCCAAACTCCAGCCTG	
Halomona-related 16S	Pse136f	TAGTAGTGGGGGATAACGTC	Lane, 1991; Nguyen et al., 2012
	1492R	GCYTACCTTGT TACGACTT	

Besides PCR-based approaches, researchers identified and quantified EBPR PAOs using fluorescent *in-situ* hybridization (FISH) probes (Tables 4 and 6) [Table 6 near here], but additional research is needed to develop FISH probes for less common PAOs and use FISH probes on samples from natural and agricultural systems. Fluorescent stains such as 4',6-diamidino-2-phenylindole (DAPI) may be useful to identify and quantify polyP granules in PAOs from engineered, natural, and agricultural systems (Table 4). As an example, DAPI staining has been used to detect and quantify intracellular polyP in stream and marine environments (e.g., Diaz et al., 2012, Rier et al., 2016) but has not been applied to agricultural systems.

Table 6. A summary of fluorescence in-situ hybridization (FISH) probes used to identify polyphosphate accumulating organism (PAO)-related organisms (updated from Seviour and Nielsen 2010). Abbreviations: not determined (ND).

Probe Name	Sequence (5'-3')	Formamide (%)	Target	Reference
ALF1b	GCTGCCTCCCGTAGGAGT	20	Alphaproteobacteria	Manz et al., 1992
BET42[a]	GCCTTCCCACTTCGTTT	35	Betaproteobacteria	Manz et al., 1992
GAM42[a]	GCCTTCCCACATCGTTT	35	Gammaproteobacteria	Manz et al., 1992
RHC175	TGCTCACAGAATATGCGG	30	*Rhodocyclus/*Accumulibacter	Hesselmann et al., 1999
RHC439	CNATTTCTTCCCCGCCGA	30	Most *Rhodocyclaceae*	Hesselmann et al., 1999
Rc988	AGGATTCCTGACATGTCAAGGG	ND	*Rhodocyclus* group	Crocetti et al., 2000
PAO462[c]	CCGTCATCTACWCAGGGTATTAAC	35	Most Accumulibacter	Crocetti et al., 2000
PAO651[c]	CCCTCTGCCAAACTCCAG	35	Most Accumulibacter	Crocetti et al., 2000
PAO846[c]	GTTAGCTACGGCACTAAAAGG	35	Most Accumulibacter	Crocetti et al., 2000
Acc-I-444	CCCAAGCAATTTCTTCCCC	35	PAO clade IA and other Type I clades	Flowers et al., 2009
Acc-II-444	CCCGTGCAATTTCTTCCCC	35	PAO clade IIA, IIC, and IID	Flowers et al., 2009
Actino-1011	TTGCGGGGCACCCATCTCT	30	*Tetrasphaera*-relatives	Liu et al., 2001
Actino-221[a]	CGCAGGTCCATCCCAGAC	30	*Tetrasphaera*-relatives	Kong et al., 2005
Actino-658[a]	TCCGGTCTCCCCTACCAT	40	*Tetrasphaera*-relatives	Kong et al., 2005
Tet1-266	CCCGTCGTCGCCTGTAGC	25	*Tetrasphaera*-relatives	Nguyen et al., 2011
Tet2-892	TAGTTAGCCTTGCGGCCG	5	*Tetrasphaera*-relatives	Nguyen et al., 2011
Tet2-174	GCTCCGTCTCGTATCCGG	20	*Tetrasphaera*-relatives	Nguyen et al., 2011
Tet3-654	GGTCTCCCCTACCATACT	35	*Tetrasphaera*-relatives	Nguyen et al., 2011
Tet3-19	CAGCGTTCGTCCTACACA	0	*Tetrasphaera*-relatives	Nguyen et al., 2011
BET135	ACGTTATCCCCCACTCAATGG	45	*Dechloromonas*-relatives	Kong et al., 2007
MIC179	GAGCAAGCTCTTCTGAAACCG	10	*Microlunatus phosphovorus*	Kawaharasaki et al., 1998
G123T	CCTTCCGATCTCTATGCA	40	*Thiothrix*-relatives	Kanagawa et al., 2000; Rubio-Rincón et al., 2017
EUB338	GCTGCCTCCCGTAGGAGT	60	Most Bacteria[b]	Amann et al., 1990
EUB338-II	GCAGCCACCCGTAGGTGT	60	Most Bacteria[b]	Daims et al., 1999
EUB338-III	GCTGCCACCCGTAGGTGT	60	Most Bacteria[b]	Daims et al., 1999

[a]Competitor probes required. [b]Use EUB338, EUB338-II, and EUB338-III together to obtain an estimate of total bacteria. [c]Use PAO462, PAO651, PAO846 together to obtain an estimate of total CAP PAOs.

Opportunities also exist for researchers to use a combination of tools: these may include combining flow cytometry and NGS technologies to design new PCR primers and FISH probes for the identification of atypically studied bacterial, eukaryotic, and archaeal PAOs and their functional genes (e.g., *ppk* and *ppx* genes). Researchers have established methods for analysing polyP in agricultural soils using ^{31}P nuclear magnetic resonance spectroscopy (^{31}P-NMR; Cade-Menun, 2015; 2017), therefore; this technique can be used in combination with other microscopy and molecular biology methods to explore the role of PAO-mediated P cycling in agricultural systems. Additionally, by combining any of the previously discussed tools (Table 4) with sensors developed to detect nutrient and environmental changes at time and spatial scales relevant to microbial processes (Blackwell et al., 2010; Pellerin et al., 2016), researchers can verify the metabolism of potential PAOs and assess their ecological role in natural and agricultural systems.

We can apply lessons learned from microbially-mediated N and C cycling studies to explore whether and how PAOs play a role in the P cycling of natural systems. Several general frameworks exist to link microbial- and ecosystem-scale processes (Bier et al., 2015; Martiny, Jones, Lennon, & Martiny, 2015; Nemergut, Shade, & Violle, 2014; Prosser, 2013; J. P. Schimel, Gulledge, Clein-Curley, Lindstrom, & Braddock, 1999; Wallenstein & Hall, 2012) but have not been applied to study microbially-mediated P cycling in natural and agricultural systems. As researchers establish studies to explore microbial P cycling in new settings, they can refer to previous work for guidance on microbial-scale ecological theories (Choudoir, Panke-Buisse, Andam, & Buckley, 2017; Prosser et al., 2007), statistical approaches (Bernhardt et al., 2017; Bier et al., 2015; Buttigieg & Ramette, 2014; Rocca et al., 2015; Schimel & Gulledge, 1998; Willis, 2016; Willis, Bunge, & Whitman, 2017), method overviews (Ekblom & Wolf, 2014; Kozich, Westcott, Baxter, Highlander, & Schloss, 2013; Pallen, 2016; Riesenfeld, Schloss, & Handelsman, 2004; Zimmerman, Izard, Klatt, Zhou, & Aronson, 2014), bioinformatics (Cock et al., 2009; Howe et al., 2014; Loman & Watson, 2013; Shade & Teal, 2015; Wilson et al., 2016), reproducible research (da Veiga Leprovost, Barbosa, Francisco, Perez-Riverol, & Carvalho, 2014; Howe & Chain, 2015; Howe, Howe, Kaleita, & Raman, n.d.; Perez-Riverol et al., 2016; Schloss, 2017; Shade & Teal, 2015; Wilson et al., 2016), and modelling (Graham et al., 2014, 2016; Manzoni, Schaeffer, Katul, Porporato, & Schimel, 2014; Powell, Welsh, Hallin, & Allison, 2015; Reed, Algar, Huber, & Dick, 2014; Todd-Brown, Hopkins, Kivlin, Talbot, & Allison, 2012; Wieder, Bonan, & Allison, 2013).

Specific to research in natural and agricultural systems, care must be taken to ensure that microbial and environmental measurement time and spatial scales are compatible (Battin, Besemer, Bengtsson, Romani, & Packmann, 2016; Bier et al., 2015; Blackwell et al., 2010), measurement bias for/against active microbial community members is understood (Carini et al., 2016; Jones & Lennon, 2010; Lennon & Jones, 2011; J. Schimel & Gulledge, 1998), and environmental variables (e.g., pH and temperature) that may influence microbial community diversity, structure, and function are accounted for in the experimental design (Battin et al., 2016; Bier et al., 2015; Dinh et al., 2016; Fierer & Jackson, 2006; Lauber, Hamady, Knight, & Fierer, 2009; Oliverio, Bradford, & Fierer, 2016; Rousk, Brookes, Glanville, & Jones, 2011; Schimel & Gulledge, 1998). Researchers must take care to design controls that consider abiotic processes may mimic PAO-mediated P cycling (e.g., reductive dissolution of Fe-P).

Studies using NGS technologies may benefit from existing environmental microbiology database tools such as RefSoil (Choi et al., 2016), Kaiju (Menzel & Krogh, 2016), and Xander (Wang et al., 2015) to name a few. Table 4 summarizes tools that can be applied to expand our knowledge of PAO metabolism, functional genes, and communities in natural and agricultural systems and also integrate these findings with research from engineered systems.

5. New Directions: Applying Research to Improve Landscape-Scale Phosphorus Management in Agricultural Systems

In the next decade, interdisciplinary soil microbiome research is positioned to increase crop yield and resilience (NASEM, 2018), but these advancements may also be extended to improve and protect water quality. More specifically, there are several reasons why agricultural systems represent an ideal setting to test and apply existing and emerging knowledge of PAO-mediated P cycling. First, the application of inorganic fertilizer and manure to farmland often contributes the largest fraction of P loading to waterbodies (Bennett et al., 2001; Dubrovsky et al., 2010). Second, soil microbial communities are diverse and rich (Bardgett & Van Der Putten, 2014; Dunbar, Barns, Ticknor, & Kuske, 2002; Fierer & Jackson, 2006; Gans, Wolinsky, & Dunbar, 2005; Hug et al., 2016; Schloss & Handelsman, 2006; Tringe et al., 2005). Therefore, besides informing research in agricultural systems, new discoveries from agricultural soils, may benefit existing PAO research in natural and engineered systems by revealing additional genetic and metabolic diversity. Third, soil P_o pools—which include microbial P such as that stored by PAOs—can be large and are understudied (*Section 1* and *3.2*). Fourth, agricultural systems integrate traits of engineered and natural systems; conventional agriculture takes place within the context of natural systems and is managed to achieve a specific goal (e.g., maximum crop yield per acre) much like WWTPs are managed to ensure treatment of water to a predetermined standard. Last, current agricultural P management strategies tend to rely on abiotic (i.e., behavioural, chemical, and physical) mechanisms to reduce P loading rather than considering a combination of abiotic and biotic P controls. Thus, there remains a large, untapped opportunity to improve water quality management of P in agricultural systems by deliberately including biotic controls.

With respect to current agricultural P management strategies, behavioural approaches encourage farmers to use the ‘4R’s’: right source, right timing, right placement, and right rate (Good and Beatty 2011; Smith et al. 2015b; Ward et al. 2018). Chemically-based management strategies utilise soil Ca, Fe, and Al amendments to adsorb DP (e.g., Buda et al. 2012). Because most of the soil P pool is bound to organic matter, clay, and minerals, physically-based P management strategies focus primarily on reducing soil and sediment transport (i.e., PP forms) through the use of vegetated buffers, wetlands, cover crops, tile drains, and reduced or no-tillage (Bergström et al., 2015; Mitsch, Cronk, Wu, Nairn, & Hey, 1995; Rittenburg et al., 2015; Sharpley & Menzel, 1987; Tomer et al., 2014; Ward et al., 2018). Chemical and physical controls can also be combined (e.g., Watts and Torbert, 2009). However, recent discussions (Christianson et al., 2016; Ford et al., 2018; Geohring et al., 2001; Jarvie et al., 2017; Williams, King, Ford, Buda, & Kennedy, 2016) concerning the unintended increases in DP loads due to tile drainage and no-till strategies, favour approaches that account for multiple P forms (i.e., DP and PP) as well as abiotic and biotic P cycling mechanisms (Ford et al., 2018; George et al., 2018; Jarvie et al., 2017).

Biologically-based agricultural P water quality management strategies typically rely on plants, rather than microbes, to reduce P transport from the field. For example, farmers may reduce P application rates so crops are forced to take up soil P (see 4Rs discussion above) or wetland vegetation may serve as a P sink (Bergström et al., 2015; Reddy et al., 1999). Also, fungi and bacteria—also known as phosphate solubilizing microbes—are often applied as amendments to agricultural fields to mineralize soil P for the benefit of the crop (Javot, Pumplin, & Harrison, 2007; Rodríguez & Fraga, 1999; Sharma, Sayyed, Trivedi, & Gobi, 2013). However, this strategy is not necessarily implemented with water quality goals in mind. Given what we know about legacy P and soil P_o pools (see *Sections 1* and *3.2*), it is important to determine whether fungal and bacterial amendments are not further exacerbating P loads (Dodd & Sharpley, 2015).

The limited application of biotic agricultural water quality management strategies for P demonstrates the need for interdisciplinary research on (1) biotic P controls such as PAO-mediated P cycling and (2) interaction between biotic and abiotic P controls. General interdisciplinary research questions addressing limitations discussed in *Section 4* and applied to agricultural systems may include:

- Are EBPR PAOs ubiquitous in agricultural systems (e.g., soils, ditches, nearby waterbodies) ?
- Can we identify unrepresented PAOs (i.e., bacteria, archaea, and eukaryotes) in agricultural systems?
- What role do PAOs play in mediating P cycling in agricultural systems?
- What is the relative importance of PAO-mediated P cycling compared to abiotic P cycling mechanism in agricultural systems?

- Can PAO-mediated P cycling be actively managed to achieve desired water quality (e.g., P retention) and crop management (e.g., adequate P availability to crops) goals?

Additionally, we also highlight three potential research directions related to pressing issues in agricultural systems. These include (1) the timing and environmental controls of microbes on ‘legacy’ soil P cycling, (2) the impact of agricultural tile drains and irrigation on microbial P cycling, and (3) the role of PAOs in field-based strategies for P recycling. We expand upon each of these example topics below and offer a number of potential research questions.

5.1 Legacy Soil Phosphorus

Legacy P loads limit the realized effectiveness of P water quality management strategies and lead to ongoing water quality issues in agricultural systems (*Section 1*). Future interdisciplinary research may link existing legacy soil P frameworks (e.g., Sharpley et al., 2013; Haygarth et al., 2014; Sharpley and Wang, 2014) with knowledge of soil microbial P pools (*Section 3.2*) and PAO metabolism (*Section 2*); including understudied or unrepresented soil microbes that accumulate P using polyP. Future legacy P research may also assess the water quality impacts of phosphate solubilizing microbes and whether these amendments utilize metabolic processes similar to EBPR PAOs. Future interdisciplinary research questions addressing legacy P may include:

- How does PAO community (or P cycling functional gene) diversity, structure, and function vary across a legacy P gradient?

- What role do PAOs play (if any) in mediating the cycling of legacy P in soils and P barriers (e.g., vegetated riparian buffers and woodchip bioreactors)?
- Under what circumstances do PAOs help/harm P management efforts? How do we cultivate PAOs in existing P management structures (e.g., vegetated riparian buffers or woodchip bioreactors) to 'catch' legacy P before it is transported to downstream water bodies?
- What is the relative importance of PAOs and abiotic P controls in mobilizing/immobilizing legacy P?
- Do phosphate solubilizing microbes in biological amendments utilize similar metabolic pathways and soil P pools as soil PAOs?
- How do phosphate solubilizing microbes interact with soil PAOs and what is the impact of both on water quality?

5.2 Impacts of Irrigation and Tile Drainage on Microbial P Cycling

Irrigation and tile drainage are implemented to moderate soil moisture and improve crop yield but may influence microbial P cycling in agricultural systems via perturbations in soil wetting/drying regimes (*Sections 1* and *3.2*). More specifically, future research may explicitly consider the role of PAOs and phosphate solubilizing bacteria in mediating the conversion of soil P pools to unintended DP loss (Jarvie et al., 2017) in irrigated and tile drained landscapes. Future interdisciplinary research questions may include:

- How does the frequency, duration, and magnitude of soil wetting/drying shape the diversity, structure, and function of PAO communities?

- What effect does the frequency, duration, and magnitude of soil wetting/drying have on microbial P pools, the role of PAOs, and P loads?
- What is the relative importance of PAOs and abiotic P controls in mobilizing/immobilizing P in irrigated and tile drained landscapes?
- How do abiotic and biotic P cycling mechanisms vary with the frequency, duration, and magnitude of precipitation events?
- How can we cultivate PAOs in existing management structures (e.g., vegetated riparian buffers or woodchip bioreactors) to ‘catch’ DP being transported through tile drains?

5.3 Phosphorus Recycling

Global mineral P stores are geographically limited (Jarvie et al., 2015) and many locations are beginning to consider P recycling programs in agricultural (Jarvie et al., 2015; Withers, Sylvester-Bradley, Jones, Healey, & Talboys, 2014) and urban (Metson & Bennett, 2015) settings. Future interdisciplinary research may link our existing understanding of legacy P and soil P_o (*Section 1*) with existing efforts in EBRP WWTPs using PAO metabolism(s) to recycle P (*Section 2*) and research in natural systems that emphasizes long-term microbial P storage in early Earth (e.g., Brown and Kornberg, 2004; Kipp and Stüeken, 2017). Future research questions may include those discussed previously with respect to legacy P as well as:

- How can we use biotic and abiotic P controls to simultaneously balance P recycling and P load reduction goals?
- Can we modify existing management structures to improve P recycling using PAOs?

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Table and Figure Captions

Table 1. A description of phosphorus (P) forms commonly used in the literature.

Table 2. A summary of polyphosphate accumulating organisms (PAOs) identified in engineered and natural systems. Abbreviations: laboratory-scale sequencing batch reactor (SBR), full-scale wastewater treatment plant (WWTP), uncharacterized wastewater (Mixed).

Table 3. Proteins associated with polyphosphate accumulating organism (PAO)-related P cycling functional genes.

Table 4. Descriptions, references, and examples of tools used to study microbially-mediated P cycling in engineered and natural systems. Abbreviations: polyphosphate accumulating organism (PAO), transmission electron microscopy (TEM), 4',6-diamidino-2-phenylindole (DAPI), fluorescence in-situ hybridization (FISH), quantitative FISH (qFISH), polymerase chain reaction (PCR), quantitative PCR (qPCR), next generation sequencing (NGS), internal transcribed spacer (ITS), nanometer-scale second ion mass spectrometry (nanoSIMS).

Table 5. A summary of polyphosphate accumulating organism (PAO) polymerase chain reaction (PCR) primer sequences.

Table 6. A summary of fluorescence *in-situ* hybridization (FISH) probes used to identify polyphosphate accumulating organism (PAO)-related organisms (updated from Seviour and Nielsen 2010). Abbreviations: not determined (ND).

Figure 1. (a) Anaerobic and (b) aerobic metabolism of model polyphosphate accumulating organism (PAO), *Candidatus* Accumulibacter phosphatis (CAP). The poly-β-hydroxyalkanoate (PHA) known as poly-β-hydroxybutyrate (PHB) is specific to CAP. Abbreviations: metal cations (M^+), phosphate (P_i). Adapted from Seviour et al. 2003, Seviour & Nielsen 2010, and Skennerton et al. 2014.

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