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INSIGHTS INTO MICROBIAL PHOSPHORUS CYCLING IN THE COLUMBIA RIVER ESTUARY

By

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CERTIFICATE OF APPROVAL

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ABBREVIATIONS

^{31}P-NMR	Phosphorus-31 nuclear magnetic resonance spectroscopy
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
DIP	Dissolved inorganic phosphorus
NH_4	Ammonium
Nitrate	Nitrate and nitrite
DIN	Dissolved inorganic nitrogen (nitrate and nitrite)
Total P	Total phosphorus
Total N	Total nitrogen
P_i	Inorganic phosphorus
P_o	Organic phosphorus
OrthoP	Orthophosphate
Monoesters	Orthophosphate monoesters
IHP	Inositol hexakisphosphate, Phytate
Diesters	Orthophosphate diesters
Poly	Polyphosphate
Pyro	Pyrophosphate
Phon	Phosphonates
PSU	Practical salinity units
CAP	*Candidatus* Accumulibacter phosphatis
16S rRNA	16 small subunit ribosomal ribonucleic acid gene
ppk1	Polyphosphate kinase 1 gene
PAOs	Polyphosphate-accumulating organisms
EBPR	Enhanced biological phosphorus removal
WWTP	Wastewater treatment plants
CRE	Columbia River estuary
BBB	Baker Bay boat launch
BBI	Baker Bay Ilwaco harbor
BBA	Baker Bay airport road
BBC	Baker Bay Chinook harbor
YBM	Youngs Bay mouth
YBB	Youngs Bay back
CBK	Cathlamet Bay knappa dock
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
LOI	Loss-on-ignition
BD	Bulk density
OM	organic matter
M3P	MehlichIII-P
B2P	Bray 2-P
ORP	Oxidation reduction potential

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ABSTRACT

Phosphorus (P) is a key nutrient required by all living organisms. In freshwater, P can be limiting as it readily adsorbs to particles; however, it is released via desorption in seawater. This makes estuaries—transition zones between land and sea—vulnerable to nutrient-driven eutrophication. However, through mineralization and biological transformations, estuaries can sequester P, rendering a valuable ecosystem service. Yet, processes involved in the retention and cycling of P in estuaries remain poorly understood. In this dissertation, I address critical knowledge gaps by characterizing P forms in sediments and by tracking changes in microbial populations involved in P storage in natural and restored wetland habitats in the Columbia River estuary.

P forms in sediments from intertidal wetlands and mudflats were characterized using solution ^{31}P nuclear magnetic resonance spectroscopy (P-NMR). Unlike many wetlands, total P was dominated by inorganic forms (mainly orthophosphate). Biologically derived inorganic polyphosphates, which constitute important storage molecules, were detected in peripheral bays, contributing as much as 10% of extracted P. In addition, several organic P forms were detected and quantified, including orthophosphate monoesters, orthosphosphate diesters, phosphonates, and inositol hexakisphosphate. The detection of various P forms in the Columbia River estuary suggests an active microbial community involved in P cycling and sequestration.

The polyphosphate-accumulating organism, *Candidatus* Accumulibacter phosphatis (CAP), discovered and described in wastewater treatment plants, was detected in emergent tidal marshes throughout the estuary at relative abundances (0.1–2.6%) similar to traditional wastewater treatment. The highest CAP abundances were found in Baker Bay in spring and summer; here, only Type I CAP was detected. In contrast, both Type I and II CAP subgroups

were present in the southern, oligohaline bays (Youngs and Cathlamet Bay). Based on *ppk1* gene copies, I estimated that CAP could account for as much as 0.09 to 18 mg kg^{-1} of sediment polyphosphate, in good agreement with the range determined by ^{31}P-NMR.

In wetlands varying by age since restoration (i.e., 0, 2, 7, 26 and 55 years after tidal reconnection) in Youngs Bay, the *ppk1* gene was detected 2 and 55 years post restoration, as well as at an undisturbed site. The largest changes in microbial community structure and sediment characteristics were observed between 7 and 26 years post-restoration, when abundances of Betaproteobacteria, which includes CAP, peaked and total sediment P and organic matter content decreased. Sediment bulk density decreased over the sequence, nearing reference levels between 7 and 26 years post-restoration. Finally, the labile P fraction (Bray-2), typically associated with active microbial P cycling, increased over the chronosequence, reaching reference levels by 26 years.

Despite the fact that the Columbia River estuary is rapidly flushed and water residence times are short, my work demonstrated active P cycling and transformations in wetland sediments. The biological sequestration and chemical retention of P are contributors to ecosystem services provided by the estuary; these services will likely be influenced by changing environmental conditions, including acidification, hypoxia, and rising temperatures. This dissertation provides important new information needed to predict how estuarine ecosystems could respond to climate change.

Chapter 1. GENERAL INTRODUCTION

The pollution of aquatic ecosystems with excess nutrients (nitrogen and phosphorus) is one of the world's most pressing environmental problems (Conley et al., 2009; Rockstrom et al., 2009; Howarth et al., 2011), putting earth systems at risk of significant perturbation (Steffen et al., 2015). For example, studies from the early 1970s demonstrated unequivocally through whole-lake manipulations that excess phosphorus (P) leads to eutrophication (Schindler, 1977). Eutrophication can lead to hypoxia, acidification, and harmful algal blooms (Paerl, 2006; Bricker et al., 2008), which result in loss of wetland habitats, benthic invertebrate and fish mass die-offs ultimately affecting human economics and health. In the last 20 years, nitrogen (N) and phosphorus (P) from agricultural runoff and from industrial and metropolitan discharges have been associated with the widespread eutrophication of estuaries and coastal oceans, including Chesapeake Bay, Long Island Sound, and the Gulf of Mexico (Millennium Ecosystem Assessment, 2005).

Ninety percent of the phosphate that is currently mined is used in fertilizers to support global food production. The greatest percentage of phosphorus mined in 2005 (17.5 million tonnes) was used in fertilizer (~14 million tonnes); yet, only 3 million tonnes made it into food, with the largest losses (~8 million tonnes) attributed to soil leaching and erosion (Cordell et al., 2009). While phosphorus in organic material is naturally recycled back to soil as orthophosphate through biogeochemical processes in unperturbed ecosystems, natural sedimentary P cycling has been overwhelmed in many systems due inputs of nutrients from agriculture and wastewater that lead to phosphorus accumulation (Sharpley et al., 2011), effectively creating a pathway from mines to the ocean (Ashley et al., 2011). This process ultimately leads to the eutrophication of rivers and coastal marine waters. At the same time, current production rates imply that global

phosphate reserves could be depleted in a century (Vaccari and Strigul, 2011). The extensive use of fertilizer to increase crop yield in agriculture combined with low efficiency of nutrient assimilation and retention is fundamentally disrupting global cycles of phosphorus and nitrogen (Elser and Bennett, 2011). As a result, there is much interest in elucidating factors that influence P sequestration within soils and sediments (Turner et al., 2006; Cade-Menun, 2015).

Phosphorus is the "backbone element" of biogeochemical ecosystems, as it is a required element for all life, resulting in a excessively large concentration in solid compared to solution forms (Sharpley et al., 2011). For example, it forms the phosphodiester bridges of DNA (deoxyribonucleic acid), makes up phospholipids in cell membranes, and transfers chemical energy through ATP (adenosine triphosphate). P species in aquatic ecosystems include: dissolved inorganic P (DIP), dissolved organic P (DOP), particulate organic P (POP), and particulate inorganic P (PIP), and these species are digested and quantified by colorimetric methods as orthophosphate. Among these, DIP is considered to be the most bioavailable. P species in sediments include organic P forms: phosphomonoesters ($R\text{-}OPO_3^{-2}$), phosphodiesters ($R_1O(RO)PO_2^-$), and phosphonates ($R\text{-}P(O)(OH)_2$). Large organic polyphosphates consist of both phosphomonoesters and polyphosphates (i.e. adenosine tripolyphosphate (ATP), and nicotinamide adenosine dinucleotide phosphate (NADH)). Inorganic species of P in sediments are most often associated with cations (e.g., Fe(II), Al(III) and Ca(II)), which form minerals such as apatite or ferric oxyhydroxides and include orthophosphate (PO_4^3), pyrophosphate, and polyphosphate (Cade-Menun, 2014). Although phosphorus is highly abundant as organic material in living organisms, it is paradoxically one of the least biologically available nutrients for plant and phytoplankton primary production (as orthophosphate) in most undisturbed ecosystems (Ashley et al., 2011).

1.1 Phosphorus cycling in estuaries

Estuaries are biologically productive transition zones between freshwater and marine systems that play a vital role in transforming, recycling, and sequestering nutrients and organic matter prior to entering the coastal ocean (Barbier et al., 2011). They are important areas of phosphorus accumulation, retention, and sequestration, which can occur via chemical, physical, or biological means (Reddy et al., 1999). For example, phosphorus is chemically "sticky" and in freshwater is most often bound to particles and sediments. However, in seawater, high ionic strength interferes with P adsorption, owing to increased competition for binding sites with other anions (Fox et al., 1986; House & Warwick, 1999; Monbet et al., 2010). This results in P efflux along salinity gradients, rendering estuaries particularly vulnerable to nutrient-driven eutrophication (House, 2003). The predominant abiotic mechanism for P removal in wetlands is by adsorption of P to sediments, including clay minerals, formation of insoluble precipitates (Ca, Mg) in alkaline soils, and oxyhydroxides (Fe, Al) in acidic soils, however seasonality in hydrology including flooding and drying result in changes in soil redox conditions, which play a governing role in transformation, storage and transport of P in wetlands (Stevenson, 1986; Reddy et al., 1999; Richardson & Reddy, 2013).

1.2 Microbial influences on P cycling

Microbes have the potential to play a dominant role in wetland P cycling. For example, microbes compete for uptake of P with comparable rates of removal by abiotic processes (Richardson et al., 1986; Kellogg et al., 2003; Noe et al., 2003), and by the general biomass of P present in microbes ranging from 25-50% of total P in wetland mineral soils (Wright et al., 2001; McDowell et al., 2003) and up to 70% of total P in hydrologically isolated soils (Williams et al., 2001). Polyphosphate (PolyP) has been demonstrated to be important in biogeochemical cycling

in the marine environment, for example diatoms can modify their physiology to make PolyP during limiting P conditions (Dyhrman et al., 2012) and it has been demonstrated that PolyP in marine diatoms plays a role in the formation of calcium phosphate minerals, effectively facilitating the sinking of P into marine sediments (Diaz et al., 2008). Excess P release from sediments associated with biogenically-formed PolyP was first observed in marine sediments in Effingham Inlet (British Columbia, Canada) and demonstrated that microbe-driven, redox-dependent production of PolyP accounted for 4 ±3% to 9 ±8% of the sediment flux of DIP in hypoxic environments within Effingham Inlet (Sannigrahi, 2005; Diaz et al., 2012).

1.3 Polyphosphate-accumulating organisms (PAO)

The sediment-water interface is a hotspot for microbial activity and potentially an important area for bacteria involved in phosphorus (P) exchange under oscillating oxygen conditions (Davelaar, 1993; Gächter & Meyer, 1993), however specific taxonomic groups capable of potentially playing an ecologically significant role in P cycling have not been identified (Hupfer et al., 2007). Polyphosphate metabolism is a mechanism allowing bacteria to survive and grow in areas of fluctuating redox conditions at the sediment-water interface (Davelaar, 1993). Davelaar (1993) proposed that PAO bacteria should be placed relative to availability of electron transfer/redox reactions rather than the anaerobic/aerobic interface, which was deemed as a vague criterion, inadequate to describe a real redox microhabitat. Figure 1.1 is adapted from Davelaar's hypothetical placement of PAO bacteria in a narrow window below true aerobic respiration and denitrification and the beginning of fermentative metabolism, where they would be situated effectively near both electron donors and acceptors. Placement of PAO bacteria within hypoxic zones denotes them as facultative anaerobes, utilizing Fe and/or Mn as final electron acceptors; Davelaar (1993) further proposed that in the presence of fully

oxygenated conditions, PAOs would not perform polyphosphate metabolism. The competitive edge for PAO bacteria therefore would be the ability to utilize Fe and/or Mn reduction rather than rely on limited oxygen availability in the narrow window afforded by redox reactions (Davelaar, 1993). Regardless of their location in the redox electron transport chain, the ability to store P as polyphosphate denotes them as fundamental components of P cycling.

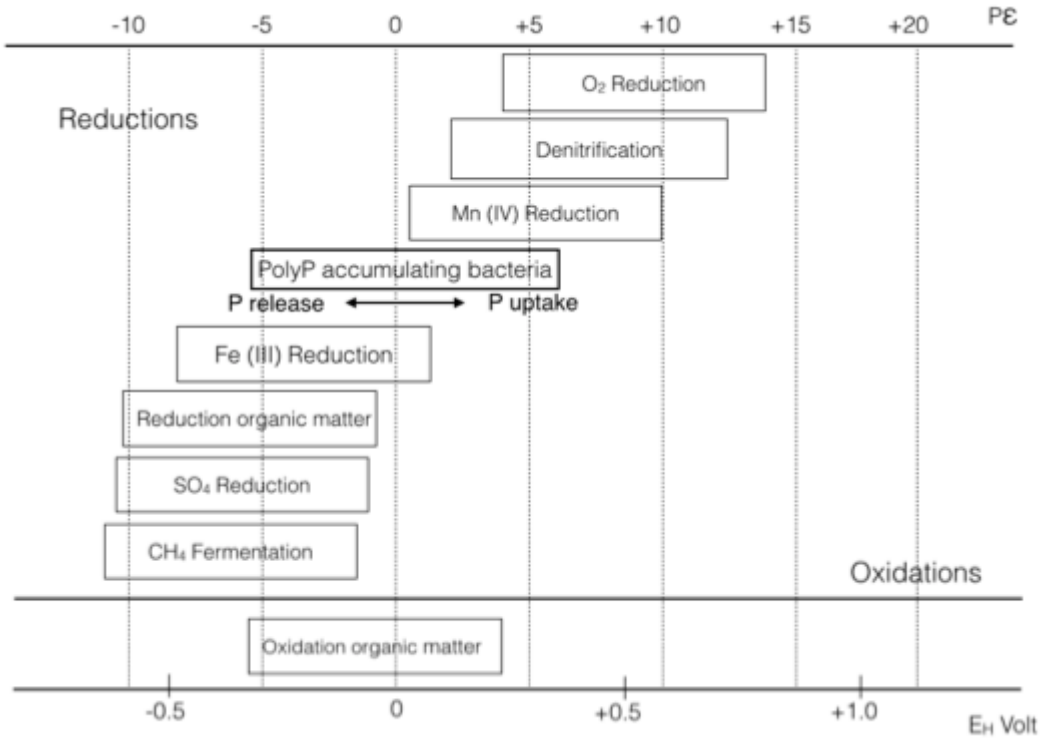

Figure 1.1 Hypothesized location of polyphosphate metabolism by polyphosphate-accumulating bacteria in the sequence of heterotrophic redox processes in aquatic sediments.

The discovery and identification of polyphosphate accumulating organisms (PAOs) in wastewater treatment supports the hypothesis that a bacterium could play an important role in biogeochemical cycling of P in a natural environment. The term PAO was used to identify organisms responsible for P removal in the Enhanced Biological Phosphorus Removal (EBPR)

process (Mino et al., 1998), and later using culture-independent methods identified *Candidatus* Accumulibacter phosphatis (CAP) a member of the Proteobacteria, as the most prominent organism responsible for successful EBPR activity (Bond et al., 1995; Hesselmann et al., 1999; Crocetti et al., 2000; Zilles et al., 2002). The foundational phenotype for EBPR operation depends upon alternating rounds of anaerobic (carbon rich, " feast") and aerobic (carbon poor, "famine") environments (Oehmen et al., 2007); under anaerobic conditions CAP takes up short-chain fatty acids (e.g. acetate, propionate) and stores them as polyhydroxyalkanoates (PHA) using energy generated from PolyP conversion to ATP (Zhou et al., 2008; Wexler et al., 2009; Oehmen et al., 2010). This is followed by aerobic PHA degradation, which readily supplies carbon and energy for growth and replenishment of PolyP stores (Mino et al., 1998).

1.4 Methods and technologies

The work presented in this dissertation required the use of advanced methods and technologies for the detection of phosphorus compounds present at low concentrations and rare microbes that carry out unique metabolisms, including ^{31}Phosphorus – nuclear magnetic resonance spectroscopy (P-NMR), and Next Generation Sequencing (NGS).

The high-energy molecule, polyphosphate is biologically synthesized across a wide-array of taxa; however, is highly dynamic in nature, being stored in some environments but rapidly degraded in others, depending upon conditions. P-NMR is one of the few methods that can be used to reliably observe and quantify polyphosphate in the natural environment.

The development of P-NMR spectroscopy for environmental analysis has provided more knowledge regarding phosphorus cycling in environmental soils than any other technique (Cade-Menun, 2015). Less than twenty published studies employed P-NMR analysis in wetland soils and only a handful of studies (<5) have characterized phosphorus in estuary wetland sediments.

Alkaline extraction followed by solution P-NMR spectroscopy is thought to be the only accurate method for quantifying contributions of organic P to total P in wetland soils (Turner et al., 2006) since colorimetric methods do not distinguish inorganic polyphosphates from organic matter. In wetlands, where biological processes often dominate, P-NMR was used to demonstrate that sediment contains a wide diversity of P forms representative of fundamental soil properties (i.e. organic matter, pH) (Cheesman et al., 2014). **Figure 1.2** summarizes the P forms that are distinguishable by P-NMR in a typical environmental soil. Table 1-1 summarizes the organic and inorganic forms identified by P-NMR and their degradability (labile vs. recalcitrant for organic P, and exchangeable and removed for inorganic P; Reddy and DeLaune, 2008).

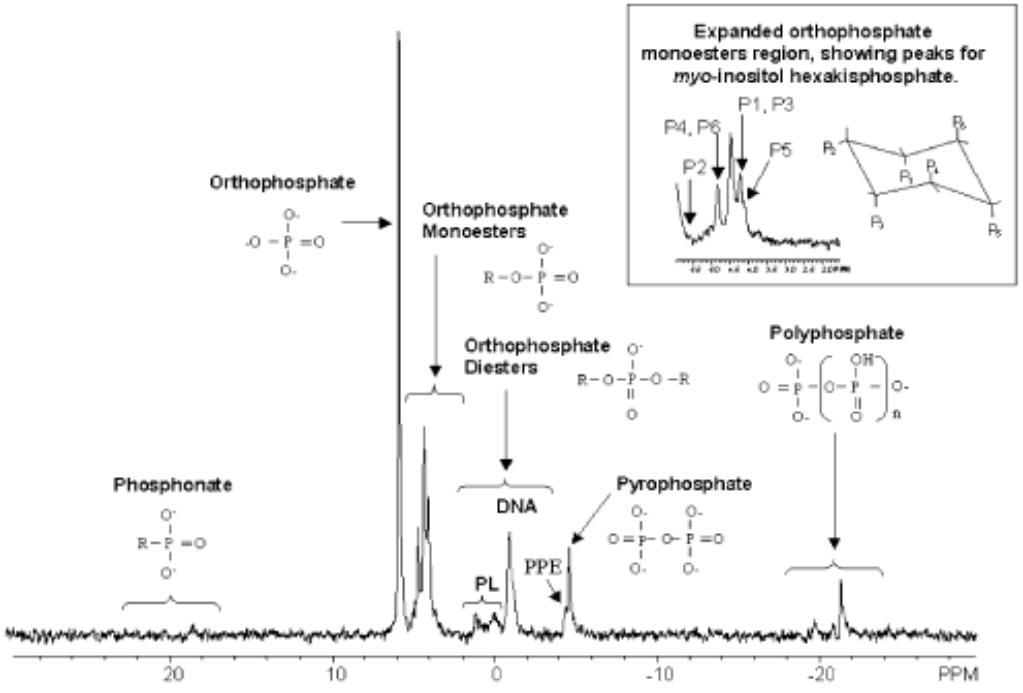

Figure 1.2 A solution ^{31}P-NMR spectrum of forest floor sediment extracted with NaOH-EDTA. This spectrum shows P form diversity typical of environmental samples including phosphonates, orthophosphate, orthophosphate monesters including DNA and phospholipids (PL), pyrophosphate and polyphosphate including the terminal P in the polyphosphate chain (PPE). The inset shows the expanded peaks and structure for myo-inositol hexakisphosphate (phytate or IHP; Cade-Menun, 2005). Elsevier and *Talanta*, 66, 2005, pg. 360, Characterizing phosphorus in

environmental and agricultural samples by ^{31}P nuclear magnetic resonance spectroscopy, Cade-Menun, B.J., Figure 1, included with permission from Elsevier (license number 4143830333113).

Table 1-1 P forms organized by organic and inorganic forms and categorized by how easily they are degraded in soils.

	Labile/Exchangeable	**Recalcitrant/Residual**
Organic	Diesters: RNA, phospholipids, sugar phosphates	Diesters: DNA Monoesters: IHP/phytate
Inorganic	P bound to salts: KCl, NaCl, NH_4Cl P bound to: Fe, Al	P bound to: Ca, Mg P bound: P minerals, crystallization

High-throughput sequencing techniques have greatly advanced knowledge about the phylogeny and diversity of traditionally non-cultivable microbiota across global environments (Caporaso et al., 2011). For example, quantifying the abundance and diversity of bacteria per gram of soil was once deemed unobtainable; however, utilization of new sequencing techniques and statistical analysis has allowed for greater accuracy with estimates of diversity, abundances of microbes, and when correlated to sediment characteristics can act as indicators of environmental land-use and perturbations (Roesch et al., 2007; Lauber et al., 2009). Use of the 16S rRNA gene for high-resolution (more and longer sequences), large-scale community (more samples) taxonomic identification has revolutionized microbial ecology and the diversity observed in varied environments (Tringe et al., 2008). The combination of high-throughput sequencing and traditional quantitative polymerase chain reactions (qPCR) allows for detection of low abundance bacteria (rare taxa) and characterizing their potential to influence biogeochemical cycling in an estuary ecosystem.

CAP is a non-cultivable bacterium using traditional microbiological methods; however, extensive studies on enrichment cultures within Enhanced Biological Phosphorus Removal (EBPR) systems have been performed using molecular methods (PCR, qPCR) and next generation sequencing (metagenomics, metatranscriptomics) to characterize population dynamics of CAP. Very little research has been done on non-enriched CAP populations in a natural environment, and molecular methods were vital to investigating its potential role in phosphorus cycling in a temperate estuary.

1.5 Study site: the Columbia River estuary

The Columbia River is the second largest by flow in the USA (Sherwood et al., 1990) and it culminates in a river-dominated, salt-wedge estuary with strong seasonality in salinity and biogeochemical properties (Chawla et al., 2008; Roegner et al., 2011). The upper extent and discharge of the Columbia River has been modified by the presence of eleven hydroelectric dams on the mainstem river and hundreds of irrigation and power dams on its tributaries (Simenstad et al., 1990), leading to the loss of shallow water habitats (Bottom et al., 2011). The Columbia River discharge (volume flux) varies with season, peaking at >10,000 m^3 s^{-1} during the snowmelt-driven spring freshet (April–June) and declining to < 2,000 m^3 s^{-1} in the dry season (July–October). In addition, more than 75% of tidal wetlands (~14,560 hectares) in the estuary have lost their hydrologic connection to the river as a result of activities aimed at supporting a diverse coastal economy including shipping, fishing, logging, tourism, and agriculture (Simenstad, 1990), although efforts in the last 10-15 years have sought to restore traditional wetlands in particular in Youngs Bay.

The estuary consists of two main channels (north and south) through which the majority of water exchange occurs, as well as four lateral bays (Baker, Youngs, Grays, and Cathlamet; **Figure**

1.3). Baker and Youngs Bays receive considerable deposits of fine sand, silts, and clays (Simenstad et al., 1990). Baker Bay on the northern side of the estuary is mesohaline, with a salinity range of 5–20 practical salinity units (PSU). In contrast, Youngs and Cathlamet Bays on the southern side of the estuary are oligohaline (0–5 PSU). The furthest upriver site, Cathlamet Bay, includes a diverse region of islands, tidal flats and marshes and is fresh (0 PSU) in surface waters. Salt intrusion into the estuary is greatest during periods of low discharge, which typically occur coincidentally with upwelling-favorable conditions between June and September (Chawla et al., 2008).

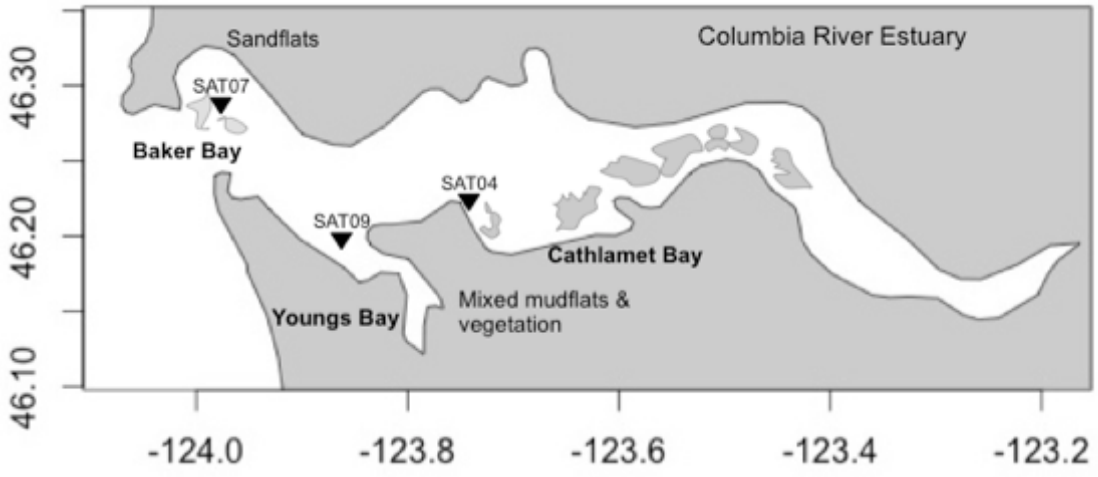

Figure 1.3 Map of lower Columbia River estuary including the three lateral bays Baker Bay in the north, and Youngs and Cathlamet Bay in the south. SATURN stations nearest lateral bays are indicated including SATURN-07 at Baker Bay, SATURN-09 at Youngs Bay, and SATURN-04 at Cathlamet Bay.

1.6 Dissertation overview

The impetus for this dissertation was previous work in the estuary that showed that water-column orthophosphate concentrations exhibited seasonal non-conservative increases along a salinity gradient, suggesting the presence of P sources that were not accounted for when orthophosphate was measured in the river and ocean end-members (Gilbert et al., 2013). It was

hypothesized that the lateral bays (Baker, Youngs and Cathlamet bays) might be a source of orthophosphate in late summer and early fall. In a metagenomic survey of estuary sediments (Smith et al., 2015) I detected sequences identified as CAP within sediments of the lateral bays (Chapter 3), and hypothesized that a PAO might be uniquely adapted to estuarine habitats and thus potentially involved in P cycling.

In order to investigate biogeochemistry cycling of P in any environment, it is imperative to identify the forms that are present, and P-NMR is one of the few techniques that can be used to identify and quantify all P forms in the environment. Chapter 2 provides a survey of P forms in the lateral bay wetlands or mudflats of the lower estuary. I chose a set of sediment samples collected in the spring to analyze for P forms using P-NMR and performed redundancy analysis to look for relationships among P forms, sediment, and water variables. Very few studies have used P-NMR to investigate P cycling in estuary wetlands and our work in Chapter 2 sets the stage for further investigation of the potential for microbial P cycling in a temperate estuary.

In Chapter 3, I identified CAP in the lateral bay sediments of the lower Columbia River estuary and characterized their abundances in space and time using qPCR targeting the single-copy polyphosphate kinase gene (*ppk1*); because there is only one copy in their genome, *ppk1* works well for estimating abundance of CAP. Although CAP is very well studied in the biotechnological EBPR process, there has been little work describing CAP in the natural environment.

Finally, in Chapter 4, I utilized targeted, high-throughput sequencing to evaluate changes in bacterial communities across a series of restored wetland sites in Youngs Bay within the lower Columbia River estuary. By targeting gene markers (i.e., CAP and other EBPR taxa in this case) in restored wetland sites, it is possible to identify changes in the capacity of the system for nutrient cycling.

1.7 Dissertation research objectives

The focus of this dissertation was the investigation of phosphorus cycling within a mesotrophic, river-dominated estuary, including the characterization of a polyphosphate-accumulating bacterium uniquely suited to environments such as estuaries, which are characterized by fluctuations in oxygen and redox state. In addition, I proposed the use of indicator taxa involved in nutrient cycling to investigate the potential for biogeochemical changes in restoration sites in an oligohaline temperate wetland.

The main objectives of my dissertation were (*i*) to characterize P forms in lateral bay sediments in the lower estuary, (*ii*) to identify and characterize CAP in estuary lateral bay sediments, and finally (*iii*) to propose a role for CAP in wetland nutrient cycling using sites undergoing restoration as a model. The research questions included:

1. What P forms are present in the peripheral wetlands and mudflats of the lower estuary?

   Hypothesis: Freshwater wetlands include a diversity of P forms, with the majority consisting of organic P. P forms and composition differ across gradients in salinity and organic matter content.

2. Can we identify and characterize spatial and seasonal abundances of CAP in sediments of the lower estuary? What environmental parameters influence CAP abundances in space and time?

   Hypothesis: CAP populations are associated with high-nutrient environments where redox conditions oscillate. Since oxygen concentrations are lowest in the summer and highest in the winter and spring, CAP will be more abundant under summer conditions when they are likely to outcompete other taxa.

3. As CAP is known for nutrient removal in wastewater treatment systems when oxygen availability oscillates, does a return to cycles of flooding —result in systematic changes in populations of phosphate accumulating organisms and their counterparts? Changes in populations of key taxa involved in nutrient cycling should indicate a shift in biogeochemical function associated with restoration.

    Hypothesis: CAP will be present in wetlands that have regular cycling of drying and wetting (i.e. oscillating redox conditions) as part of a microbiome involved in nutrient recycling. Changes in populations of certain taxa will accompany the return of nutrient cycling functions within the wetland.

Specific objectives for each chapter include:

Objectives for Chapter 2:

- Identify and quantify P forms in the lateral bay sediments of the lower estuary using solution ^{31}P-NMR.
- Relate environmental conditions within lateral bays to retention/sequestration of P forms using redundancy analysis.

Objectives for Chapter 3:

- Identify CAP in estuarine sediments using culture-independent molecular tools.
- Characterize the spatial and seasonal abundances of CAP bacteria at sites in lateral bays and correlate with seasonal environmental conditions.

Objectives for Chapter 4:

- Evaluate sediment development across restored sites to provide physicochemical context for microbial communities.
- Compare microbial communities across sites utilizing the EBPR core microbiome as a metric for nutrient removal functions.
- Survey for CAP bacteria as a potential indicator or assessment of the return of nutrient cycling functions within restored wetlands.

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Chapter 2. PHOSPHORUS FORMS IN SEDIMENTS OF THE COLUMBIA RIVER ESTUARY[1]

2.1 Introduction

Phosphorus (P) is an essential but often limiting nutrient in aquatic and terrestrial ecosystems (Daniel et al., 1998). Dwindling global mineable P deposits and losses from watersheds render upstream systems at risk of P limitation, while downstream systems are increasingly under threat from nutrient-stimulated eutrophication (Conley et al., 2009; Tiessen et al., 2011). Watershed losses result from inefficient recycling or poor sequestration and lead to P transport from soils to surface waters; ultimately, excess P ends up in estuaries and coastal systems (Ashley et al., 2011). Eutrophication can lead to hypoxia, acidification, and harmful algal blooms (Paerl, 2006; Bricker et al., 2007) and represents a growing worldwide threat to human health and well-being (Millenium Ecosystem Assessment, 2005).

Estuaries are biologically productive transition zones between freshwater and marine systems that play a vital role in transforming, recycling, and sequestering nutrients and organic matter (OM) prior to entering the coastal ocean (Barbier, et al., 2011). They often comprise a variety of habitats, including wetlands (transitions between land and water that are inundated either permanently or seasonally), mudflats, and seagrass beds. Tidal mixing of fresh and saltwater within estuaries leads to particle flocculation (Sholkovitz, 1976) and the localized resuspension of bottom material in turbidity maxima (Van Beusekom and Brockmann, 1998; Small and Prahl, 2004). Combined with downstream transport in rivers, estuaries are vulnerable to nutrient pollution because dissolved inorganic P (DIP) desorption from sediment surfaces

[1] ^{31}P-NMR analysis was done by B. Cade-Menun at the Saskatchewan Structural Sciences Centre at the University of Saskatchewan, with support funding from Agriculture & Agri-Food Canada. This chapter has been submitted for publication in the Journal of Environmental Quality.
[2] Sediment data was contributed by S. Kidd as part of her dissertation work at Portland State University (Kidd, 2017).

occurs when anions in seawater compete with phosphate anions for binding sites, leading to P efflux (Fox et al., 1986; Froelich, 1988; House and Warwick, 1999; Monbet et al., 2010). Moreover, the reducing conditions associated with intense OM remineralization increase P desorption, setting up a positive feedback loop whereby eutrophic conditions contribute to P mobilization and limit nutrient sequestration (Howarth et al., 2011; Li et al., 2013). This impairs the critical function of estuaries and wetlands, which is to reduce nutrient loads before they reach coastal waters (Turner et al., 2006).

Phosphorus retention, or sequestration, in wetlands can occur via chemical, physical, or biological means. For example, many P forms can be adsorbed to sediments (e.g., clay minerals) and/or form insoluble mineral precipitates with calcium (Ca) and/or magnesium (Mg) under alkaline conditions and (hydr)oxides with iron (Fe) and/or aluminum (Al) under acidic conditions (Stevenson, 1986; Reddy et al., 1999; Richardson and Reddy, 2013). Microbial activities will also influence P sequestration, including uptake of inorganic P and its conversion to organic P or polyphosphates, and the formation of Ca-phosphate minerals from diatom-derived polyphosphates (Diaz et al., 2008). Although P can be retained in organic (e.g., orthophosphate diesters) and inorganic (e.g., polyphosphate) forms (Cheesman et al., 2010, 2014), organic compounds are thought to account for large fractions of sequestered P (Reddy et al., 1999; Vaithiyanathan and Richardson, 1997). Within the organic fraction, the specific form influences its sequestration potential (Condron et al., 2005). For example, Turner et al. (2006) detected more labile orthophosphate diesters in cattail marsh sediment than in mineral soil, where organic P was present predominantly as inositol hexakisphosphates (IHP). Typically, IHPs have much longer turnover times than orthophosphate diesters due to increased sorption to minerals (Giles et al., 2011).

Since P retention depends on chemical form, characterizing the latter is critical for estimating P retention and sequestration potential and identifying important biogeochemical processes involved in P cycling (Celi and Barberis, 2005; Condron et al., 2005; Quiquampoix and Mousain, 2005). The goal of this study was to characterize P forms in sediments of the Columbia River estuary, a mesotidal, river-dominated system characterized by rapid flushing and short water residence times (~1-5 d; Chawla et al., 2008). Previous work in the estuary showed that water-column DIP exhibited seasonal non-conservative increases along a salinity gradient, suggesting the presence of P sources that were not accounted for when orthophosphate was measured in the river and ocean end-members (Gilbert et al., 2013). It is likely that P can be either released or sequestered in the peripheral wetlands and mudflats, depending on environmental conditions. To better understand the P cycle in these ecosystems we used solution ^{31}P-nuclear magnetic resonance spectroscopy (P-NMR) to determine P forms in sediment extracts collected from seven sites within the estuary's lateral bays in spring 2014 and summer 2014 and 2016.

2.2 Materials and Methods

2.2.1 *Field sites*

The Columbia River is the second largest by flow in the USA (Sherwood et al., 1990) and it culminates in a river-dominated, salt-wedge estuary with strong seasonality in salinity and biogeochemical properties (Roegner et al., 2011). The U.S. Pacific Northwest coast is strongly influenced by summer upwelling when northerly winds promote the offshore transport of surface waters, which are replaced by nutrient-rich subsurface oceanic water (Huyer, 1983; Grantham et al., 2004). The Columbia River discharge varies with season, peaking at >10,000 m^3 s^{-1} during the snowmelt-driven spring freshet (April–June) and declining to < 2,000 m^3 s^{-1} in the dry season

(July–October). Salt intrusion into the estuary is greatest during periods of low discharge, which typically occur coincidentally with upwelling-favorable conditions between June and September (Chawla et al. 2008).

The estuary consists of two main channels (north and south) through which the majority of water exchange occurs, as well as four lateral bays (Baker, Youngs, Grays, and Cathlamet; **Figure 2.1**). Baker and Youngs Bays receive considerable deposits of fine sand, silts, and clays (Simenstad et al., 1990). Baker Bay on the northern side of the estuary is mesohaline (i.e., brackish), with a salinity range of 5–20 practical salinity units (PSU). In contrast, Youngs and Cathlamet Bays on the southern side of the estuary are oligohaline (0–5 PSU). The furthest upriver site, Cathlamet Bay, includes a diverse region of islands, tidal flats and marshes and is fresh (0 PSU) in surface waters.

2.2.2 *Sample collection and chemical analysis*

Sediment samples were collected 1–2 h before low tide in spring 2014 (March-May) at sites within Baker Bay (three sites: BBB, BBI, BBC), Youngs Bay (two sites: YBB, YBM) and Cathlamet Bay (one site: CBK; **Figure 2.1**). Additional samples collected in August 2014 and 2016 from Baker Bay (BBA and BBB) were analyzed to provide preliminary data on seasonal and diurnal variation in P forms. The samples were obtained by coring sterile polypropylene Falcon tubes (15 and 50 mL) into the sediment to a depth of 0–10 cm. Following collection, the samples were stored in a cooler on dry ice for transport back to the laboratory (~2–3 h) where they were stored at -20°C pending analysis.

In situ sediment characteristics were determined to provide a snapshot of conditions at the time of collection. These included dissolved oxygen (DO), salinity, redox potential (oxidation-reduction potential, ORP), pH and temperature data using Extech soil (Extech, FLIR

Commercial Systems, Inc., NH) and Vernier probes (Vernier Software and Technology, Inc, OR).

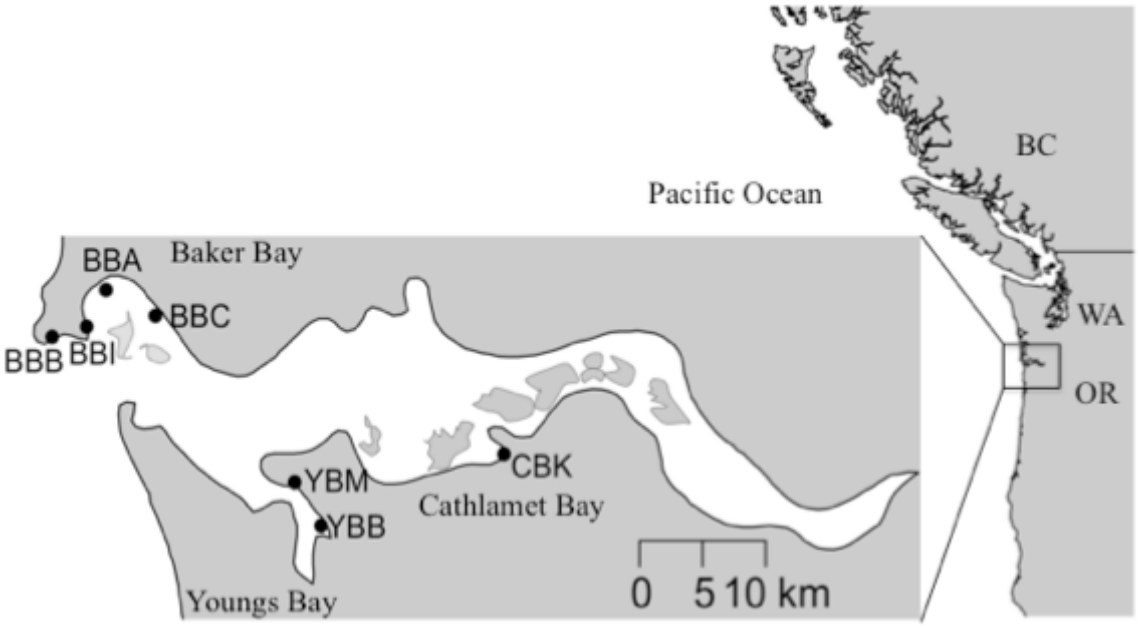

Figure 2.1 Map of the Columbia River estuary, sampling sites within tidal wetlands from three lateral bays: Baker Bay (BB), Cathlamet Bay (CB), and Youngs Bay (YB). BBB = Baker Bay, Boat Launch, BBI = Baker Bay, Ilwaco harbor, BBA = Baker Bay, airport road, BBC = Baker Bay, Chinook Harbor, YBM = Youngs Bay, Mouth, YBB = Youngs Bay, Back, and CBK = Cathlamet Bay, Knapp dock.

Water samples were collected just above the sediments and were kept on dry ice in a cooler during transportation to the laboratory where they were stored at -20^{o}C pending processing. Total nitrogen (N) and total P concentrations were determined on unfiltered water samples; the remaining water samples were filtered through combusted (4 h at 450°C) 0.7 μm, 25 mm GF/F filters (Whatman, NY) into 60 mL acid-cleaned HDPE bottles (Nalgene). A rapid flow analyzer (Astoria Analyzer, Astoria-Pacific) was used for colorimetric determination of dissolved nitrate, nitrite, ammonium, and molybdate-reactive P (hereafter described as DIP) concentrations (Antweiler et al., 1996) and for measurement of total N and P after alkaline persulfate digestion (Patton and Kryskalla, 2003). Sediment Total P concentrations were determined by ignition followed by

extraction with dilute H_2SO_4; P in extracts was determined colorimetrically (SmartChem 170, Unity Scientific, MA; Richardson and Reddy 2013) and total OM content was estimated by loss on ignition (LOI) at 500^{o}C (Nelson and Summers, 1996). Soil texture was analyzed using the hydrometer method (AgSource Laboratories, OR).

Physical and biogeochemical measurements of water column properties from in situ sensor platforms in the Columbia River were obtained from a publically available online database (www.stccmop.org) maintained by the Center for Coastal Margin Observation and Prediction (Baptista et.al., 2015).

2.2.3 *Extraction of phosphorus from sediments*

Using a modification of the Cade-Menun and Preston (1996) procedure, approximately 3.0 g of air-dried sediment was suspended in 30 mL of NaOH-EDTA (0.25 M NaOH, 50 mM Na_2EDTA) and sonicated (Branson Digital, Cleveland, OH) for 1 min at 20 kHz (~55 W) in an ice bath followed by shaking at 150 rpm for 4 h. Samples were centrifuged for 10 min at 1200 x *g* and the supernatant was removed, neutralized with 10% HCl and frozen at -80°C prior to lyophilization. An aliquot of each NaOH-EDTA extract was analyzed for Al, Ca, Fe, Mg, manganese (Mn), and P (mg L^{-1}) by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using a Plasma 400 emission spectrophotometer (PerkinElmer, Waltham, MA).

2.2.4 *Solution P-NMR*

Lyophilized extracts were redissolved in a solution combining 0.6–0.65 mL each of D_2O, H_2O, NaOH-EDTA solution, and 10 M NaOH, vortexed, centrifuged at 1500 x *g* for 20 min, and stored at 4°C within 12 h of analysis. If precipitate formed (observed in two samples from BBA, BBC), it was filtered out using 0.2-µm polycarbonate syringe filters prior to analysis. Spectra were collected on a Bruker Avance 500 MHz NMR spectrometer with a 10-mm broadband probe. The

NMR delay times were determined based on the ratio of concentrations of P/(Fe+Mn) within the extracts (McDowell et al., 2006). A temperature of 21°C and a 45° pulse were used, with a pulse delay of 4.5 s. Acquisition time was 0.5 s and 2800–5830 scans (~4–8 h) were collected. Samples were analyzed with no spinning and no proton decoupling.

2.2.5 *Data analysis and statistics*

The NMR spectra were processed using NMR Utility Transform Software (NUTS; Acorn NMR, Livermore, CA, 2000 edition). Processing included standardizing the orthophosphate peak for each sample at 6 ppm. Phosphorus forms were identified by their chemical shifts using published libraries for known peaks, with spiking for confirmation (Cade-Menun, 2015). Spectra were processed with 7–10 Hz line broadening for full spectra and 3 Hz line broadening to identify peaks in the orthophosphate monoester and diester regions (Cade-Menun and Liu, 2014). Corrections were made to account for orthophosphate diester degradation by summing the peak areas associated with degradation products (α- and β-glycerophosphate, mononucleotides, degraded from phospholipids and RNA respectively), subtracting this sum from the total monoester area, and adding it to the total diester area (Young et al., 2013, Schneider et al., 2016).

Phosphorus forms and environmental data were normalized with centered log ratio (clr) transformations (Abdi et al., 2015). Ordination of transformed data was performed using redundancy analysis (RDA) in order to relate variation in a set of response variables (here, clr-transformed P forms) to variation in a set of explanatory variables (here, log-transformed, scaled environmental characteristics). Statistical analyses were performed with R using the vegan package (R Core Team 2015). When two variables were found to be correlated, one was eliminated from each RDA.

2.3 Results

2.3.1 *Chemical analysis*

Physicochemical characteristics associated with each sample are shown in **Table 2-1**. The range in total sediment P concentrations was more than two-fold among sites in the spring (March–May), with the highest value observed at CBK (1366 mg kg^{-1}) and the lowest at BBC (601 mg kg^{-1}). The OM content mirrored total sediment P (11.3% at CBK, 2.2% at BBC). The lowest sediment P and OM concentrations were observed at the highest water temperature (~15°C; Table 1). Four of six sites where DO was measured had concentrations below the threshold for hypoxia (2 mL L^{-1}) at the time of collection; however, there was no relationship between DO and total P in sediments or the water column.

In Baker Bay, sediment and waters samples were collected both in spring and summer, making it possible to perform some limited seasonal comparisons of conditions that might influence P forms. Overall, there was similar spatial variability in characteristics of sediments and overlying waters in spring and summer (**Table 2-1**). In overlying waters, higher salinity (17–18 PSU vs. 4–6 PSU) and higher DIP concentrations were observed in the summer and total P concentrations were at the high end of those observed in spring. Although ammonium concentrations were higher in the summer than in the spring, nitrate (NO_3+NO_2) and total N concentrations did not differ. The two Baker Bay samples collected at dusk (BBBd) and sunrise (BBBs) were collected at low tide and had high ORP values, indicating oxidizing conditions in the sediments (107, 157; respectively). The pH values of sediments measured at dusk (8.8) were higher than those measured at sunrise (7.8).

The P recovery following sediment NaOH-EDTA extraction was 5.7–24.5% in spring samples and 1.5–17.4% in summer samples (**Table 2-1**). Greater P recovery was achieved at

Table 2-1 Physicochemical characteristics of surface water and sediments sampled from the estuary collected and analyzed for solution P-NMR in 2014.

	Site	Date (Time)	Water † DIP µM	TotP µM	NO_3+NO_2 µM	TotN µM	NH_4	Salinity PSU	Temp °C§	Sediment ‡ Total P mg kg^{-1}	LOI %	DO ml L^{-1}	pH	Clay+Silt %
SPRING	CBK	4/29/14 (1:10)	0.59	1.5	27.0	39.3	3.14	0	11.7	1366	11.3	3.8	7.4	13.4
	YBM	3/28/14 (18:55)	0.30	1.2	1.64	21.7	1.68	3	10.8	712	3.8	0.4	7.2	35.0
	YBB	3/28/14 (19:19)	0.94	5.1	36.4	80.7	4.10	1	10.8	1221	9.6	0.7	6.5	-
	BBC	5/29/14 (10:15)	0.98	2.9	1.20	29.8	3.25	6	15.1	601	2.2	7.5	7.5	11.8
	BBI	4/29/14 (7:30)	0.85	1.9	5.64	30.2	4.82	4	13.8	630	2.5	1.5	7.5	15.8
	BBB	3/28/14 (7:04)	0.54	13.7	24.7	114.7	2.61	4	9.4	853	6.5	0.7	7.2	42.4
SUMMER	BBA	8/25/14 (7:50)	4.79	10.5	0.10	56.9	16.0	18	17.9	752	1.2	6.8	7.7	13.4
	BBB dusk	8/30/16 (19:00)	1.20	0.9	6.64	17.4	3.87	18	17.4	1315	7.0	(107)	8.8	-
	BBB sunrise	8/31/16 (7:00)	1.04	1.3	7.30	17.8	5.56	15	18.1	1126	6.4	(157)	7.8	

†Water, in the water column above the sediments; DIP, dissolved inorganic phosphorus (P); TotP, total P; DIN, NO_3^-+NO_2^-; TotN, total dissolved nitrate/nitrite; NH_4^+, ammonium; PSU, practical salinity units; and Temp, water temperature collected by SATURN sensors.

‡ Total P (sediment) was analyzed by ignition followed by colorimetric analysis, and organic matter was calculated as loss-on-ignition (LOI); DO, dissolved oxygen and pH measurements were collected by in situ probes at one time point (Jan, 2014), BBBd and BBBs measurements were made when sediments were collected; ORP, oxidation reduction potential values; Clay and Silt were analyzed for sediments collected in August 2013.

§Temperature of sediment was measured at sample sites with Extech soil probes (BBB dawn and BBB ebb) and for water from local

Table 2-2 Concentrations of P and selected cations in alkaline sediment extracts from estuary lateral bay sediment samples.

Season	Location	Sample	Date (Time)	NaOH-EDTA extracts P	Fe	Mn	Al	Mg	Ca	P Recovery†
				----------mg kg^{-1}----------						%
Spring	Cathlamet Bay	CBK	4/29/14 (1:10)	111.0	34.1	60.3	77.0	24.5	1553.5	8.1
	Youngs Bay	YBM	3/28/14 (18:55)	47.7	1.69	0.56	13.4	36.3	193.6	6.7
		YBB	3/28/14 (19:19)	298.8	73.7	34.0	205.1	16.1	1077.7	24.5
	Baker Bay	BBC	5/29/14 (10:15)	33.9	0.45	0.34	16.7	10.6	109.7	5.7
		BBI	4/29/14 (7:30)	100.1	3.29	1.39	49.6	87.6	575.5	15.9
		BBB	3/29/14 (7:04)	204.3	12.0	6.01	58.5	73.3	1044.1	23.9
		BBA	8/25/14 (7:50)	11.1	1.50	0.19	16.5	12.9	79.4	1.5
Summer		BBB dusk	8/30/16 (19:00)	195.7	1.1	1.9	25.0	50.4	714.9	17.4
		BBB sunrise	8/31/16 (7:00)	173.5	1.9	3.8	26.5	51.2	2077.4	13.2

† P Recovery: Percentage of total sediment P extracted by NaOH-EDTA

sites with higher OM and total sediment P (e.g., YBB) compared to those with lower concentrations of OM and total P (e.g., BBC; Table 2-1). The samples with low OM also had the lowest Fe and Mn concentrations in the extracts (e.g., 0.45 and 0.34 mg kg^{-1}, respectively at BBC; Table 2-2), and therefore may have required longer delay times to be fully quantitative for extracted P (McDowell et al., 2006). Of the cations extracted, Ca was most abundant, ranging from 110–2077 mg kg^{-1} across samples (Table 2-2), while Al concentrations ranged from 13.4 mg kg^{-1} (YBM) to 205 mg kg^{-1} (YBB).

2.3.2 *Phosphorus forms and their contributions to sediment P*

Figure 2.2 shows example P-NMR spectra, which demonstrate peaks associated with P forms detected in estuarine sediments of the Columbia River; the remaining spectra are included as supplemental figures at the end of the chapter (Figure 2.5 and Figure 2.6). Chemical shifts associated with the peaks are summarized in Table 2-4and the percentages of total P accounted for by identified P forms are in Table 2-6.

Total extracted P was dominated by inorganic forms (P_i; Figure 2.3), which exceeded contributions from organic P (P_o) by 2–6 times. Orthophosphate, which varied five-fold across sites (34.7–172.4 mg kg^{-1} at YBM and BBB, respectively), accounted for 70.7–84.4% of total P [$S(P_i + P_o)$] and 86–99% of P_i (). Both pyrophosphate and polyphosphate were detected at all sites except BBA in the summer (for which the P concentration was low). For most samples there was little variation in concentration (Figure 2.2), with pyrophosphate and polyphosphate accounting for as much as 5.5 and 10.0% of P_i, and up to 4.7 and 5.8% of total P, respectively (Table 2-5).

Table 2-3 Phosphorus forms as percent of total P by solution P-NMR spectroscopy.

Season	Location	Sample	Date (Time)	**Inorganic†**				**Organic‡**			
				----------% of Total P----------							
				OrthoP[¥]	Pyro	Poly	**Total Pi**	cMono	cDiester	Phon	**Total Po**
	Cathlamet Bay	CBK	4/29/14 (1:10)	76.9	0.4	0.3	77.6	13.3	8.4	0.7	22.4
	Youngs Bay	YBM	3/28/14 (18:55)	72.7	4.3	2.8	79.8	10.6	8.9	0.7	20.2
		YBB	3/28/14 (19:19)	77.4	1.0	1.0	79.4	10.3	9.6	0.7	20.6
Spring		BBC	5/29/14 (10:15)	70.7	4.5	6.1	81.3	11.0	6.3	1.4	18.7
		BBI	4/29/14 (7:30)	69.5	1.2	5.4	76.1	11.6	11.6	0.7	23.9
		BBB	3/28/14 (7:04)	84.4	1.1	1.0	86.5	5.4	7.4	0.7	13.5
	Baker Bay	BBA	8/25/14 (7:50)	72.6	0.0	0.8	73.4	22.6	3.2	0.8	26.6
Summer		BBB dusk	8/30/16 (19:00)	78.0	1.2	1.8	81.0	11.7	6.6	0.7	19.0
		BBB sunrise	8/31/16 (7:00)	77.7	0.6	0.9	79.2	10.9	9.2	0.7	20.8

†OrthoP, orthophosphate; Pyro, pyrophosphate; Poly, polyphosphate; Total Pi, sum of all inorganic P forms.
‡MonoP, sum of orthophosphate monesters; Diester P, sum of orthophosphate diesters; PhosP, phosphonates; Total Po, sum of all organic P forms.

The largest total polyphosphate (pyrophosphate + polyphosphate) concentrations were observed in Baker Bay in the north (6.2 mg kg^{-1}, BBI), while the lowest were observed in Cathlamet Bay to the south (CBK, 0.8 mg kg^{-1}). Concentrations of P_o ranged from 9.6 to 61.6 mg kg^{-1}, accounting for 13.5–22.4% of total extracted P in sediments in the spring (). Orthophosphate monoesters (SMono) made the largest contribution to P_o; SMono accounted for 50–85% of P_o and 5.4–13.3% of extracted P, with larger contributions to P_o in the summer (66.3 ± 16.8%) than in the spring (54 ± 4.5%; Table 2-5).

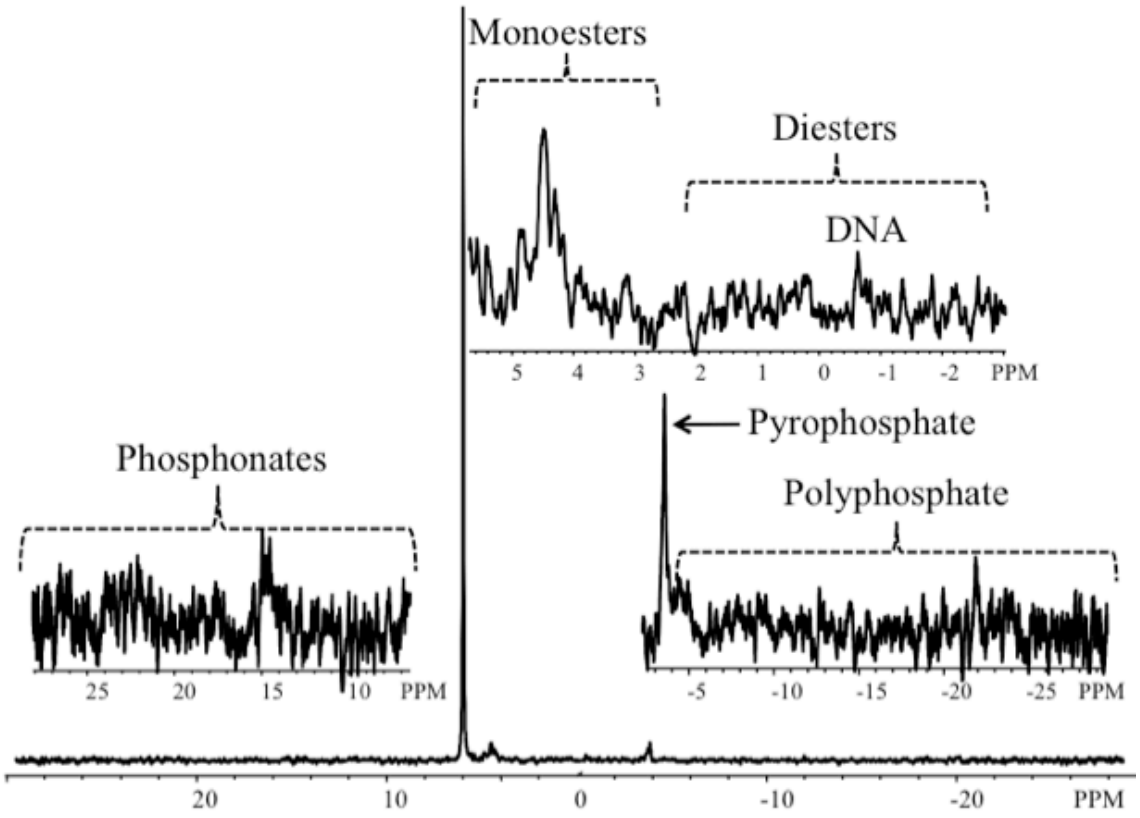

Figure 2.2 Representative P-NMR spectrum from a sediment sample collected in Youngs Bay (YBM). The full spectrum, scaled to the orthophosphate peak at 7 Hz line-broadening, is shown at the bottom; the upper right inset shows the orthophosphate monoester and orthophosphate diester regions, including a peak corresponding to DNA, the bottom right inset shows the pyrophosphate and polyphosphate region, and the bottom left inset shows the phosphonate region.

Table 2-4 Chemical shifts of peaks detected in ^{31}P-NMR spectra.

Category	P Form or Compound Class	Chemical Shift (ppm)
Inorganic P		
	Orthophosphate	6.00 ± 0.00
	Pyrophosphate	-4.14 ± 0.06
	Polyphosphates	-3.94 ± 0.08, -4.61 ± 0.20, -5.56 ± 0.22, -6.85 ± 0.38, -8.56 ± 0.38, -9.55 ± 0.38, -10.84 ± 0.43, -12.90 ± 0.78, -15.10 ± 0.54, -17.81 ± 0.20, -18.92 ± 0.36, -20.78 ± 0.49, -21.79 ± 0.43, -23.49 ± 0.88, -25.23 ± 0.34, -26.69 ± 0.37
Organic P		
	Phosphonates	26.66 ± 0.63, 22.86 ± 0.52, 21.24 ± 0.74, 17.17 ± 0.57, 16.05 ± 0.90, 14.69 ± 0.16, 13.15 ± 0.29, 11.16 ± 0.88, 9.33 ± 0.33
Orthophosphate Monoesters		
Phytate	*myo*-IHP	5.55 ± 0.04, 4.57 ± 0.04, 4.21 ± 0.04, 4.15 ± 0.03
	scyllo-IHP	3.75 ± 0.03
	neo-IHP	6.44 ± 0.03, 4.33 ± 0.06
	D-*chiro*-IHP 4e/2a	6.66 ± 0.08, 5.37 ± 0.02, 4.04 ± 0.06
	D-*chiro*-IHP 4a/2e	6.25 ± 0.04, 4.76 ± 0.05, 4.35 ± 0.04
Degradable	Glucose 6-phosphate	5.26 ± 0.02
	α-glycerophosphate	4.86 ± 0.03
	ß-glycerophosphate	4.53 ± 0.03
	Mononucleotides	4.47 ± 0.01, 4.43 ± 0.01, 4.37 ± 0.04, 4.28 ± 0.02
	Choline phosphate	3.91 ± 0.04
	Monoester 1	6.89 ± 0.04, 6.77 ± 0.05, 6.59 ± 0.05, 6.34 ± 0.04, 6.17 ± 0.04
	Monoester 2	5.83 ± 0.03, 5.74 ± 0.01, 5.67 ± 0.01, 5.12 ± 0.05, 5.01 ± 0.06, 4.67 ± 0.03
	Monoester 3	3.62 ± 0.08, 3.39 ± 0.06, 3.24 ± 0.06, 2.97 ± 0.11, 2.67 ± 0.07
Orthophosphate Diesters		
	Other Diester 1	2.42 ± 0.06, 1.89 ± 0.12, 1.44 ± 0.05, 1.23 ± 0.03, 1.05 ± 0.09, 0.72 ± 0.04, 0.55 ± 0.04, 0.23 ± 0.08, -0.38 ± 0.09
	DNA	-0.70 ± 0.10, -0.91 ± 0.07
	Other Diester 2	-1.24 ± 0.10, -1.90 ± 0.08, -2.23 ± 0.10, -2.83 ± 0.18

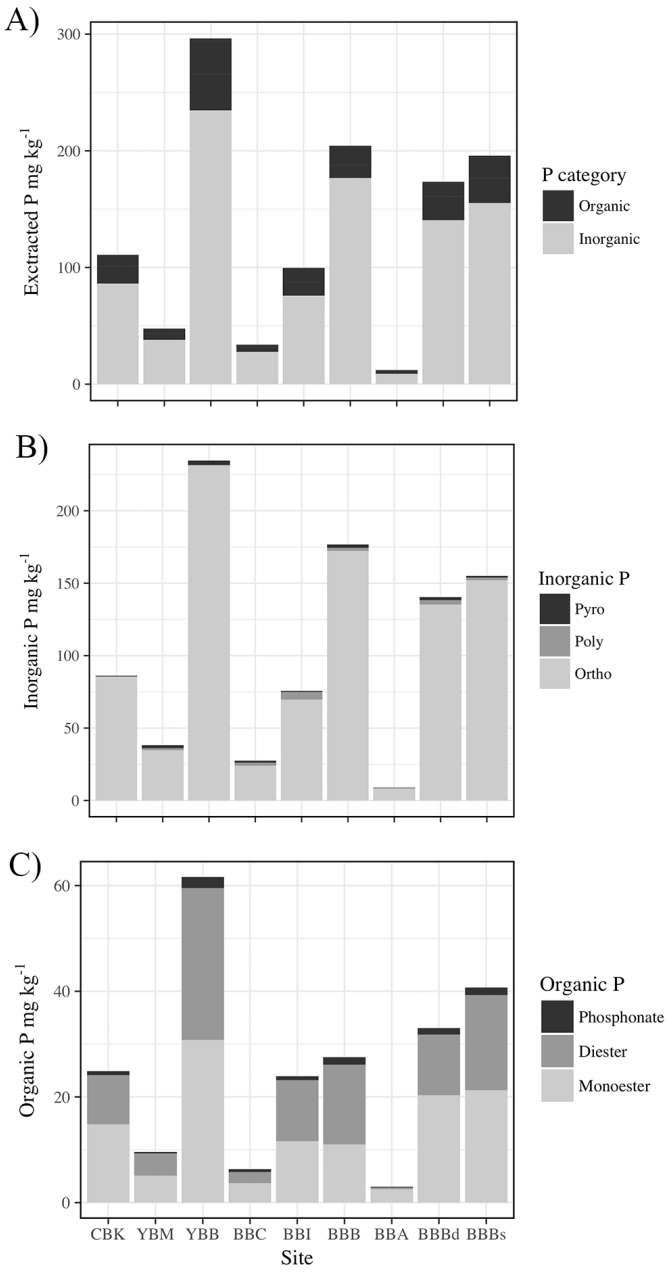

Figure 2.3 P forms in sediment extracts from sites in the Columbia River estuary collected in the spring (CBK, YBM, YBB, BBC, BBI, BBB) and summer (BBA, BBBd, and BBBs). BBBd and BBBs were both collected at Baker Bay boat launch; BBBd and BBBs were collected at dusk and sunrise, respectively. (A) Total organic and total inorganic P; (B) inorganic phosphorus forms (Pyro = pyrophosphate; Poly = polyphosphate; Ortho = orthophosphate); and C) organic phosphorus forms (phosphonate; Diester = orthophosphate diester; Monoester = orthophosphate monoester).

Identified monoesters comprised stereoisomers of inositol hexakisphosphate (IHP), including *chiro, myo, neo, scyllo*-IHP, as well as α- and β-glycerophosphate and mononucleotides. The IHP stereoisomers were broadly detected in estuarine sediments at concentrations that varied considerably (1.4–54.1 mg kg^{-1}) over small distances, indicating high local heterogeneity (**Table 2-5**). For example, the lowest and highest concentrations were observed within Baker Bay (BBB and BBI, respectively). Stereoisomers of IHP (*chiro, myo, neo, scyllo*) contributed approximately equally to IHP in the spring with the exception of BBB, where contributions from *myo*-IHP were small (**Table 2-5**). The data are consistent with a downstream increase in IHP, with values at Youngs (YBB) exceeding those at Cathlamet Bay, which were more than three-fold lower than at BBI (**Table 2-5**).

Orthophosphate diesters (Diester) made up 6.3–11.6% of extracted P, contributing 2.1–28.7 mg kg^{-1} of P in the spring (Table 2-4; Figure 2.3). The highest concentration of total orthophosphate diesters was observed at YBB (28.7 mg kg^{-1}). DNA was detected throughout the estuary, contributing as little as 0.2 mg kg^{-1} of P (BBC) and as much as 4.5 mg kg^{-1} of P (YBB), which also had the highest concentration of diester degradation products (α-and β-glycerophosphate and mononucleotides; Table 2-5). Though present at low concentrations, phosphonates were detected throughout estuarine sediments (0.7–1.4% P), ranging from 0.33–2.1 mg kg^{-1} of total extracted sediment P (Table 2-5; Figure 2.3).

2.3.3 *Diel differences in environmental conditions and P forms*

Although the ratio of pyrophosphate to polyphosphate was similar in dusk and dawn samples at BBB in Baker Bay (Table 2-5), total polyphosphate concentrations were higher at dusk (5.2 mg kg^{-1}) than at dawn (2.9 mg kg^{-1}). The sunrise (dawn) sample had more total orthophosphate diesters, *myo*-IHP, and degradable P forms than the dusk sample but less DNA.

With only two samples, however, it is difficult to confirm whether differences were due to spatial heterogeneity, tidal influences, or biogeochemical processes.

2.3.4 *Redundancy analysis*

Redundancy analysis (RDA) was performed to identify relationships among P forms and environmental characteristics within sediments and overlying waters at each of the sample sites (Fig. 4). The first biplot (Figure 2.4) displays relationships among sites and sediment characteristics (TotalP, LOI, DO, Fe, Mn, Ca, and Al). Variation among sites was explained by Ca (RDA1) and LOI (RDA2) concentrations, with one summer Baker Bay site (BBBs) separating from the others due to an association with a high extracted Ca concentration and a high pH and another (BBB) separating due to an association with low dissolved oxygen (Figure 2.4). The RDA analysis also revealed that Baker Bay (BBC, BBBd) sites differed along a gradient of extracted Mn concentration, differences among Youngs Bay sites were driven by differences in extracted Fe content, and Cathlamet Bay sites differed in extracted Al. When sites and P forms were analyzed by RDA, most sites clustered along a gradient of polyphosphate and diesters (not shown).

Table 2-5 Phosphorus forms as percent of total P by solution P-NMR spectroscopy.

Season	Location	Sample	Date (Time)	**Inorganic†**				**Organic‡**			
				----------% of Total P----------							
				OrthoP[¥]	Pyro	Poly	**Total Pi**	cMono	cDiester	Phon	**Total Po**
	Cathlamet Bay	CBK	4/29/14 (1:10)	76.9	0.4	0.3	77.6	13.3	8.4	0.7	22.4
	Youngs Bay	YBM	3/28/14 (18:55)	72.7	4.3	2.8	79.8	10.6	8.9	0.7	20.2
		YBB	3/28/14 (19:19)	77.4	1.0	1.0	79.4	10.3	9.6	0.7	20.6
Spring		BBC	5/29/14 (10:15)	70.7	4.5	6.1	81.3	11.0	6.3	1.4	18.7
		BBI	4/29/14 (7:30)	69.5	1.2	5.4	76.1	11.6	11.6	0.7	23.9
	Baker Bay	BBB	3/28/14 (7:04)	84.4	1.1	1.0	86.5	5.4	7.4	0.7	13.5
		BBA	8/25/14 (7:50)	72.6	0.0	0.8	73.4	22.6	3.2	0.8	26.6
Summer		BBB dusk	8/30/16 (19:00)	78.0	1.2	1.8	81.0	11.7	6.6	0.7	19.0
		BBB sunrise	8/31/16 (7:00)	77.7	0.6	0.9	79.2	10.9	9.2	0.7	20.8

†OrthoP, orthophosphate; Pyro, pyrophosphate; Poly, polyphosphate; Total Pi, sum of all inorganic P forms.
‡MonoP, sum of orthophosphate monesters; Diester P, sum of orthophosphate diesters; PhosP, phosphonates; Total Po, sum of all organic P forms.

Table 2-6 Comparisons of phosphorus form contributions across the estuary lateral bay sample sites.

Season	Location	Samples	Inorg: Org†	Mono: Di	cMono	cDi	Total IHP	cM:D	Myo:other IHP	Deg	DNA	Total Poly	Pyro: Poly
					----------mg kg^{-1}--------					----------mg kg^{-1}----------			
Spring	Cathlamet Bay	CBK	3.5	5.2	14.8	9.3	7.8	1.6	0.7	5.4	1.7	0.8	1.3
	Youngs Bay	YBM	4.0	3.5	5.1	4.2	2.5	1.2	0.6	2.2	0.5	3.4	1.5
		YBB	3.8	3.2	30.8	28.7	16.4	1.0	1.0	14.3	4.5	3.3	10.0
		BBC	2.1	9.7	3.7	2.1	2.3	1.8	0.4	0.9	0.2	3.6	0.7
		BBI	3.2	2.1	11.6	11.6	54.1	1.0	0.5	4.2	4.0	6.2	0.15
		BBB	6.4	2.8	11.0	15.1	1.4	0.7	0	8.2	2.3	4.3	1.1
Summer	Baker Bay	BBA	3.0	31.3	2.5	0.4	0	7.1	0	0.3	0.02	0.9	0
		BBB dusk	4.3	7.3	20.3	11.5	11.5	1.8	0.3	7.6	0.5	5.2	0.7
		BBB sunrise	3.8	12.4	21.3	18.0	9.8	1.2	0.8	15.1	0.0	2.9	0.7

† Inorg:Org, ratio of inorganic to organic P; Mono:Di, ratio of Orthophosphate monoesters to diesters without correction for degradation; cMono, cDi, total orthophosphate monoester and diesters corrected for degradation; Total IHP, sum of *myo*-, *scyllo*-, *neo*- and D-*chiro*-inositol hexakisphosphate ; *Myo*:other IHP, ratio of sum of *myo*-inositol hexakisphosphate to all sterioisomers (*scyllo-, neo-,* D-*chiro-inositol hexakisphosphate*); Deg, degradation compounds include α-glycerophosphate, β-glycerophosphate, and mononucleotides; DNA, sum of identified DNA peaks; Total Poly, sum of pyrophosphate and polyphosphate; Pyro:Poly, ratio of pyrophosphate to polyphosphate.

A second biplot (Figure 2.4) demonstrated relationships among P forms and characteristics of extracted sediments (Total P, DO, LOI, pH, Fe, Al, Mn, Ca). Eigenvector values associated with RDA1 suggested that variance was best explained by DNA (among I forms) and extracted Ca (among environmental variables). The eigenvector values associate with RDA2 indicated that variance in the data set was best explained by total IHP (among P forms) and LOI (among environmental variables; Figure 2.4). Total IHP, orthophosphate, a phosphonates were highest when dissolved oxygen was low. Differences in water column characteristics (TP, nitrate, DIP, and temperature) did not explain patterns in P forms (total polyphosphates, IHP, total monoesters, total diesters, orthophosphate, DNA and phosphona since most of the P forms clustered together and were not associated with characteristics of overlying waters (data not shown).

2.4 Discussion

Despite relatively low recovery of P in NaOH-EDTA extracts, we detected a range o forms in estuarine sediments. The recovery rates reported here for NaOH-EDTA extracted I 24%) are comparable to other P-NMR studies in wetlands with similar sediment characteris For example, P recovery was 6–16% in calcareous soils and 16-30% in sediments with low and neutral pH (Cheesman, et al., 2014). In contrast, higher recoveries (25–84%) have been reported in wetland sediments that are acidic (pH 3.6–4.8) or that have high OM (56–94%; Turner and Newman, 2005; Cheesman et al., 2014). It is likely that the residual P that was n extracted in our protocol was inorganic, alkali-stable, and associated with Ca or Mg minera since these forms are not readily extracted using NaOH-EDTA (Turner and Newman, 2005 Defforey et al., 2017).

In the Columbia River estuary, sediment extracts were dominated by inorganic phosphorus forms, mainly as orthophosphate. The dominance of inorganic P is somewhat unusual for wetlands, which typically have high OM and increased biological activity (Richardson and Reddy 2013). However, the short water residence times in the Columbia (~1-5 d; Neal, 1972; Chawla et al., 2008; Kärnä et al. 2016) likely limit OM accumulation; together with seawater, this influences sediment redox state and pH. In addition, tidal exchange (in both brackish and freshwater environments) limits the extent of reducing conditions due to regular cycles of wetting and drying, which influences the preservation of organic P forms (Turner, et al., 2005; Cheesman et al., 2014).

Polyphosphates and pyrophosphates were detected in all estuarine sediment samples, where they contributed as much as 10% of inorganic P (6% of extracted P). In almost half the samples, polyphosphate concentrations exceeded pyrophosphate, in contrast to other studies that reported higher pyrophosphate concentrations relative to polyphosphate (Turner and Newman, 2005; Zhang et al., 2009; Cheesman et al., 2010). We included a neutralization step prior to lyophilization, which increases the stability of polyphosphate (Cade-Menun et al., 2006), unlike the previous work cited. Some P forms are susceptible to degradation during sample preparation (extraction) and degrade to orthophosphate. For example, if polyphosphate alone was lyophilized in NaOH-EDTA, peaks were detected at 6.0 (orthophosphate, 44%), -4.0 (polyphosphate end, 44%), and -17.9 ppm (polyphosphate long-chain end, 12%) indicating almost half of the polyphosphate was degraded to orthophosphate (Cade-Menun et al., 2006), neutralizing the extractant prior to lyophilization reduced the degradation of orthophosphate to 2%.

Degradation of polyphosphate to pyrophosphate (either in situ or during sediment extraction) may also account for the fact that only a handful of studies report detection of

polyphosphate in wetlands (Zhang et al., 2009; Cheesman et al., 2014). Polyphosphate is biologically produced across the domains of life, including by bacteria, fungi, protozoa, plants, and mammals (Kornberg, 1995; Brown and Kornberg, 2004; Kulaev et al., 2004) and is thought to be indicative of microbial activity rather than linked to particular soil or sediment types (Cheesman et al., 2014).

Interestingly, the largest contribution to sediment P by polyphosphate in the Columbia was in brackish sediments in Baker Bay where OM was <10% and total sediment P was relatively low. The detection of polyphosphate in the Columbia suggests a greater potential for microbial P sequestration and cycling in low nutrient, low organic content sediments than has generally been assumed. Further investigation of this is warranted.

Although extracted P was dominated by inorganic forms, organic P accounted for ~30% of total P and was comprised of P forms that are considered both labile (e.g., RNA, phospholipids) and stable (e.g., IHP), despite rapid flushing and a low contribution of organic P compared to other freshwater wetland (Turner and Newman, 2005; Turner et al., 2006; Cheesman et al., 2010; Chessman et al., 2014) or lake sediments (Carman et al., 2000). The dominant organic P form in these estuarine sediments was the monoester, *myo*-inositol hexakisphosphate (*myo*-IHP), which was detected in all but one sample.

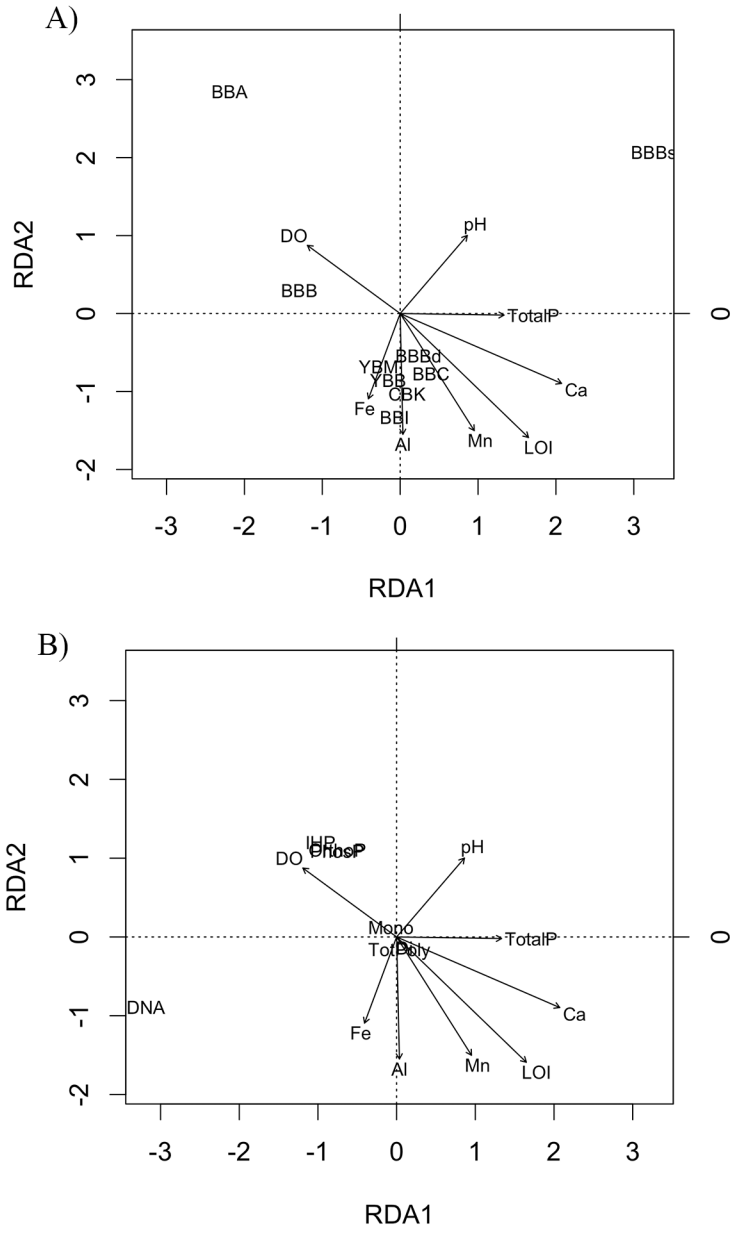

Figure 2.4 Biplots produced from redundancy analysis showing relationships between sediment P forms and environmental characteristics in samples from the Columbia River estuary. (A) Sample sites and sediment characteristics (DO = dissolved oxygen, pH, LOI = loss-on-ignition, Ca, Fe, Al, Mn, Total P = total sediment P); (B) P forms (TotPoly = total of pyrophosphates and polyphosphates; IHP = inositol hexakisphosphates; Mono = monesters, Di = diesters, OrthoP = orthophosphate, DNA = deoxyribonucleic acid, and PhosP = phosphonate) and sediment characteristics. P forms IHP, PhosP and OrthoP overlap next to DO in the upper left quadrant.

Derived from plants, inositol phosphates (*myo*-IHP or phytate, and sterioisomers *scyllo*-, neo-, and D-*chiro*), accrue in terrestrial soils and in aquatic sediments, where concentrations are positively influenced by high clay concentrations and low pH and phytate is the dominant form (Turner et al., 2002b). Phytate is known to form insoluble complexes with Fe oxides in acidic soils and in sediments with high mineral content, making it resistant to microbial phytases (Turner et al., 2002b; Turner and Newman, 2005; Turner and Weckström 2009; Cheesman et al., 2014) as long as conditions are not anoxic (Suzumura et al., 1995a). Although sediments of the lower Columbia were not acidic, they had high mineral content relative to OM, suggesting that preservation of phytate could be high.

Sedimentary phytate concentrations tend to be lower in the presence of salt (Suzumura et al., 1995a, 1995b), with salinities greater than 10 PSU destabilizing phytate complexes and leading to P release back to overlying waters (Gardolinksi, et al., 2004). For the most part, waters overlying the Columbia River sediments were less than 10 PSU; however, in the summer salinities reached 15-18 PSU. Yet, there was no difference in the concentration of phytate among the samples, suggesting either that there is a continuous source of phytate from upstream environments, that phytate may be preserved in low-oxygen conditions, or that phytate stability is not as strongly depressed in the presence of seawater as has been assumed. The negative relationship identified between DO and phytate (IHP) suggests that low-oxygen environments may stabilize this P form within estuary sediments. It is noteworthy that the highest variability in IHP concentrations was observed in Baker Bay, which is characterized by high spatial heterogeneity in environmental conditions; it is likely that this heterogeneity influences IHP sequestration potential in estuarine sediments.

In addition to sequestration of organic forms, P retention is influenced by abiotic buffering processes, which limit fluctuations in DIP through mineral precipitation with Fe, Al, Ca, and Mn (e.g. Fe oxyhydroxides, Ca mineral precipitates; Richardson and Marshall, 1986; Froelich, 1988; Reddy et al., 1993; Bridgham et al., 2001; Li et al., 2013). Retention also occurs through abiotic adsorption to sediment surfaces, particularly on fine particles (Li et al., 2013). The relatively low and similar DIP concentrations in waters overlying Columbia River estuary sediments despite variations in total P within the sediments may occur due to P buffering. Sites on the southern side of the estuary with the greatest freshwater influence (Youngs and Cathlamet Bay) had greater concentrations of extractable cations, OM, and sedimentary P, and higher proportions of silt and clay compared to sites sampled in Baker Bay; this suggests a higher abiotic buffering capacity for P in the southern lateral bays. In addition, Youngs and Cathlamet Bay sites had high concentrations of extractable Ca^{2+}, which readily forms mineral precipitates with P (e.g., $CaHPO_4$ from Ca^{+2} and HPO_4^{-2}) at rates comparable rates with sediment P sorption (Reddy et al., 1993; Li et al., 2013).

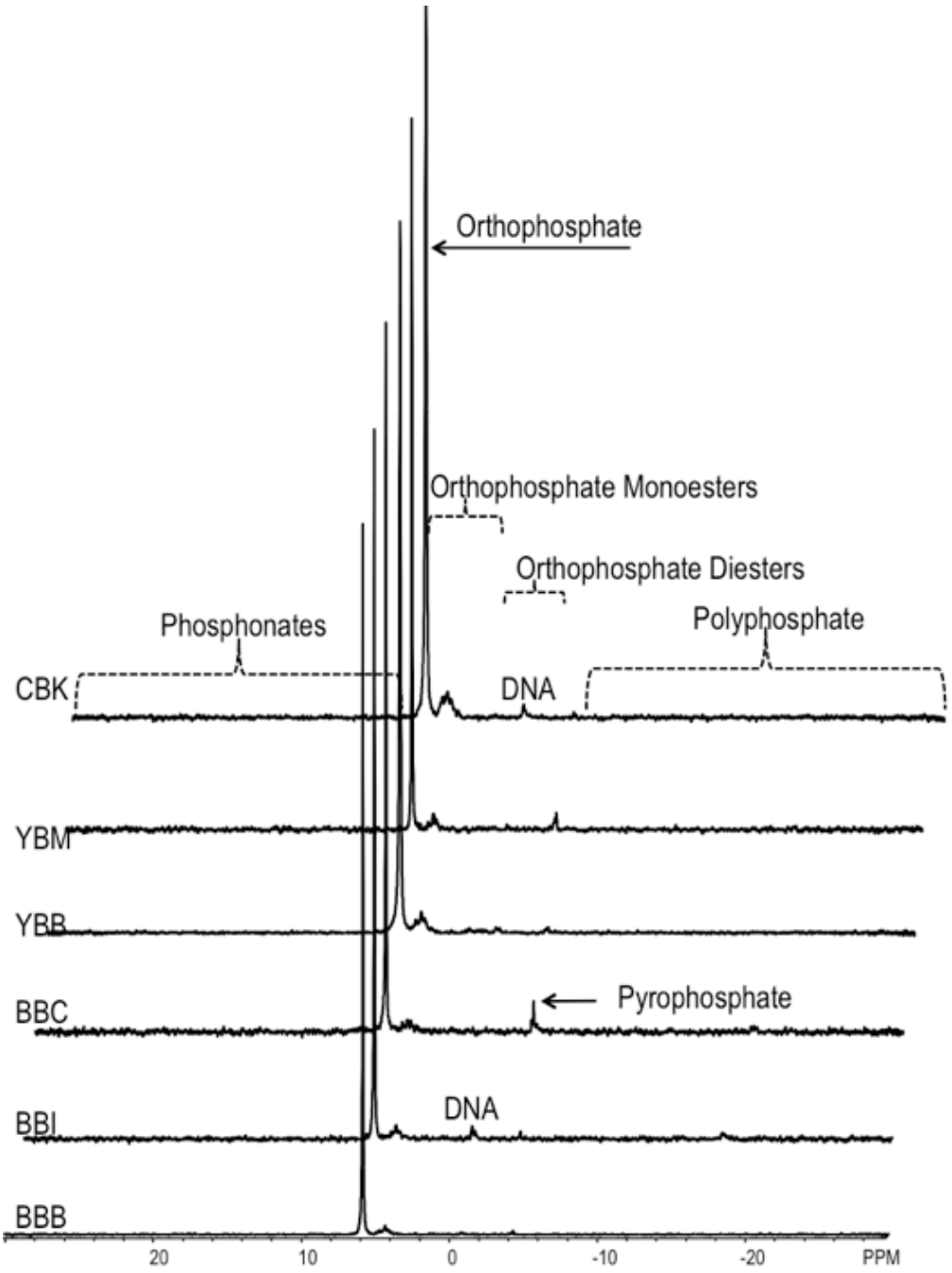

Figure 2.5 P-NMR full spectra from 6 spring sample lateral bay sites from the mouth of the estuary at Baker Bay (BBB) to furthest upriver at Cathlamet Bay (CBK) scaled to the orthophosphate peak at 7Hz line-broadening. Chemical shift areas include phosphonates, 25.0 to 7.0 ppm; orthophosphate, 6.0 ppm; orthophosphate monesters, 7.0 to 2.5 ppm; orthophosphate diesters, 2.5 to -4.0 ppm; and pyrophosphate plus polyphosphates, -4.0 to -25 ppm.

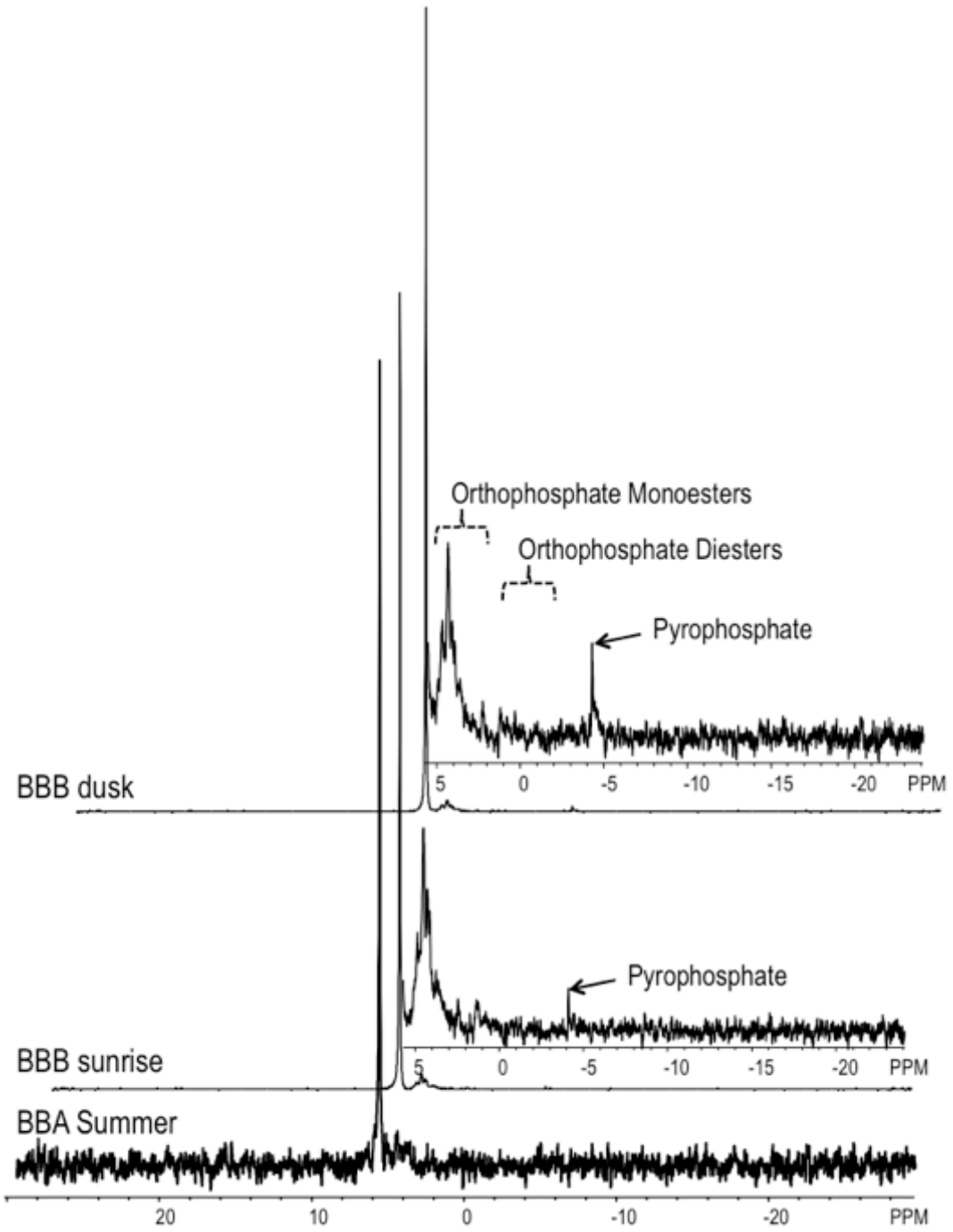

Figure 2.6 P-NMR full spectra from 3 summer samples from the mouth of the estuary at Baker Bay (BBA, BBB) scaled to the orthophosphate peak at 7Hz line-broadening. Samples at BBB were collected during a 24-hour sampling at dusk (~7:00pm) and at sunrise (~7:00am). Spectra at BBB are zoomed into the orthophosphate monoester and orthophosphate diester region and the pyrophosphate and polyphosphate regions.

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CHAPTER 3. DISTRIBUTION OF *CANDIDATUS* ACCUMULIBACTER PHOSPHATIS IN SEDIMENTS OF THE COLUMBIA RIVER ESTUARY

3.1 Introduction

Phosphorus is a key nutrient in aquatic systems, where it is often limiting to growth of primary producers (Daniel et al., 1998). However, in excess, it contributes to eutrophication and can lead to harmful algal blooms and fish kills (Paerl, 2006; Bricker et al., 2007). To address this problem, wastewater treatment incorporates P removal from activated sludge in a process called Enhanced Biological Phosphorus Removal (EBPR). EBPR exploits the fact that certain microbes in activated sludge, known as polyphosphate accumulating microorganisms (PAOs; Mino et al., 1998), are able to over-accumulate inorganic phosphate under aerobic conditions and store it as polyphosphate (polyP), linear polymers of orthophosphate, when carbon concentrations are low (Potgieter & Evans, 1983; Santos et al., 1999). The process involves alternating rounds of anaerobic (carbon rich, "feast") and aerobic (carbon poor, "famine") environments (Oehmen et al., 2007). Under anaerobic conditions, these organisms take up short-chain fatty acids (e.g. acetate, propionate) and store them as polyhydroxyalkanoates (PHA) using energy generated from polyP conversion to adenosine tri-phosphate (ATP; Zhou et al., 2008; Wexler et al., 2009; Oehmen et al., 2010). Conversely, during aerobic conditions, energy for metabolism and growth comes from PHA degradation (Mino et al., 1998).

The core EBPR microbiome includes members that perform key processes involved in nutrient transformation, including denitrification, nitrification, and phosphorus removal (Nielsen et al., 2010; Nielsen et al., 2012). The term polyphosphate accumulating organisms (PAOs) applies to organisms responsible for P removal (Mino et al., 1998). Using culture-independent methods, *Candidatus* Accumulibacter phosphatis (CAP) was identified as the dominant organism responsible for EBPR, although other PAOs exist (Bond et al., 1995; Hesselmann et al., 1999;

Crocetti et al., 2000; Zilles et al., 2002). Based on the phylogeny of polyphosphate kinase (*ppk1*), CAP falls into two major types (I and II), each having multiple subdivisions (IA–IE, and IIA–IIF) (McMahon et al., 2002; He et al., 2007; Peterson et al., 2008); recent studies have increased the number of clades within Type II to 9 (Mao et al., 2016), which are as distant from each other as they are to the Type I clades (Skennerton et al., 2015). Other PAOs in EBPR include *Tetrasphaera* (phylogenetically divided into 3 clades (Nguyen et al., 2011), *Gemmatimonas aurantiaca* (Zhang et al., 2012), and *Candidatus* Halomonas phosphatis (Nguyen et al., 2012). The phylogenetic diversity observed in clades within EBPR reactors supports the idea that CAP is capable of occupying very different ecological niches. However, little work has been done on CAP in the natural environment, other than freshwater ecosystems located near EBPR wastewater plants (Kunin et al., 2008; Peterson et al., 2008).

The CAP genome is large (~5.31 Mbp), and recent metagenomic analyses suggest that they are able to carry out a wide variety of metabolisms (Garcia-Martin et al., 2006). Because of their discovery in WWTP, CAP has generally been associated with high-nutrient environments. However, an ability to fix carbon and the presence of high-affinity phosphate transporters suggest that CAP would be most competitive under low-nutrient environments (Skennerton et al., 2015). Although it has been argued that the term PAO should only be used in the context of EBPR, since key carbon and P transformations occur over short time frames and at high rates (McMahon and Read, 2013), a natural environmental niche for PAOs should occur at the sediment-water interface where large diel fluctuations in redox conditions can occur (Hupfer et al., 2007).

Using solution ^{31}P-nuclear magnetic resonance spectroscopy (P-NMR), polyphosphate was detected throughout lateral bays of the Columbia River estuary, where it contributed up to

10% of extracted P in sediments (Chapter 2; Watson et al., in review). The accumulation of organic matter at the sediment-water interface make these environments a hotspot for microbial activity, while steep gradients and rapid fluctuations in environmental conditions lead to enhanced microbial interaction with sediment P (Hupfer et al., 2007). High microbial activity at the sediment-water interface is often characterized by fluctuations in redox conditions and oxygen concentrations (Davelaar, 1993), which produces a unique ecological niche. This niche has been exploited by organisms adapted to withstand dramatic oscillations, including those that carry out the intracellular accumulation of phosphate in polyphosphate molecules (PAOs) (Davelaar, 1993; Gächter & Meyer, 1993).

Estuaries are dynamic and biologically productive ecosystems that are characterized by large fluctuations in environmental conditions. Thus, estuaries constitute an ideal setting to evaluate the conceptual model of PAO metabolism. The main goal of this study was to test the hypothesis that CAP occurs in surface sediments of the Columbia River estuary, where environmental conditions change daily and seasonally. We further hypothesized that CAP abundance is correlated with seasonal and spatial differences in environmental characteristics. The objectives were to: (*i*) look for CAP in estuarine sediments using culture-independent molecular tools; and to (*ii*) characterize the spatial and seasonal distributions of CAP within lateral bays of the Columbia River estuary (**Figure 3.1**).

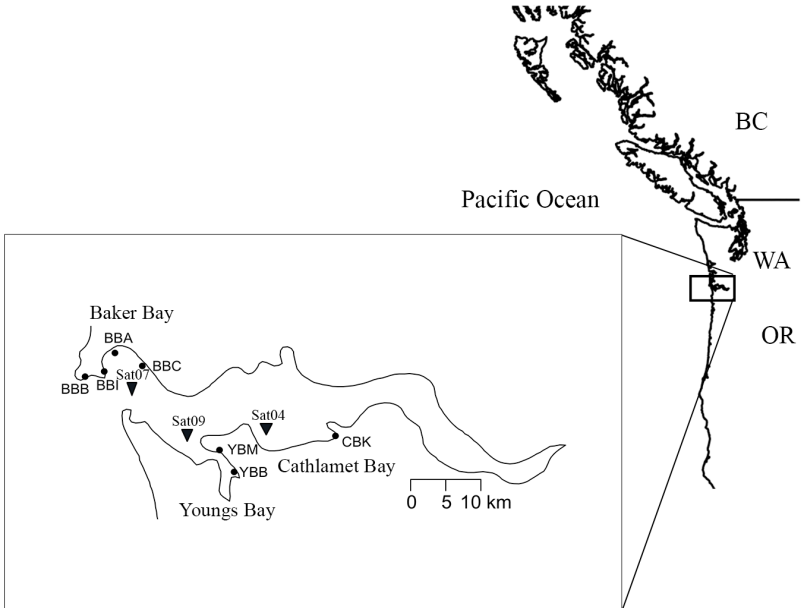

Figure 3.1 Map of the Columbia River estuary including lateral bay sample sites and SATURN continuous sensor observatories (Sat07; SATURN 07, Sat09; SATURN09; Sat04, SATURN 04).

3.2 Methods

3.2.1 *Field sites*

The Columbia River is the second largest by flow in the U.S. (Sherwood et al., 1990) and it culminates in a river-dominated estuary with strong seasonality in salinity (Chawla et al., 2008) and biogeochemical properties (Roegner et al., 2011). The estuary consists of two main channels through which the majority of water exchange occurs and includes four lateral bays (Baker, Young's, Grays, and Cathlamet). The sampling sites were located in tidal emergent marsh sediments in three of these bays: Baker, Young's, and Cathlamet (**Figure 3.1**). Baker Bay on the northern side of the estuary is mesohaline, with a salinity range of 5–20 practical salinity units (PSU). Young's and Cathlamet Bay is oligohaline (0–5 PSU) at sites close to the mainstem, while Cathlamet Bay is oligohaline in its deeper waters (~8 m). Both Baker Bay and Young's

Bay receive considerable deposition of fine particles (silts and clays) (Simenstad, et al., 1992), while Cathlamet Bay receives deposition of sand, silt and clays. The samples were collected in mudflats with minimal plant cover.

The Columbia River discharge varies with season, peaking at >10,000 m^3 s^{-1} during the snowmelt-driven spring freshet (April–June) and declining to < 2,000 m^3 s^{-1} in the dry season (July–October; **Figure 3.2**A). Salt intrusion into the estuary is greatest during periods of low discharge, which typically coincides with upwelling-favorable conditions on the coast between June and August (Huyer, 1983; Chawla et al., 2008).

3.2.2 *Sample collection and nutrient analysis*

Sediment samples were collected during low tide between September 2013 and August 2014 at sites within Baker Bay (four sites: BBB, BBI, BBA, BBC), Young's Bay (two sites: YBB, YBM) and Cathlamet Bay (one site: CBK; **Figure 3.1**). The samples were obtained by coring sterile polypropylene Falcon tubes (15 and 50 ml) into the sediment (0-5 cm). Following collection, the samples were stored in a cooler on dry ice for transport back to the laboratory (~2-3 h) where they were stored at -80^{o}C pending analysis. Sediment characteristics of lateral bay sample sites are summarized in Chapter 2.

Surface water samples were collected in waters overlying the sediments for determination of dissolved and total nutrient concentrations. The water samples were filtered through combusted (4 h at 450^{o}C) 25 mm glass fiber filters (Whatman, NY) into 60 ml acid-cleaned HDPE bottles (Nalgene). Unfiltered water samples were collected for the determination of total nitrogen and total phosphorus concentrations. All samples were kept on dry ice in a cooler during transportation to the laboratory where they were stored at -20^{o}C pending processing.

A rapid flow analyzer (Astoria Analyzer, Astoria-Pacific) was used for colorimetric determination of surface water dissolved nitrate, nitrite, ammonium, and orthophosphate concentrations (Antweiler et al., 1996). Total phosphorus and total nitrogen was determined colorimetrically following alkaline persulfate digestion (Patton and Kryskalla, 2003).

3.2.3 *Continuous sensor data*

Real-time data on physical and chemical parameters at or near the field sites were obtained from in situ sensor platforms maintained by the Center for Coastal Margin Observation and Prediction (CMOP). SATURN-07, -09 and -04 in particular capture conditions in surface waters of the major lateral bays (Baker Bay, Young's Bay and Cathlamet Bay, respectively). These stations include sensors to measure temperature, salinity, dissolved oxygen, turbidity, chlorophyll a fluorescence, and colored dissolved organic matter (Baptista et al., 2015).

3.2.4 *DNA isolation and Polymerase Chain Reaction (PCR)*

DNA was extracted using the FastDNA spin kit for soil (MO Bio Laboratories, Inc., Carlsbad, CA). DNA was quantified and checked for purity using a Nanodrop UV-Vis spectrophotometer (Thermo Scientific, Nanodrop 2000). The 16S rRNA gene was targeted to survey the lateral bays for the presence of CAP. Primers targeting the V4 region (438f/846r) produced a 408 bp product (Kunin et al.,2008). PCR reactions to amplify CAP 16S rRNA gene sequences were performed in a 25 µl reaction mixture including the following: 10x Ex Taq Buffer, Deoxynucleoside triphosphates (dNTP) mix (2.5 mM each), 0.4 µM primers, and 0.5U μl^{-1} of Takara Ex Taq Polymerase (Takara Bio Inc., Mountain View, CA). PCR reactions were carried out on a Thermal Cycler (Bio-Rad Laboratories, Inc.) with the following conditions;

initial denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 30 s with a final extension at 72°C for 3 min. To minimize interferences from environmental inhibitors a dilution series was performed with the genomic DNA extracted from sediment samples. A 10x dilution (~25 ng μl^{-1}) of extracted DNA from sediment sites produced a PCR product with a single band seen in a 1% agarose gel of the expected size. The *ppk1* gene was targeted to compare abundances of CAP across lateral bays and seasons. Primers ppk-893F/997R (*ppk1 gene*) produced a 104 bp product (He et al., 2007) and were chosen based on the least number of mismatches against the complete genome of Accumulibacter Clade IIA UW-1 (referred to as CAP IIA UW-1; AJ224937) (Garcia Martin et al., 2006) *ppk1* gene. PCR reactions were performed as described above with the following conditions: an initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 61°C for 45 s, and 72°C for 30 s with a final extension at 72°C for 3 min. DNA isolated from activated sludge from a wastewater treatment plant (wwtp) that performs EBPR (Durham wwtp, Tigard, OR) was used as a positive control. Table 3-1 summarizes the primers used to amplify 16S rRNA and the *ppk1* genes in estuarine sediment and activated sludge.

Table 3-1 Primers used in end-point PCR to amplify 16S rRNA and *ppk1* genes from CAP in sediments of the Columbia River estuary.

Target	Primer	Sequence (5'–3')	Amplicon size (bp)	Reference
16S rRNA (CAP)	438f	GGTTAATACCCTGWGTAGAT	409	Kunin et al., 2008
	846r	GTTAGCTACGGCACTAAAAGG		
16S rRNA (Universal)	341f	CCTACGGGAGGCAGCAG	194	Mao et al., 2015
	534r	ATTACCGCGGCTGCTGG		
Acc-IIC *ppk1*[1]	254f	TCACCACCGACGGCAAGAC	1123	McMahon et al., 2007, Peterson et al., 2008
	1376r	ACGATCATCAGCATCTTGGC		

[1]Acc-*ppk1*-254f and Acc-ppk1-1376r are primers that target total "*Candidatus* Accumulibacter phosphatis" (CAP) *ppk1* genes (McMahon et al., 2007)

3.2.5 *Clone library construction and sequence analysis*

Amplified PCR products from sediment samples were purified using a QIAquick PCR purification kit (QIAGEN Sciences, MD), ligated into a plasmid, and cloned into chemically competent cells with a TOPO 2.1 TA cloning kit (Thermo Fisher Scientific, Grand Island, NY) according to manufacturer's instructions. Transformed cells were grown up on LB–ampicillin plates using blue-white screening. Individual white colonies were picked and inoculated in LB–ampicillin liquid media overnight at 37^{o}C. Plasmids were extracted using a QIAprep Spin Miniprep Kit (Qiagen, CA). Fifty random colonies from the LB plates for each field site were picked and re-streaked, incubated overnight, and stored at 4^{o}C until sequencing was performed. Of these, 10–12 were selected randomly for sequencing, which was performed either at the Molecular and Cell Biology Core at Oregon Health & Science University or at Beckman Coulter Genetics, Inc., MA by the dideoxy (chain-termination) method (Sanger et al., 1977).

The sequences were aligned against the nucleotide database within NCBI (National Center for Biotechnology Information, NCBI http://blast.ncbi.nlm.nih.gov/) using BLAST (Basic Local Alignment Search Tool). Operational taxonomic units (OTUs) were assigned to sequences sharing >97% similarity. OTUs, as well as sequences previously identified in literature as CAP Type I and Type II subgroups (He et al., 2007), were aligned for comparison in Geneious 4.0. Samples retrieved from lateral bays were compared initially within sites and grouped by similarity. Representatives from each group within sites were then aligned between sites and with sequences from the literature, and trimmed to eliminate unmatched ends. A progressive multiple sequence alignment was performed in Geneious with ClustalW. A phylogenetic tree

was constructed by Bayesian inference (Huelsenbeck and Ronquist, 2001), following the ClustalW alignment using the genetic distance HKY model, with gamma rate variation and a subsampling frequency of 500.

3.2.6 *Quantitative PCR (qPCR)*

qPCR was conducted on a StepOnePlus real time machine (Applied Biosystems, Carlsbad, CA, USA) using Applied Biosystems SYBR green PCR Master Mix (Warrington, UK) with a total reaction volume of 20 µl. Programs consisted of an initial denaturation at 95^{o}C for 3 min, followed by 45 cycles of denaturation for 30 s at 94^{o}C, annealing for 45 s at 61^{o}C, and extension for 30 s at 72^{o}C followed by a melting curve calculation. Reactions were performed in triplicate with 1 µl diluted (50–75x) genomic DNA template, 0.5 µM forward (ppk-893f) and reverse (ppk-997r) primers (He et al., 2007; Table 3-1), 100 ng µl^{-1} bovine serum albumin, and 1.5% DMSO. Reactions were run in 96-well plates sealed with adhesive optical covers and centrifuged for 3 min at 1000 rpm prior to analysis.

qPCR standards were made from purified *ppk1* and 16S rRNA amplicons from activated sludge. The plasmids were linearized by EcoRV digestion (New England BioLabs, Ipswich, MA) and purified with a QIAquick PCR purification kit (QIAGEN). Five to six-point calibration curves were created by 10-fold serial dilution of standard controls in triplicate with each qPCR reaction ranging from 10^3 to 10^8 target copies. The concentration of all standards was determined with Picogreen using a Nanodrop Fluorospectrophotometer 3300 (ThermoFisher Scientific, Wilmington, DE). Copy numbers were calculated based on concentration and molecular weight of the plasmid and *ppk1* insert. Each reaction included a no-template control to check for contamination and primer-dimer formation and genomic DNA extracted from activated sludge was included in each assay as positive control. Detection limits were approximately 10^3 copies

and no-template controls (blanks) were below detection limits. DNA extracts from estuary sediments were diluted 20–75x prior to amplification to avoid PCR inhibition (Table 3-2). Dilution increased efficiency of the qPCR reactions as evidence by detectable levels of the *ppk1* gene and ranged from 20 – 75x (**Table 3-2**). The calibration curves had PCR efficiencies of 87–99% (R^2 = 0.96–0.99) (**Table 3-2**).

Table 3-2 A summary of dilution factors for sediments, qPCR slopes, efficiencies and correlation coefficients for reactions carried out with the ppk1 gene.

Lateral bay sediment - qPCR assays					
Gene	Site - dilution factor	Slope	Intercept	R^2	PCR Efficiency (%)
	BBB - 75x	-3.453	37.225	0.996	94.8
	BBI - 75x	-3.531	37.031	0.998	91.9
ppk	BBA – 75x	-3.692	37.796	0.992	86.6
	BBC – 20x	-3.350	36.713	0.992	98.8
	YBM – 50x	-3.685	35.881	0.998	86.8
	YBB – 75x	-3.665	39.793	0.961	87.4
	CBK – 50x	-3.561	37.213	0.995	98.6
	BBB – 20x	-3.553	37.083	0.998	91.2
16S	BBA – 20x	-3.402	36.901	0.998	96.8
rRNA	YBM – 20x	-3.474	37.209	0.994	94.0
	CBK – 20x	-3.367	35.510	0.999	98.2
	BBI, BBC, YBB – 20x	-3.574	37.777	0.997	90.4

3.3 Results

3.3.1 *Environmental conditions within lateral bays*

River discharge peaked in the late spring (May–June) during the freshet (**Figure 3.2**A). Within the water column, pH and dissolved oxygen were highest during the spring, which is typically associated with high rates of primary production (**Figure 3.2**B,D). In contrast, dissolved oxygen levels reached a minimum in the summer months (July–September; **Figure**

3.2D), including oscillations into hypoxia (≤ 2 ml L^{-1}) in the summer at depth (Figure 3.2E). Salinity reached maximum values from July–October (**Figure 3.2**F).

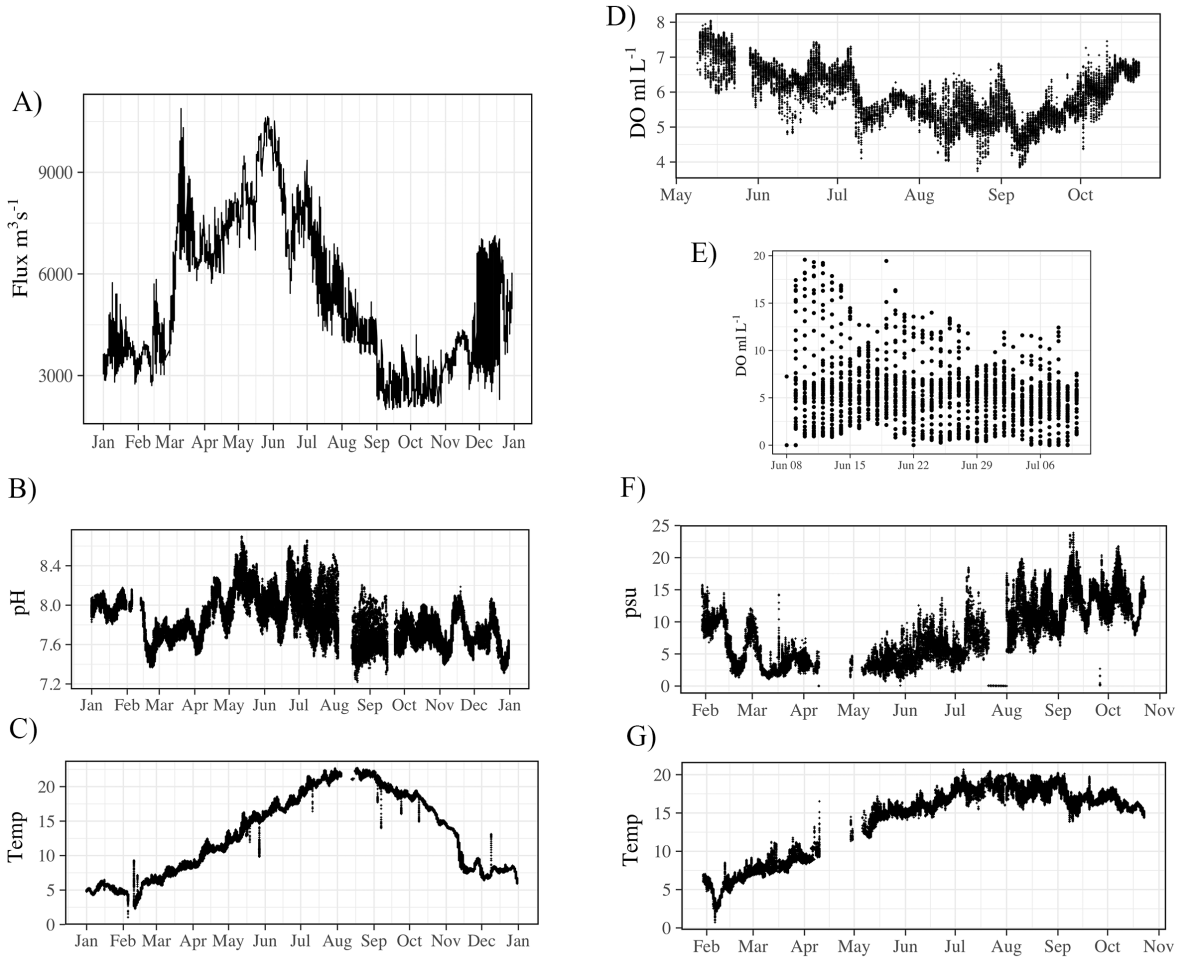

Figure 3.2 Environmental conditions in the lower Columbia River estuary including A) River discharge (m^3 s^{-1}) determined at Bonneville Dam from January–December 2014, B) pH at Saturn-04 at Cathlamet Bay, C) Temp; water temperature (◦C) at Saturn-04, D) DO; dissolved oxygen (ml L^{-1}) at Saturn-07, Baker Bay; E) DO from stream bed channel in Baker Bay; F) psu; practical salinity units from Saturn-07; and G) Temp, water temperature (^{o}C) at Saturn-07.

Orthophosphate and dissolved ammonium (NH_4^+) were highest during the summer months (June–September), particularly in Baker Bay (Figure 3.3A,C), while dissolved nitrate+nitrite (DIN), total phosphorus (TP), and total nitrogen (TN) were highest during the

winter (January–April; Figure 3.3B,D,E). Dissolved inorganic nitrogen, TP, and TN concentrations were higher in Young's and Cathlamet Bays compared to Baker Bay.

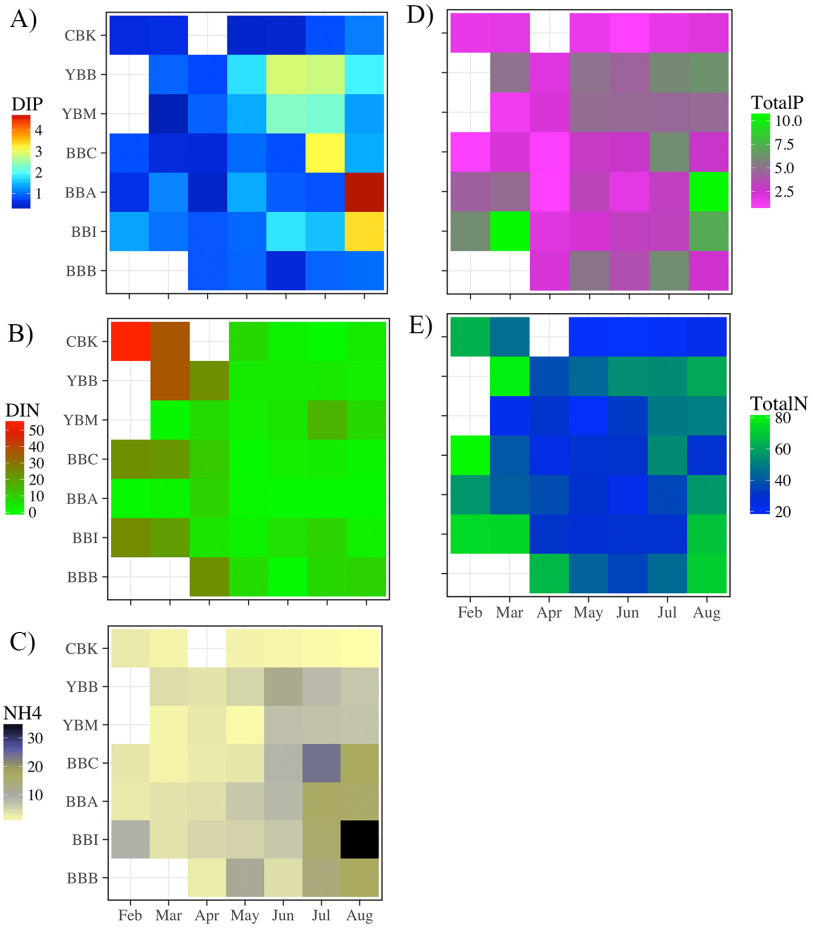

Figure 3.3 Nutrient data collected from surface water of lateral bays from January–September 2014 including; A) DIP, orthophosphate, B) dissolved inorganic nitrogen (nitrite+nitrate), C) ammonium, NH4, D) total phosphorus, and E) total nitrogen all values reported in µM. Sites are listed from upriver (freshwater) site (CBK) at the top of the y-axis to the mesohaline sites (BBB) at the bottom of the y-axis.

3.3.2 *Phylogenetic diversity of CAP in lateral bays*

Amplicons produced through end-point PCR using CAP-specific 16S rRNA primers shared >97% similarity with CAP 16S rRNA gene sequences deposited in GenBank. Both the

environmental sequences and sequences isolated from EBPR samples at a wastewater treatment plant in Durham, Oregon included representatives from the two major lineages identified as Type I and Type II (He et al., 2007). The majority of CAP-affiliated 16S rRNA gene sequences detected in lateral bay sediments throughout the Columbia River estuary were Type I rather than Type II (13 vs. 5; Fig. 3.4). Cathlamet Bay (CBK) had four unique OTUs while most of the Baker Bay sites had only two different OTUs. Within the WWTP sample out of the 10-12 OTUs sampled 4 unique sequences were detected two of each Type I and II. Sequences from the Type I subgroup were detected in all of the environmental samples, while those from the Type II subgroup were only detected in the southern lateral bays where there was no influence of seawater (Cathlamet Bay and Young's Bay). Within Baker Bay, the sequences from one site (BBA) were more similar (bootstrap value of 1) to Type I OTUs from Cathlamet Bay than the other Baker Bay sites.

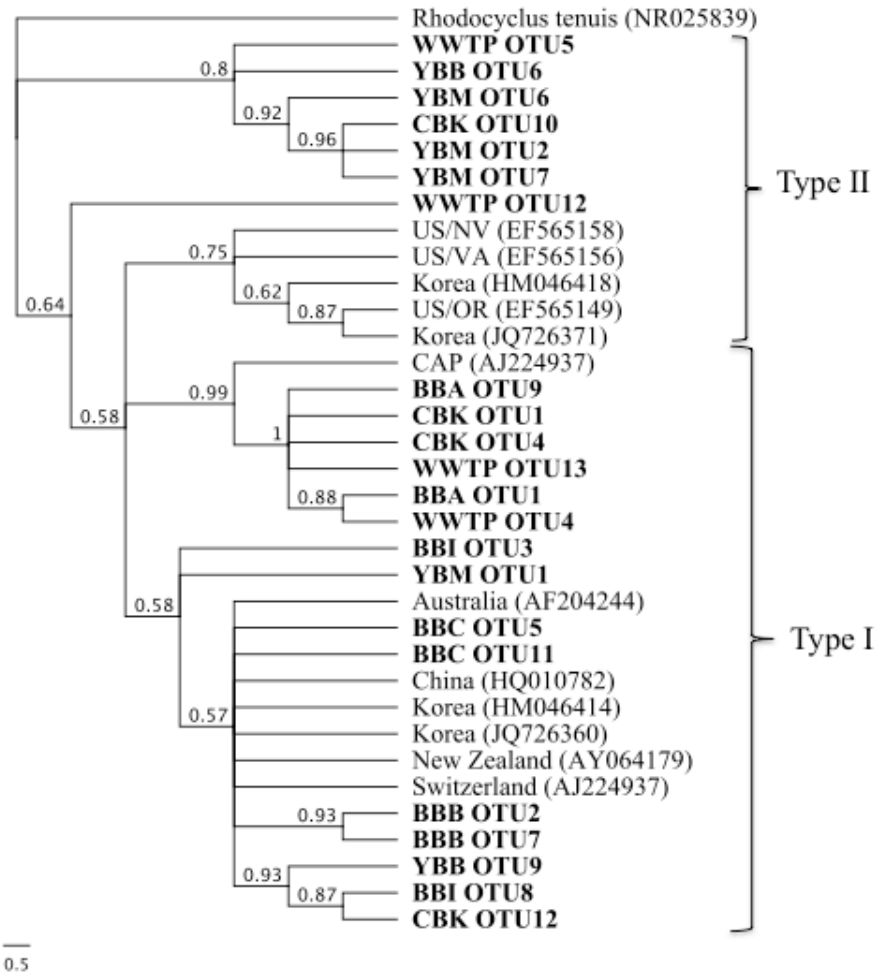

Figure 3.4 Phylogenetic analysis of 16S rRNA gene sequences attributable to *Candidatus* Accumulibacter phosphatis. Sequences recovered in this study (bold face type) were compared with reference sequences in the NCBI GenBank database and are listed here including country of EBPR facility and accession number. Node values represent support (bootstrap) values and the scale bar indicates the number of changes per nucleotide base.

3.3.3 *ppk1 gene abundances*

The complete genome of Clade IIA of CAP (UW-1) (Garcia-Martin et al., 2006) and draft genomes of Clade IA and IC (Flowers et al., 2013; Skennerton et al., 2015) demonstrated that both Type I and II subgroups carry a single copy of *ppk1*, making it a good indicator of CAP abundance. Abundances of CAP were estimated by quantifying the number of copies of *ppk1* in sediment samples by quantitative PCR. In addition, abundances of the whole bacterial

community were estimated from the number of 16S rRNA genes, assuming there were 4 copies cell^{-1} (Mao, et al., 2015), although soil bacteria are thought to range from 1 to 5 copies cell^{-1} depending upon growth rates (Klappenbach et al., 2000). With this assumption, CAP accounted for 6% (±1.6) of the total bacterial community in activated sludge from the Durham EBPR WWTP (Table 3-4).

Among the environmental samples, the greatest number of *ppk1* gene copies (Figure 3.5) and highest relative abundances (**Figure 3.6**) were observed at a site within Baker Bay (BBB) in June, where CAP accounted for 2.6% of the total bacterial community (~1.5 x 10^6 copies g^{-1}). Although absolute abundances were similar between June and July at BBB, CAP accounted for a much lower percentage (0.3–1%) of the bacterial community in July. The number of copies of *ppk1* g^{-1} peaked during periods of low-to-moderate flow at BBA (early February and mid-July, while at BBB and CBK, copies of *ppk1* were most abundant from March to May when river flow was high. At YBB, absolute abundances of *ppk1* did not vary among the four samples where it was detected (February, March, April, and July).

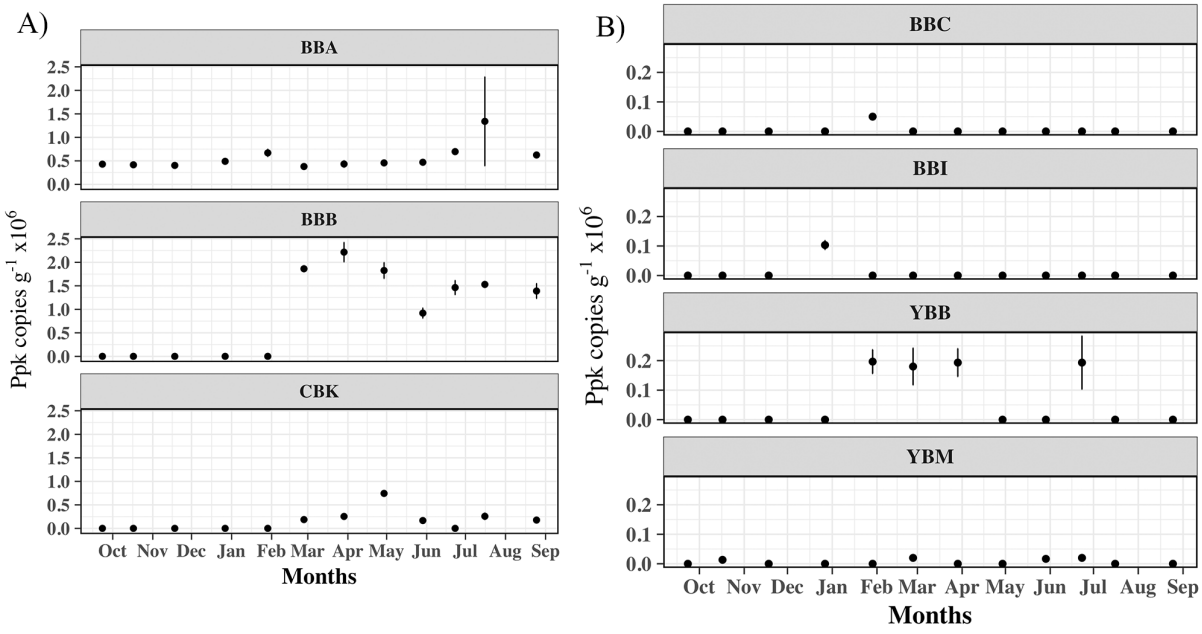

Figure 3.5 Abundances of the *ppk* gene (copy g^{-1} x 10^6) from lateral bay sample sites across seasons. Highest abundances are grouped together in A) BBA, BBB, and CBK, and lower abundances observed in B) BBC, BBI, YBB and YBM.

Although CAP was present at much lower absolute abundances at the other Baker Bay sites relative to BBB, the relative abundances were similar between BBA and BBB (**Figure 3.5**). In addition, whereas they were only detected in the spring at BBB, they were found throughout the year at BBA. The highest relative abundances of CAP at BBA were observed during autumn and winter (September–February), as well as July (**Figure 3.5**). These time periods correspond to those where local flow dominated, rather than snowmelt-driven runoff (**Figure 3.2**). The other two sites (Ilwaco and Chinook harbor, BBI and BBC, respectively) had low relative abundances of CAP (~0.1% of the total bacterial community) and they were detected only in one sample each in the winter months (December at BBI; January at BBC).

Both absolute and relative abundances of CAP were higher in Baker Bay sediments (BBB, BBA) compared to Cathlamet Bay (CBK) and Youngs Bay (YBB, YBM). With the exception of June, Cathlamet Bay sediments had detectable *ppk1* from February through August, reaching 1.4% relative abundance in April (7.4 x 10^5 copies g^{-1}). The lowest relative abundances were observed at the two Youngs Bay sites (YBB, YBM), where they accounted for 0.01–0.32% of the bacterial community and a maximum of 1.9 x 10^5 copies g^{-1} (at YBB in June). Abundances were near the level of detection throughout the year at YBM; at YBB, CAP was detected only in the winter months (January–March).

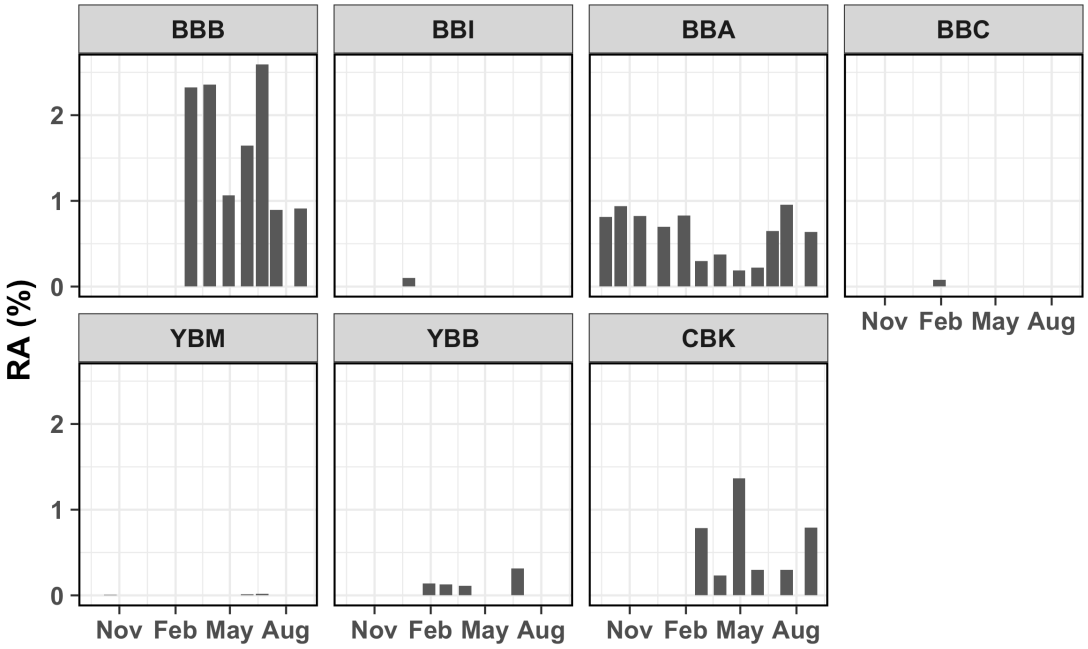

Figure 3.6 Relative abundance (%) of the *ppk1* gene at lateral bay sample sites.

3.4 Discussion

Relative abundances of CAP (*ppk1* genes) in sediments of the Columbia River estuary ranged from 0.01–2.6%, which rivals abundances reported in conventional activated sludge

(0.02–7.0%) (Mao et al., 2015). Within EBPR systems, CAP tends to account for higher percentages of the microbial community (4–25%) (Oehmen et al., 2007; He and McMahon, 2011; Nielsen et al., 2012) , where they fall into one of two types (Type I or Type II; He et al., 2007). The two types are thought to occupy different ecological niches, with Type I found predominantly when phosphorus is plentiful, while Type II dominates when P is limiting (Acevedo et al., 2012; Welles et al., 2016).

Most of the CAP-affiliated 16S rRNA gene sequences detected in sediments fell into the Type I category, indicating although the Columbia River does not possess particularly high nutrient concentrations in the water column (e.g., Gilbert et al., 2013), sediments in the estuary may undergoing rapid P cycling. Although the highest abundances of *ppk1* were observed between March and September within mesohaline sediments of Baker Bay (mainly at BBB), concentrations of orthophosphate and other P forms were not particularly high. Sequences attributed to Type I subgroups were similarly observed in estuarine sediments from a California stream, which included representatives from three novel clades not observed in samples from nearby freshwater environments (IB, ID, and IE; Peterson et al., 2008). It is possible that this finding is related to the fact that Type I CAP is able to take up and use nitrate in addition to oxygen as an electron acceptor for P uptake (Dabert et al., 2001; Zeng et al., 2003; Kong et al., 2004; Oehmen et al., 2007), while Type II is not (Garcia Martin et al., 2006; Carvalho et al., 2007; Flowers et al., 2009). During the winter, spring, and early summer, nitrate concentrations tend to exceed those of ammonium in the Columbia River estuary (Gilbert et al., 2013). Only in the summer months do ammonium levels increase substantially; notably, it was during the late summer months that abundances of *ppk1* affiliated with Type I CAP decreased significantly while ammonium concentrations were at their annual peak.

In addition, there is significant seasonal variation in the salinity regime in the Columbia River estuary, with winters dominated by freshwater and summers characterized by brackish to saline waters (5–25 PSU; Chawla et al., 2008). Results from a short-term bench reactor study revealed that exposure to high salinity (5% NaCl) inhibits aerobic metabolism in PAOs, leading to a shift from P accumulation to glucose accumulation in CAP populations (Welles and Hooijmans, 2014; Welles et al., 2015). This finding suggests that salinity may influence metabolic processes, community structure, or both. In addition, glucose-accumulating organisms (GAOs) have been shown to compete with PAOs for volatile fatty acid uptake under anaerobic conditions (Lopez-Vazquez et al., 2009). This is significant, since hypoxia is much more likely to occur in the Columbia River estuary during the summer months when flood tides deliver hypoxic waters from the shelf to the estuary (Roegner et al., 2011). It is possible that either GAOs outcompete PAOs under these summer conditions, or that PAOs shift to a glucose accumulating metabolism, as has been observed in previous studies (Barat et al., 2006; Barat et al., 2008; Erdal et al., 2008; Zhou et al., 2008).

We estimated the potential contribution by CAP to sedimentary P using volume-based measurements of polyphosphate determined using Raman Spectroscopy in full-scale EBPR plants (Majed et al., 2012). In that study, polyphosphate was estimated as ⅔ of the cell volume of CAP, which are typically, 3-5 μm in diameter, at the end of an aerobic cycle. Taking the volume of a sphere ($4/3\ \pi\ r^3$), converting the resulting volume to grams ($1\ x10^{12}\ \mu m^3 = 1\ g$), and multiplying by ⅔, we get an estimate of cell-specific P; multiplying by the number of *ppk1* copies g^{-1} yields an estimated contribution of the sum of cell-specific P to total polyphosphate (mg kg^{-1}; **Table 3-3**). For example, at BBB we estimated the contribution from CAP-produced polyphosphate to be ~18 mg kg^{-1}. This is in close agreement with measurement of polyphosphate

made using ^{31}P-NMR (Chapter 2), which would have been 17.9 mg kg^{-1} if full recovery of polyphosphate was in sediment extracts was achieved (i.e., the measured 4.3 mg kg^{-1} polyphosphate multiplied by 1/recovery rate of 24%, or 100/24). However, estimates from BBA and YBB were almost an order of magnitude different than the measured polyphosphate concentration; this discrepancy could reflect physiological differences in polyphosphate accumulation under different environmental conditions, for example those favoring glucose accumulation instead of phosphorus accumulation (Lopez-Vazquez et al., 2008). Indeed, large variations in the contribution of polyphosphate to P cycling have been observed in EBPR studies using Raman spectroscopy (Majed et al., 2012). Differences observed in polyphosphates in sediments could also be attributed to contributions from other taxa, since it is biologically produced across the domains of life, including by bacteria, fungi, protozoa, plants, and mammals (Kornberg, 1995; Brown and Kornberg, 2004; Kulaev et al., 2004).

Table 3-3 Estimate of contribution to sediment P by polyphosphate. Estimate is based on cell volume calculation, P-NMR is polyphosphate measured, Recovery is the rate of total P recovered in sediment samples, and Actual is the P-NMR measurement divided by recovery rate.

Site and date	*ppk1* (copies g^{-1})	Estimate (mg kg^{-1})	P-NMR (mg kg^{-1})	Recovery (%)	Actual (mg kg^{-1})
BBB (3/28/14)	2.2 x 10^{6}	18	4.3	23.9	17.9
BBA (8/25/14)	6.3 x 10^{5}	5.7	0.8	1.5	53.3
YBB (3/28/14)	2.0 x 10^{5}	1.8	3.3	24.5	13.5
CBK (4/29/14)	7.4 x 10^{5}	6.7	0.8	8.1	9.8

In the marine environment, polyphosphate can be an important component of the P cycle, since it provides a mode of P storage in certain taxa [e.g., the marine cyanobacterium, *Trichodesmium* (Orchard et al., 2010), and in marine diatoms (Dyhrman et al., 2012)], which is particularly important when P is present in limited supply. In *Trichodesmium*, for example,

polyphosphate can account for up to 25% of cellular P following a pulsed supply of P in oligotrophic waters (Orchard et al, 2010). In a seasonal system, such as the Columbia River estuary, the potential for CAP to remove P would likely occur during the seasonal pulse of orthophosphate that occurs during the spring freshet. Excess P could be taken up by CAP, and with increased photosynthesis resulting in longer periods of oxygen store P as polyphosphate. Shorter periods of respiration or hypoxic conditions would result in excess P storage and the potential for removing P as cellular polyphosphate. In this scenario, CAP could facilitate the removal of P from the estuary by assisting apatite (Ca-P mineral) formation such as observed by the large sulfur bacterium, *Thiomargarita namibiensis* (Schulz and Schulz, 2005), which accumulates P within the cell and facilitates formation of phosphorite in ocean shelf sediments (Goldhammer et al., 2010).

Table 3-4 qPCR data from Sept. 2013–Aug. 2014 sediment sampling in the lateral bays of the estuary. Data is provided for samples in which *ppk1* was detectable in samples, missing months means ppk was below detection limit. Wastewater treatment plant assays are averaged across 6 qPCR experiments (*). Rel Abd = relative abundance.

		Samples	*ppk1*		16S rRNA		Rel Abd
			Copies g^{-1}	s.d.	Copies g^{-1}	s.d.	(%)
2014	26 - Feb	BBB	1.86E+06	6.85E+04	3.21E+08	1.80E+07	2.32
	29 - March	BBB	2.21E+06	2.09E+05	3.76E+08	1.26E+07	2.36
	29 - April	BBB	1.83E+06	1.68E+05	6.96E+08	1.35E+08	1.07
	29 - May	BBB	9.22E+05	1.05E+05	2.26E+08	3.55E+07	1.64
	23 - June	BBB	1.46E+06	1.53E+05	2.27E+08	1.15E+07	2.59
	16 - July	BBB	1.53E+06	6.67E+04	6.81E+08	4.01E+07	0.90
	25 - Aug	BBB	1.39E+06	1.58E+05	6.41E+08	2.08E+08	0.91
2013	23 – Sept	BBA	4.92E+05	1.09E+05	1.96E+08	2.96E+07	1.05
	17 – Oct	BBA	4.15E+05	1.97E+04	1.78E+08	1.15E+07	0.94
	18 – Nov	BBA	4.03E+05	5.43E+04	1.98E+08	3.30E+07	0.82
	27 - Dec	BBA	4.90E+05	3.26E+04	2.82E+08	1.08E+07	0.70
2014	29 - Jan	BBA	6.69E+05	7.86E+04	3.23E+08	3.04E+07	0.83
	26 - Feb	BBA	3.80E+05	1.05E+04	5.09E+08	1.25E+07	0.30
	29 - March	BBA	4.34E+05	7.26E+04	4.62E+08	9.42E+07	0.38
	29 - April	BBA	4.56E+05	5.67E+04	9.78E+08	5.54E+07	0.19
	29 - May	BBA	4.70E+05	3.20E+04	8.50E+08	9.48E+07	0.22

	23 - June	BBA	6.97E+05	4.81E+04	4.33E+08	4.34E+07	0.65
	16 - July	BBA	1.34E+06	9.46E+05	6.29E+08	1.32E+08	0.95
	25 - Aug	BBA	6.26E+05	2.79E+04	5.84E+08	8.33E+07	0.34
2013	27 - Dec	BBI	1.05E+05	1.23E+04	4.30E+08	2.80E+07	0.10
2014	29 - Jan	BBC	4.96E+04	1.95E+03	2.51E+08	1.96E+07	0.08
2013	17 - Oct	YBM	1.31E+04	1.85E+03	4.07E+08	2.22E+07	0.01
2014	26 - Feb	YBM	1.78E+04	2.51E+03	2.01E+09	3.01E+08	0.00
	29 - May	YBM	1.70E+04	2.18E+03	5.00E+08	3.74E+07	0.01
	23 - June	YBM	1.88E+04	7.96E+02	4.77E+08	1.40E+07	0.02
2014	29 - Jan	YBB	1.95E+05	4.29E+04	5.45E+08	6.75E+07	0.14
	26 - Feb	YBB	1.80E+05	6.17E+04	5.66E+08	4.30E+07	0.13
	29 - March	YBB	1.97E+05	4.76E+04	6.93E+08	4.05E+07	0.11
	23 - June	YBB	1.93E+05	8.57E+04	2.42E+08	1.12E_07	0.32
2014	26 - Feb	CBK	1.87E+05	1.70E+04	9.59E+07	1.29E+07	0.78
	29 - March	CBK	2.53E+05	5.88E+04	4.42E+08	2.68E+06	0.23
	29 - April	CBK	7.41E+05	4.04E+04	2.19E+08	2.61E+07	1.36
	29 - May	CBK	1.68E+05	2.29E+04	2.22E+08	1.45E+07	0.30
	16 - July	CBK	2.57E+05	6.69E+04	3.47E+08	1.96E+07	0.29
	25 - Aug	CBK	1.76E+05	1.33E+04	9.14E+07	2.21E+07	0.79
		WWTP*	4.43E+06	4.56E+05	3.01E+08	1.40E+07	5.97*

3.5 Conclusions

Nucleotide sequences attributable to *Candidatus* Accumulibacter phosphatis (CAP) were detected in wetland sediments of the Columbia River estuary in varying abundance. The highest densities of CAP were observed in mesohaline sediments of Baker Bay in the spring and summer months. According to phylogenetic analysis, only Type I CAP were found in Baker Bay; in contrast, both Type I and Type II CAP were detected in the southern peripheral bays of the Columbia River estuary, but at much lower abundances. Based on estimates of cellular polyphosphate content derived from previous studies, we estimate that almost all of the sedimentary polyphosphate could come from production by CAP in mesohaline sediments in Baker Bay, but not in oligohaline and freshwater sediments of the southern peripheral bays (Youngs and Cathlamet Bays).

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Chapter 4. RESPONSE OF MICROBIAL ASSEMBLAGES TO TIDAL RECONNECTION IN RESTORED HABITATS OF THE COLUMBIA RIVER ESTUARY[2]

4.1 Introduction

Over 40% of the global population resides along the coast (UNEP, 2006), which provide more ecosystem services than any other environment (Gedan et al., 2009). Wetlands constitute natural filters that purify water entering from rivers, runoff, rain, and groundwater as nutrients are recycled by biota (Coveney et al., 2002; Dunne et al., 2012); this is facilitated by shallow depths, the presence of emergent vegetation, and relatively long water residence times typical of wetland environments (Barbier et al., 2011). These ecosystem services can be financially significant; in the Mississippi delta, a reduction of nitrogen (N) and phosphorus (P) loads of 100 and 66%, respectively, was observed from inflow to outflow through coastal wetlands, with estimated savings of $500,000–$2.6 million in water treatment costs for coastal communities (Day et al., 2005). The global decline in area of estuarine and coastal wetlands has led to an estimated 63% loss of filtering and detoxification, leading to impaired water quality as evidenced by increases in harmful algal blooms, fish kills, shellfish closures, and oxygen depletion events (Paerl, 2006; Worm et al., 2006).

A successful wetland restoration project includes planning, implementation, performance assessment, adaptive management, and dissemination of results; however, many project focus on implementation, since assessment, management, and dissemination require integration of various specialists such as plant and animal ecologists, soil scientists, hydrologists, and disturbance ecologists among others (Thom et al., 2010). Often, very little follow-up is done to assess the success of restoration actions, and when evaluation is performed it often focuses on changes in

[2] Sediment data was contributed by S. Kidd as part of her dissertation work at Portland State University (Kidd, 2017).

habitat, vegetation, fauna, and geomorphology, while changes in biogeochemical processes in soils and sediments are often ignored, depending upon restoration goals (Blackwell et al., 2004). In the Columbia River estuary (CRE), the primary goal of restoration projects has been to repair connectivity among aquatic habitats to regain areas of refuge and rearing for juvenile salmonids that are listed as threatened or endangered under the Endangered Species Act (Roegner et al., 2009). Monitoring metrics for CRE restoration projects include targets for hydrological variables (water surface elevation), water quality (temperature, salinity), elevation (topography), lands*cap*e features, plant community (composition, cover), and fish community (Roegner et al., 2009); the metrics do not include targets for biogeochemical processes such as nutrient transformation or removal. Yet, through their roles in transforming and sequestering or removing nutrients, microbes strongly influence biological productivity and nutrient fluxes to the coastal ocean (Lotze, 2006), rendering this component of restoration vitally important.

The functional recovery of impaired wetland habitats depends not only on restoring physical and structural habitat features such as large woody debris and pools (Nickelson et al., 1992). Effective stream improvement techniques in Oregon coastal streams are aimed at target species, juvenile coho salmon (Oncorhynchus kisutch) to create suitable summer and winter rearing habitat, but because of the role microbes play in nutrient cycling recovery also depends on the composition and metabolic activities of microbial communities (Hartman et al., 2008; He et al., 2015). Microorganisms respond quickly to changes in physical and chemical conditions, making them excellent environmental indicators (Fortunato et al., 2013) that could illuminate the effects of interventions such as wetland restoration. Despite this, the use of individual microbes or particular communities as indicators of wetland restoration is rare (Sims et al., 2013); instead, nutrient removal rates are often reported, which fail to isolate key processes involved in nutrient

transformations (Gutknecht et al., 2006). Nutrient removal rates can also be influenced by the presence of legacy sources in restored habitats, which leaves the false impression that natural settings have lower nutrient filtration capacities than restored sites (Blackwell et al., 2010). Further, nutrient removal rates do not capture the transformations that occur, for example between inorganic forms such as nitrate to more complex organic forms, such as a pools of dissolved organic nitrogen (DON). An investigation two years post restoration demonstrated that wetlands retained or transformed 97% of NO_3-N and 53% of soluble reactive phosphorus (SRP; orthophosphate); however, 20% more DON and 13% more total P (inorganic, organic, and particulate P) was exported because of the restoration action (Ardon et al., 2010a).

The upper extent and discharge of the Columbia River has been modified by the installation of eleven hydroelectric dams on the mainstem river and hundreds of irrigation and power dams on its tributaries (Simenstad et al., 1990). Changes in the annual hydrograph have resulted in significant losses of wetland habitat downstream (Sherwood et al., 1990). In addition, more than 75% of tidal wetlands (~14,560 hectares) in the Columbia River estuary have lost their hydrologic connection to the river as a result of activities aimed at supporting a diverse coastal economy including shipping, fishing, logging, tourism, and agriculture (Simenstad et al., 1990). In Youngs Bay, one of two peripheral bays along the southern shore of the Columbia River estuary and one of its only oligohaline tidal wetlands (Bottom et al., 2011), has lost an estimated 97% of historical tidal wetland habitat since the 1900s (Thomas, 1983). In the 1990s, 12 stocks (ecologically significant units, or ESUs) of Columbia River salmonids were listed as endangered, spurring a movement by major power-producing industries (Bonneville Power Administration, Northwest Power and Conservation Council) to facilitate recovery by focusing on habitat restoration in former wetlands (Johnson et al., 2003).

A natural experimental construct exists in historical Youngs Bay wetlands to investigate microbial communities in restored wetlands and their potential to remove nutrients. The oligohaline wetlands of Youngs Bay were diked and drained primarily to provide land for agriculture; today they are primarily used as cattle/dairy pastures and hay fields, although since 2015 efforts have been underway to restore much of the area back to wetlands (Kidd, 2017). The use of land for agriculture generally leads to an increase in nutrient loading (i.e., of nitrate and phosphate), which often results in eutrophication of downstream aquatic systems (Withers and Haygarth, 2007; Elser and Bennett, 2011; Sharpley et al., 2014).

In effort to assess a return of nutrient removal and transformation to restored wetlands, this study targeted microbes that perform nutrient transformations. For example, Enhanced Biological Phosphorus Removal (EBPR) provides a well-characterized model system with which to study nutrient removal potential by microbial communities (Nielsen et al., 2010; Nielsen et al., 2012). Wastewater treatment relies on a number of core microbial taxa to remove nitrogen, phosphorus, and carbon; a detailed study of 25 Danish treatment plants revealed a strong similarity among them (Nielsen et al., 2010). EBPR communities perform key metabolic functions including denitrification, nitrification, fermentation, hydrolysis and phosphorus removal (Nielsen et al., 2010; Nielsen et al., 2012). The primary organisms responsible for P accumulation are known as polyphosphate accumulating organisms, or PAOs. The dominant PAOs in most EBPR systems belong to *Candidatus* Accumulibacter phosphatis (CAP), an taxon within the Betaproteobacteria (Bond et al., 1995; Hesselmann et al., 1999; Crocetti et al., 2000; Zilles et al., 2002). A previous study showed that in addition to orthophsphate, a variety of organic P forms are present in the Columbia River estuary (Watson et al., in review; Chapter 2),

including polyphosphates (pyrophosphate and polyphosphate), which contributed up to 10% of extracted P in sediments.

Since estuarine wetlands play a vital role in transforming, recycling, and sequestering nutrients and organic matter prior to entering the coastal ocean, they can be considered the wastewater treatment plants of natural aquatic ecosystems (Barbier et al., 2011); thus, it is useful to look for members from the EBPR core microbiome to infer nutrient removal potential in natural systems. For example, the abundance and presence of *Candidatus* Accumulibacter phosphatis (CAP), the most prominent organism for successful EBPR activity and P removal (Bond et al., 1995; Hesselmann et al., 1999; Crocetti et al., 2000; Zilles et al., 2002) should serve as an indicator of a P removal potential as well evidence of alternating cycles of aerobic/anaerobic conditions—required for the accumulation of polyphosphate by PAOs, including CAP (Oehman et al., 2007)—in the natural environment.

Because CAP has been well characterized and has been detected in the Columbia River estuary (Chapter 3), the goal of this work was to track changes in its abundance in response to restoration actions to better understand how they influence microbial populations and the cycling and transformation of nutrients. Active and passive restoration practices in Youngs Bay have resulted in a chronological series of aged restoration wetland sites (a chronosequence) from 1 to 55 years that includes reference sites of natural wetlands with no historical diking, and current diked wetlands used for agriculture (0-year or pasture). The objectives of the study were to analyze microbial community composition in aged restoration sites, to determine whether microorganisms that form part of the core EBPR microbiome are present in the natural environment, and to survey for CAP specifically as a potential indicator of restoration development stage. CAP was chosen because the group possesses a well-characterized single

copy gene involved in polyphosphate synthesis (*ppk1*; He et al., 2007) and therefore gene copy numbers provide a good estimate for cell densities in CAP taxa.

4.2 Methods

4.2.1 *Field sites*

The Columbia River is the second largest by flow in the U.S. (Sherwood et al., 1990) and culminates in a river-dominated estuary with strong seasonality in salinity (Chawla et al., 2008) and biogeochemical properties (Roegner et al., 2011). A set of tidal wetland restoration sites in Youngs Bay watershed (Astoria, OR) were chosen for sediment analysis including 1, 2, 3, 4, 5, 6, 9, 25, 34, and 54 years after tidal reconnection ("post-restoration") and compared with 1 current pasture (0-year, diked and pre-restoration), and 2 reference wetland sites (no historical diking; **Figure 4.1**). A subsample of these sites were chosen to sample microbial communities including years 2, 7, 26, and 55 post-tidal reconnection, and reference sites including two natural wetlands (no diking) and one site currently used as a pasture (0-year). Youngs Bay receives a considerable amount of clays, silts and sands from the river and experiences salinity of 0-5 practical salinity units (PSU) depending upon season, river flow, and tidal cycle (Bottom et al., 2011).

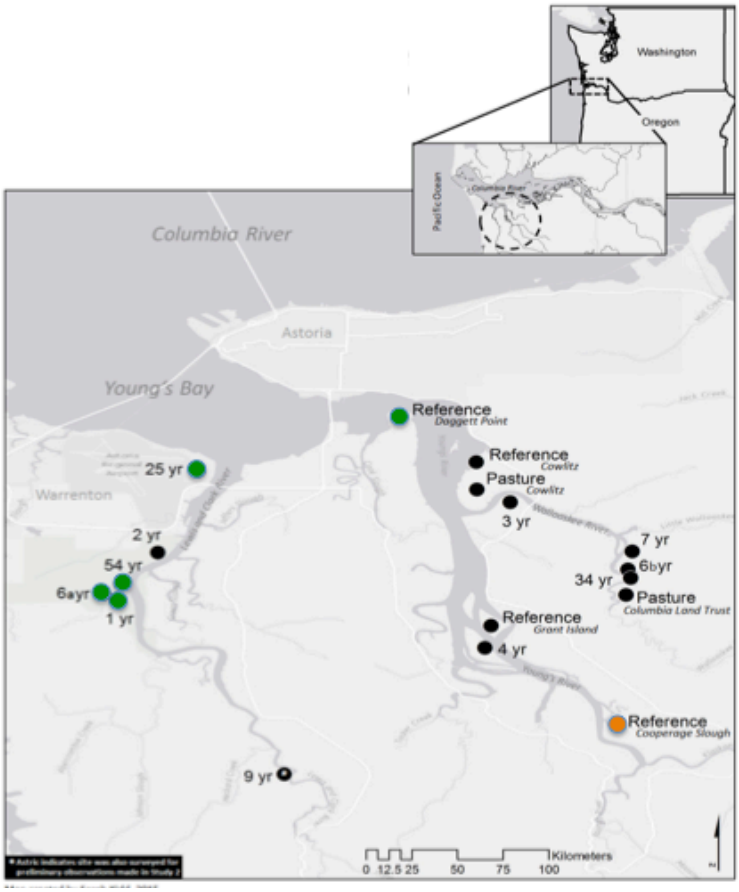

Figure 4.1 Youngs bay restoration and reference sites, green dots indicate restoration sites and two reference sites (green sampled in 2014 and yellow in 2015) sampled for microbial analysis. Map provided by S. Kidd.

4.2.2 *Soil survey*

Surface sediments were collected in restored, pasture, and natural wetland sites during the summer months of 2013 and 2014. Twelve to sixteen soil core samples (8.5 cm width x 30 cm depth) were collected every 20–30 m at transects randomly established within each site parallel to the tidal wetland elevation gradient and alongside vegetation transects. Soil samples from low and mid-elevation were analyzed to provide context for the characterization of microbial communities at the corresponding elevations.

Total phosphorus (P) and nitrogen (N) in sediments were analyzed by the Kjeldahl method (Anderson, 1976; Bremner, 1996). Sediments were further analyzed by the Mehlich III procedure using an acidic solution for multi-elemental extraction of P, K, Ca, Mg, Cu, Fe, Mn and Zn (Mehlich, 1984). A Bray-2 extraction was also done to extract easily exchangeable, acid-soluble P, including P bound to salts (NH_4Cl, KCl) (Bray and Kurtz, 1945). Following extraction, N was analyzed as NH_4^+ and P as PO_4^{3-} using colorimetric methods (US EPA, 1993).

Physicochemical characteristics of the soil samples were determined to contextualize microbial observations in restored and reference sites. Briefly, soil water content was measured by weighing wet sediment sample, drying the sediment in a constant temperature oven, and then reweighing the sample to determine the amount of water removed by weight (Reddy et al., 2013). Soil water content (%) is calculated by dividing the difference between the wet and dry weights by the weight of the wet sample, ultimately reporting the ratio of the mass of water to the mass of wet soil. Bulk density refers to the ratio of the mass of dry solids to the bulk volume of soil and was measured after drying a measured volume of soil ($g\,cm^{-3}$; Blake and Hartge, 1986). Soil pH was measured on dry soil with commercially available electrodes in a 1:1 soil/water slurry (Thomas, 1996). Particle size analysis was done to determine soil texture and is based on the percentages of sand, silt, and clay and making up sediments and was performed by the hydrometer method (Gee & Bauder, 1986). Textures included clay (<0.002 mm), silt (0.002–0.02 mm), and fine sand (0.02–0.25 mm). Finally, loss-on-ignition (LOI, or ashing) was performed to measure the amount of organic matter present in sediments and was done by combusting sediments at high temperatures to oxidize the organic matter and weighing the soil before and after to determine a ratio of lost organic matter (Nelson and Sommers, 1996).

4.2.3 *Sample collection for microbial analysis*

Sediment for microbial analysis was collected in low, low-to-mid, and mid-elevation areas within each sample site described here as (*i*) low elevation with no vegetation, (*ii*) low-mid elevation with sparse vegetation (just above low), and (*iii*) mid elevation with full vegetation. Samples in 2014 were collected at low-to-mid elevation in the top 5 cm of sediment from 3 randomly selected areas within a 5 m radius, homogenized, and sieved to remove large organic materials. Sediments were partitioned into triplicate lysis matrix tubes provided by the FastDNA spin kit for soil (MO Bio Laboratories, Inc., Carlsbad, CA) and flash frozen in liquid nitrogen in the field. Samples were stored on dry ice until return to the lab and stored at -80°C until analysis. Sediment samples collected in the second year (2015) were collected at mid-elevation, perpendicular to the tidal wetland elevation gradient. Three random areas were chosen along a 50 m transect within the top 5 cm of soil, sieved to remove large organic matter and partitioned into triplicate lysis matrix tubes, flash frozen and stored on dry ice until return to the lab and stored at -80°C until analysis.

4.2.4 *DNA isolation and Polymerase Chain Reactions (PCR)*

DNA was extracted (0.5–1.0g) using the FastDNA spin kit for soil (MO Bio Laboratories, Inc., Carlsbad, CA). DNA was quantified and checked for purity using a Nanodrop UV-Vis spectrophotometer (Thermo Scientific, Nanodrop 2000).

PCR for the polyphosphate kinase gene (*ppk1*) associated with CAP was performed in a 25 µl reaction mixture including the following: 10x Ex Taq Buffer, deoxynucleoside triphosphates (dNTP) mix (2.5 mM each), 0.4 µM primers, and 0.5U μl^{-1} of Takara Ex Taq Polymerase (Takara Bio Inc., Mountain View, CA). PCR reactions were carried out on a MyCycler thermal cycler (Bio-Rad Laboratories, Inc.)

The *ppk1* gene was targeted to compare abundances of CAP across chronologically aged restoration sites. Primers ppk-893F/997R produced a 104 bp product (He et al., 2007) and were chosen based on the least number of mismatches against *ppk1* from the completed genome of Accumulibacter Clade IIA UW-1 (referred to as CAP IIA UW-1; AJ224937) (Garcia Martin et al., 2006). PCR reactions were performed as described above with the following conditions: an initial denaturation at 95^{o}C for 5 min, followed by 35 cycles of 94^{o}C for 30 s, 61^{o}C for 45 s, and 72^{o}C for 30 s with a final extension at 72^{o}C for 3 min. DNA isolated from WWTP activated sludge from a wastewater treatment plant that performs EBPR (Durham WWTP, Tigard, OR) was used as a positive control for all PCR reactions for both 16S rRNA and *ppk1* genes.

Amplified PCR products were purified with a QIAquick PCR purification kit (QIAGEN Sciences, MD), ligated into a plasmid and cloned into chemically competent cells with a TOPO 2.1 TA cloning kit (Thermo Fisher Scientific, Grand Island, NY) according to manufacturer's instructions. Transformed cells were grown up on ampicillin–LB plates using blue-white screening. Individual white colonies were picked and inoculated in LB-ampicillin liquid media overnight at 37^{o}C. Plasmids were extracted using a QIAprep Spin Miniprep Kit (Qiagen, CA.) Individual samples were sequenced at Molecular and Cell Biology Core at Oregon Health & Science University (West Campus, Beaverton, Oregon). Operational taxonomic units (OTUs) were aligned against the nucleotide database within NCBI (National Center for Biotechnology Information, NCBI http://blast.ncbi.nlm.nih.gov/).

4.2.5 *MiSeq library preparation and data analysis*

High-throughput sequencing of small subunit ribosomal RNA (16S rRNA) was performed using the PCR primers 515F (5'-GTG CCA GCM GCC GCG GTA A-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3'), which targeted the V4 region of the 16S rRNA

gene (Caporaso et al., 2011). The reverse primer was equipped with an 8 bp barcode, multiplex primers (v. 2.0), and staggered adaptors (added to 806R primer) to increase amplicon diversity and to desynchronize sequence data (JGI-MGM workshop). Both primers contained sequencer adaptor regions (Illumina adaptor P5-forward and P7-reverse).

Sample preparation was performed as described at http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/. Each sample was amplified in triplicate 25 µl PCR reactions, pooled, and cleaned with the QIAquick PCR purification kit (QIAGEN). PCR reactions contained 13 µl MO BIO PCR grade water, 10 µl 5-Prime Hot Master Mix, 0.5 µl each of the forward and unique reverse primers (10 µM final concentration), and 1.0 µl genomic DNA. PCR conditions were 94°C for 3 min, 35 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, with a final extension at 72°C for 10 min. Samples were analyzed by MiSeq (Illumina) sequencing at the Molecular and Cell Biology Core at Oregon Health & Science University (West Campus, Beaverton, Oregon).

The QIIME software package was used to analyze sequence data following MiSeq sequencing (Caporaso et al., 2011). OTU picking and checks for chimeras were performed with usearch6 (Edgar, 2010). Alignment and assignment of taxonomy was done with PyNAST and the Greengenes core alignment (Caporaso et al., 2010; DeSantis et al., 2006; Werner et al., 2012). Statistical analysis was performed in R (R Core Team, 2015) using the vegan package.

4.2.6 *Quantitative-PCR for* CAP *ppk1 gene*

Quantitative-PCR was conducted on a StepOnePlus real time machine (Applied Biosystems, Carlsbad, CA, USA) using Applied Biosystems SYBR green PCR Master Mix (Warrington, UK) with a total reaction volume of 20 µl. Programs consisted of an initial denaturation at 95°C for 3 min, followed by 45 cycles of denaturation for 30 s at 94°C, annealing

for 45 s at 61°C, and extension for 30 s at 72°C, followed by a melting curve calculation. Reactions were performed in triplicate with 1 µl diluted (50–5x) genomic DNA template, 0.5 µM forward (*ppk*-893F) and reverse (*ppk*-997R) primers (He et al., 2007), 100 ng μl^{-1} bovine serum albumin, and 1.5% DMSO. Reactions were run in 96-well plates sealed with adhesive optical covers and centrifuged for 3 min at 1000 rpm prior to analysis.

Five to six-point calibration curves were created by 10-fold serial dilution of triplicate standards for each qPCR reaction ranging 10^3 to 10^8 target copies. Standards were generated using *ppk1* amplicons from activated sludge from the Durham WWTP. Cloning, purification, and sequence analysis for the 104 bp *ppk1* product was performed as described above. The plasmid was linearized with EcoRV digestion (New England BioLabs, Ipswich, MA), purified with a QIAquick PCR purification kit (QIAGEN), and checked for size by gel electrophoresis. Concentrations of all standards were determined with Picogreen using a Nanodrop Fluorospectrophotometer 3300 (ThermoFisher Scientific, Wilmington, DE). Copy numbers were calculated based on the concentration and molecular weight of the plasmid and *ppk1* insert. Each reaction also included a no-template control to check for contamination and primer-dimer formation. Genomic DNA extracted from the EBPR wastewater plant was included in each assay as a positive control.

4.3 Results

4.3.1 *Physical sediment characteristics*

Clay and silt accounted for 21% and 62%, respectively, of soils at the 26-year site, while sand made the largest contribution (47%) at the 0-year site (**Table 4-1**). The pH of restored wetland sediments increased over the chronosequence, ranging from 4.5 at the newly restored (0-year) site to 5.1 at the 55-year site. The pH at the natural reference site was 5.5.

The water content (%) of the sediments increased with age since restoration, ranging from 51.3% at the 2-year site to 71.8% at the 55-year site (**Figure 4.2**B), with the largest intra-site variation in samples occurring at the newly restored site. Water content in sediments at the natural reference site averaged 67%. Organic matter (OM) content generally decreased over the chronosequence, with the highest percentage occurring at the newly restored site (24.4%) and the lowest occurring at the 2-year site (10.4%; **Figure 4.2**C). OM content was 14.2% at the reference site. Finally, bulk density decreased with restoration age, with the highest volume measured at the 2-year site (0.7 g cm^{-3}), and the lowest at the 55-year site (0.4 g cm^{-3}); bulk density at the reference site (0.45 g cm^{-3}) was similar to the 26 and 55-year sites (**Figure 4.2**D).

4.3.2 *Chemical analysis*

Physicochemical characteristics of samples from the restored and reference sites are summarized in **Table 4-2** and **Table 4-3**. Of the cations extracted with Mehlich III, sodium (Na) concentrations were highest, averaging 1655 mg kg^{-1} with the highest concentration occurring at the 7-year site (2355 mg kg^{-1}) and the lowest at the newly restored (0-year) site (541 mg kg^{-1}; Table 1). The reference site concentration was between the two (1625 mg kg^{-1}). Extracted calcium (Ca) concentrations were also relatively high, ranging from 727 mg kg^{-1} at the newly restored (0-year) site to 1073 mg kg^{-1} at the 55-year site; the concentration of Ca at the natural reference site was close the maximum observed (936 mg kg^{-1}). The cation present at lowest abundance was copper (Cu), which ranged from 1.1 mg kg^{-1} (newly restored site) to 3.2 mg kg^{-1} (55-year and natural reference sites).

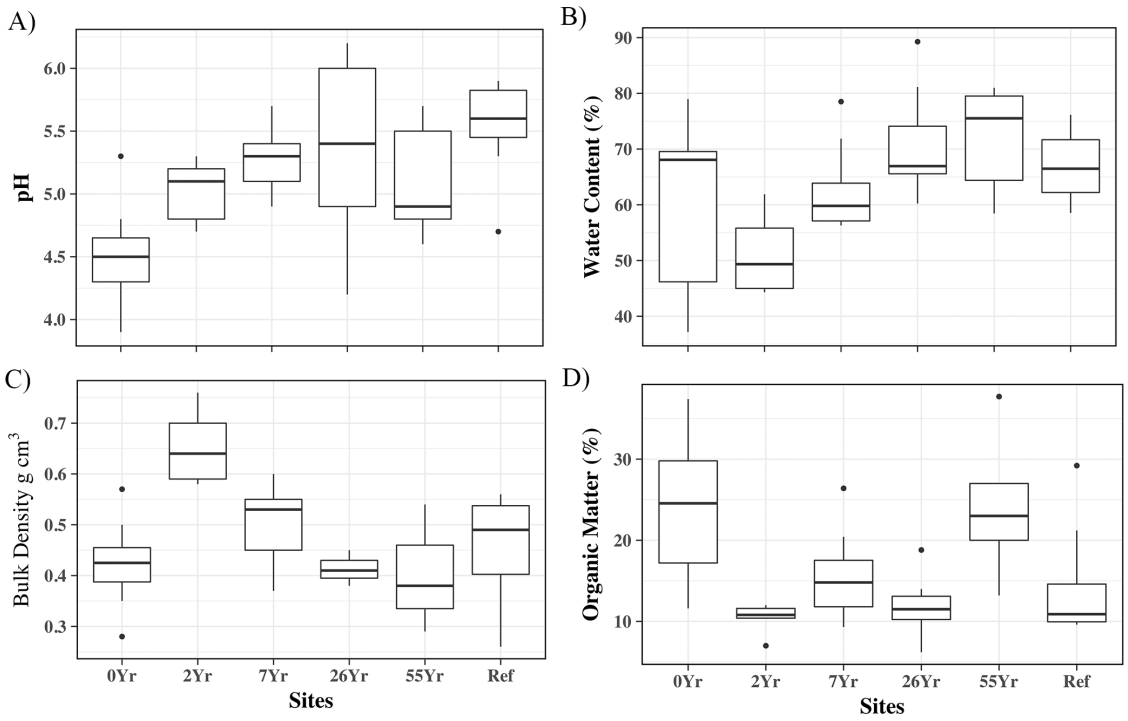

Figure 4.2 Box plots representing the spread of the data collected for sediment characteristics from restored and reference wetland. The bottom of the box represents the first quartile and the top is the third quartile of the data, the whiskers are calculated by multiplying the interquartile range by 1.5 and subtracting it from the first quartile (bottom whisker) and adding it to the third quartile (top whisker) representing a 95% confidence interval around the quartile values, the vertical band is the median, and points outside the box plot are outliers beyond the whiskers. Sediment characteristics include (A) pH, (B) water content (%), (C) bulk density (g cm^3), and (D) organic matter.

Sediment salinities ranged from 3.8 to 5.1 PSU across the sample sites (Table 4.3). Sediment temperatures ranged from 17.2°C (55-year site) to 20°C (newly restored, 0-year site). Oxidation-reduction potential (ORP) ranged from 88 (oxidizing) at the newly restored site to -16 (reducing) at the 2-year site, compared to 20 at the natural reference site (Table 4.3).

Sediment nutrient concentrations from the newly restored and natural reference wetland sites are shown in Figure 4.3. Total P ranged from 642 mg kg^{-1} at the 0-year site to 1686 mg kg^{-1} at the 7-year site, the two highest concentrations occurring at the 2 and 7-year sites (1491 and

1686 mg kg^{-1}, respectively; Fig. 4.3A). The 55-year site had similar average total P concentrations to the reference site (718 and 809 mg kg^{-1}; respectively). P concentrations in the Mehlich III extracts increased from 20 mg kg^{-1} to 46 mg kg^{-1} over the chronosequence, with similar concentrations observed at the reference and 55-year sites (Figure 4.3B). Bray-2 P ranged from 55.6 mg kg^{-1} at the newly restored site to 108 mg kg^{-1} at the 26-year site, which was similar to the concentration at the reference site (106 mg kg^{-1}; Fig. 4.3C); there was a gradual increase in Bray-2 P concentrations over the chronosequence.

The residual P remaining after concentrations of Mehlich III and Bray-2 P are removed is considered highly resistant, or recalcitrant. Residual P concentrations mirrored Total P, where the lowest concentrations were found at the newly restored and 26-year sites (545.3, 541.1 mg kg^{-1}; respectively) (Figure 4.3D). The highest concentration of residual P was found at the 7-year site (1566.5 mg kg^{-1}).

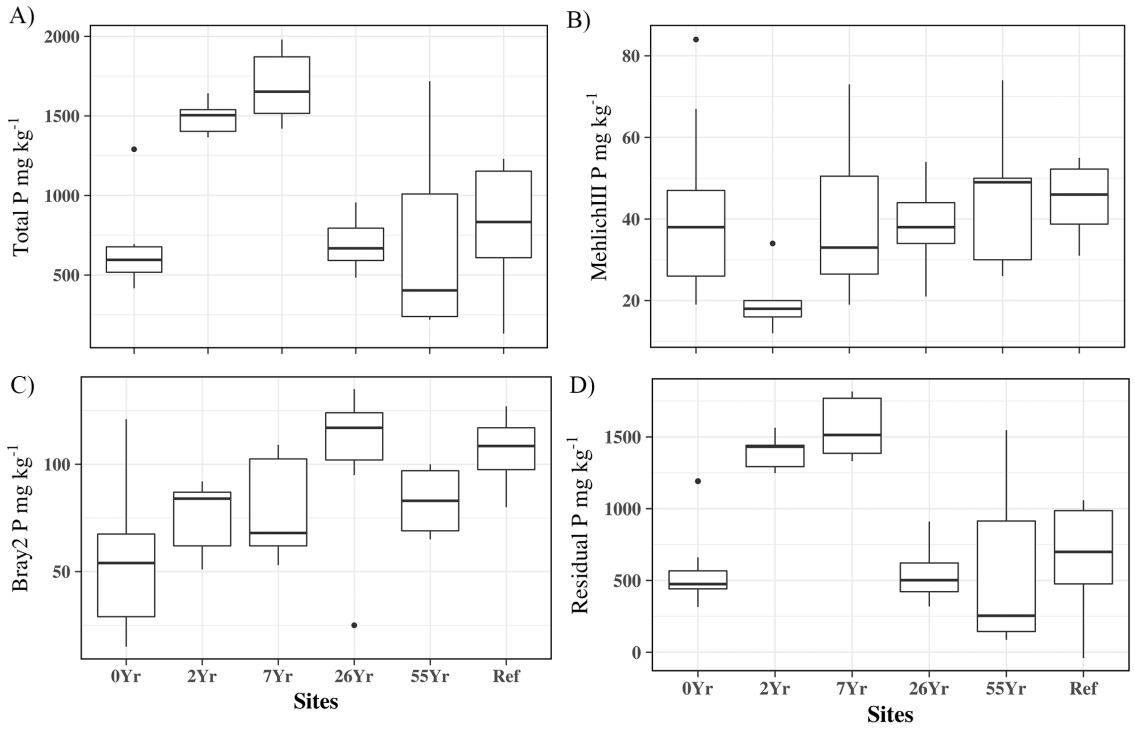

Figure 4.3 Box-and-whisker plots showing the spread of data collected for sediment P concentrations from the restored and reference sites. The bottom of the box represents the first quartile and the top is the third quartile of the data, the whiskers are calculated by multiplying the interquartile range by 1.5 and subtracting it from the first quartile (bottom whisker) and adding it to the third quartile (top whisker) representing a 95% confidence interval around the quartile values, the vertical band is the median, and points outside the box plot are outliers beyond the whiskers. (A) Total P; (B) Mehlich III; (C) Bray-2; (D) residual P.

Table 4-1 Concentrations of cations present in extracts from the Mehlich III phosphorus procedure.

Site	Mehlich III - extracted minerals (mg kg^{-1})								
	Ca	Mg	K	Fe	Al	Na	Mn	Cu	Zn
0 years	727 ±197	541 ±304	190 ±126	502 ±58	1203 ±257	541 ±526	32.6 ±23	1.1±0.4	8.7±4.2
2 years	1063 ±181	889 ±111	281 ±39	531 ±86	890 ±85	1533 ±468	95.2 ±52	2.6±1.0	15.2±3.6
7 years	952 ±136	1012 ±124	303 ±41	467 ±56	1025 ±111	2335 ±538	61.7 ±32	2.1±1.1	9.2±3.2
26 years	862 ±256	822 ±258	272 ±81	473 ±55	639 ±217	1968 ±668	50.3 ±34	2.8±1.9	11.6±4.4
55 years	1073 ±69	1035 ±80	298 ±63	388 ±51	767 ±50	1929 ±458	114 ±42	3.2±0.9	9.3±1.8
Reference	936 ±101	948 ±83	292 ±50	414 ±117	794 ±60	1625 ±303	57.6 ±24	3.2±1.1	11.6±2.6

Table 4-2 Water and sediment characteristics collected during sediment collection for microbial analysis. Soil texture (clay, silt and sand) was analyzed from sediments collected for physical characteristics.

Site	Sediment					
	ORP	Temp (°C)	Salinity (PSU)	Clay (%)	Silt (%)	Sand (%)
0 years	88 ±132	20.1±0.4	4.5±1.6	12.8 ±6	40.5 ±9.0	46.8 ±12
2 years	-16±41	17.5±0.2	4.6±1.2	8.0 ±3.3	56.7 ±9.2	35.3 ±11.9
7 years	37±68	18.3±1.5	4.2±0.8	7.1 ±3.1	54.7 ±11.5	41.2 ±8.8
26 years	78±28	18.1±0.4	5.1±1.6	21 ±0.8	61.9 ±4.5	17.2 ±5.0
55 years	25±34	17.2±0.3	3.8±0.6	9.2 ±2.0	46.9 ±18	43.9 ±16.1
Reference	20±107	19.2±0.8	4.9±1.8	11 ±4.4	54.5 ±9.5	34.5 ±9.0

4.3.3 *MiSeq analysis*

On average, 8561 unique operational taxonomic units (OTUs) were retrieved across five sites, ranging from 7697 OTUs at the 26-year site to 9234 OTUs at the 55-year site. Sequence analysis failed at the natural reference site (only 61 sequences were retrieved), a therefore the sample was not considered in this analysis. Instead,

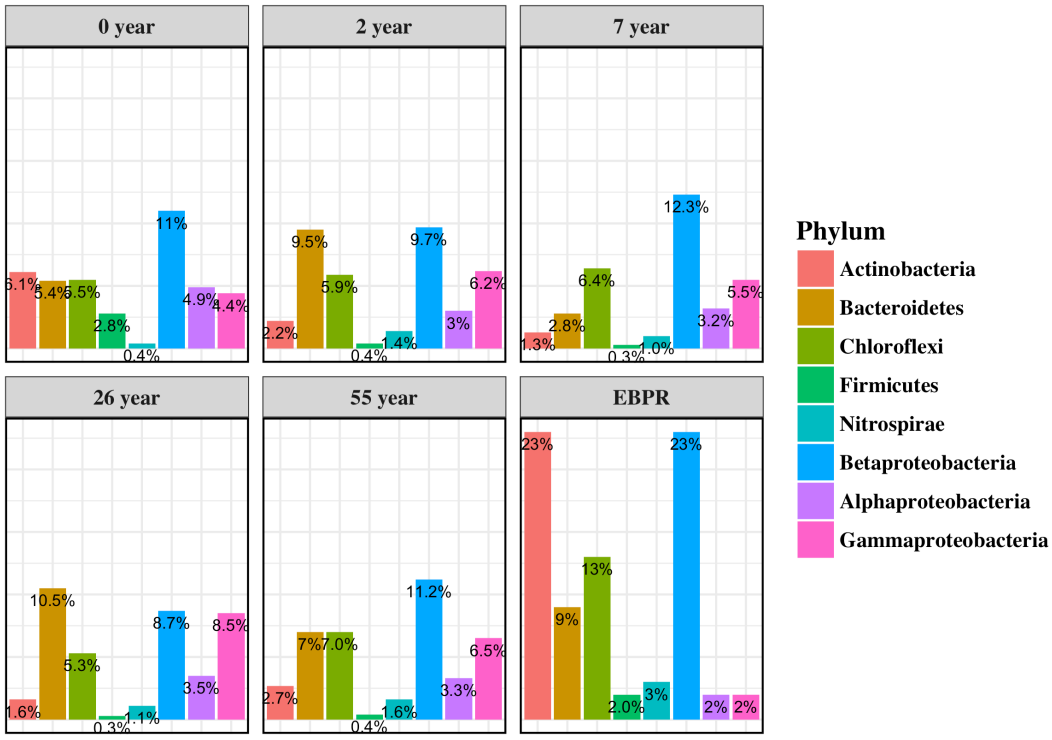

Figure 4.3 Abundances (% of total OTUs) of the core EBPR microbiome phyla from each restored wetland site, and EBPR abundances based on 25 full-scale Danish EBPR plants.

the 55-year sample was considered a "reference" site for comparison. The following phyla were previously identified as core members of a typical EBPR microbiome: Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Nitrospirae, and within the Proteobacteria, classes Betaproteobacteria, Alphaproteobacteria, and Gammaproteobacteria (Nielsen et al., 2012); their average abundances from 25 full-scale Danish EBPR plants are included in Figure 4.4 for comparison to restored wetlands. Percent abundances of the phyla sequenced in restored and reference wetlands were calculated by summing the number of OTUs assigned to each phylum divided by the total OTUs identified in each sample.

In a full-scale EBPR system, the core microbiome makes up 77% of the total bacterial community (Nielsen et al., 2012); the same groups accounted for only 33–41% of the total bacterial community in the restored wetland sites (Figure 4.4). The phyla that showed the greatest differences between restoration sites and EBPR systems were Actinobacteria and the Proteobacteria, which include Betaproteobacteria, Gammaproteobacteria, and Alphaproteobacteria. Actinobacteria represented <3% of the restored site community compared with 23% in EBPR. Betaproteobacteria had the highest abundances in the restoration sites, accounting for 8.7–12.3% of the total community, compared to 23% in EBPR plants. Alphaproteobacteria and Gammaproteobacteria both had greater contributions in restored wetlands (3–3.3% and 6–8.5%, respectively) than observed in EBPR where they contribute to only 2% of the core community.

The phyla that were closer in abundance between restoration sites and EBPR were Bacteroidetes, Chloroflexi, Firmicutes, and Nitrospirae. Bacteroidetes had similar abundances to EBPR microbiomes (9%) in 2 and 26-year sites (9.5 and 10.5%, respectively) but were less abundant in the 7-year site (2.8%). Chloroflexi ranged between (5.3–6.4%) across all restored

wetland sites but less than EBPR (13%). Firmicutes were small contributors in restored wetlands (0.3–0.4%) as well as in EBPR plants (2%). Contributions from Nitrospirae were small (1.4–1.6%) across all restored wetland sites and in EBPR (3%). The pasture had similar abundances in Bacteroidetes, Chloroflexi, Firmicutes, and Nitrospirae, but differed in Actinobacteria, Betaproteobacteria, Alphaproteobacteria, and Gammaproteobacteria.

Through MiSeq analysis, we identified two unique 16S rRNA OTUs belonging to the genus *Candidatus* Accumulibacter (CAP) in the class Betaproteobacteria. CAP OTU2468 was present in samples from 0, 2, and 26-year sites, in addition CAP OTU9316 was present in 0, 2, 26-year as well as the 7-year site. Abundances of CAP OTUs from the MiSeq analysis accounted for <1% of the community, with 0.03% at the newly restored site, 0.04% at the 2-year site, 0.01% at 7 years, 0.05% at 26 years, and no CAP OTUs identified at the 55-year site.

4.3.4 *Quantitative PCR*

Extracted genomic DNA from restored site sediments contained contaminants that inhibited PCR and therefore extracts were diluted in series (20–75x) prior to amplification with primer sets targeting the *ppk1* gene, which increased efficiencies. Standard curves had correlation coefficients of 0.98–0.99 and PCR efficiencies of 87–93% (Table 4.3) indicating that qPCR was accurate and inhibitors were negligible following dilution of extracted DNA. Detection limits were approximately 10^3 copies and no template controls (blanks) were below detection limits.

Table 4-3 Quantitative PCR reaction characteristics for sediment samples from Youngs Bay restoration and reference sites.

Youngs Bay sediment - qPCR assays					
Gene	Site - dilution factor	Slope	Intercept	R^2	PCR Efficiency (%)
ppk1	2014 – Low (50x)	-3.582	38.1	0.997	90.2%
	2014 – Low-Mid (50x)	-3.512	38.6	0.991	92.6%
	2015 – Mid (50x)	-3.672	37.9	0.987	87.2%
	2015 – Mid (50x)	-3.678	38.4	0.984	87.1%

Environmental abundances of CAP (*ppk1*) were determined by qPCR targeting *ppk1* as a proxy for abundance. The completed genome of CAP (Clade IIA UW-1; Garcia-Martin et al., 2006) and draft genomes of Clade IA and IC (Flowers et al., 2013; Skennerton et al., 2015) indicate that both Type I and II subgroups carry a single copy of *ppk1*, and therefore its quantification is a good proxy for cell density. Detectable abundances of CAP were observed at the 2 and 55-year restored and natural site at low and low-mid elevations. However, they were only observed at the natural site at mid-elevation. Abundances of CAP ranged from 6.8×10^4 copies g^{-1} measured at the low-elevation, 55-year site to 2.1×10^5 copies g^{-1} observed at the 2-year site at low-mid elevation (Figure 4.5).

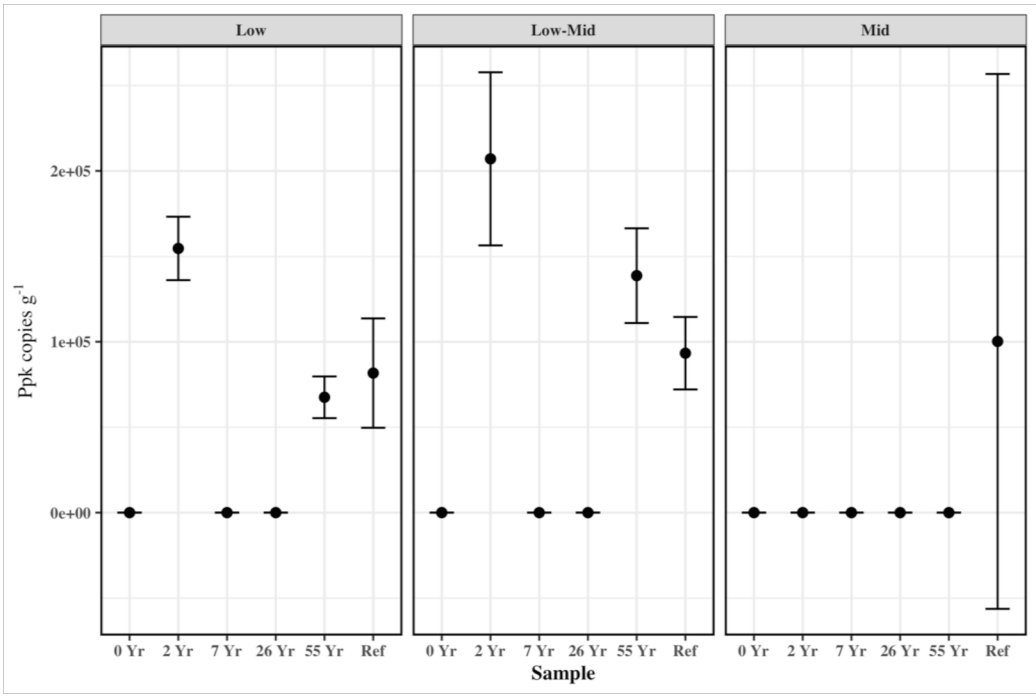

Figure 4.4 Abundances of *ppk1* gene from restoration and reference sites at low, low-mid and mid elevation.

4.3.5 *Redundancy Analysis*

Redundancy analysis (RDA) was performed to identify relationships among sediment P concentrations, sediment characteristics, and EBPR phyla at each of the restoration and reference sites (Figure 4.6). The first biplot displays relationships among the sites and sediment characteristics (Total P, Mehlich III-P, Bray 2-P, water content, bulk density, organic matter, and pH). Variation among sites was explained by pH (RDA1) and Total P (RDA2) concentrations, with reference site 0-year separating from the others due to an association with a high pH and 7-year separating due to an association with high Total P (Figure 4.6A). The RDA analysis also revealed that 2 and 26-year restored sites differed along a gradient of extracted Bray 2-P concentration.

A second biplot (Figure 4.6B) demonstrated relationships among EBPR phyla and characteristics of sediments (Total P, MehlichIII-P, Bray 2-P, water content, bulk density, organic matter, and pH). Eigenvector values associated with RDA1 suggested that variance was best explained by abundances of sequences attributable to the phylum Firmicutes and extracted pH (among environmental variables). The eigenvector values associated with RDA2 indicated that variance in the data set was best explained by Bacteroidetes and Total P (among environmental variables; Figure 4.6B). The EBPR phyla that separated out from the others were Firmicutes, Actinobacteria and Bacteroidetes.

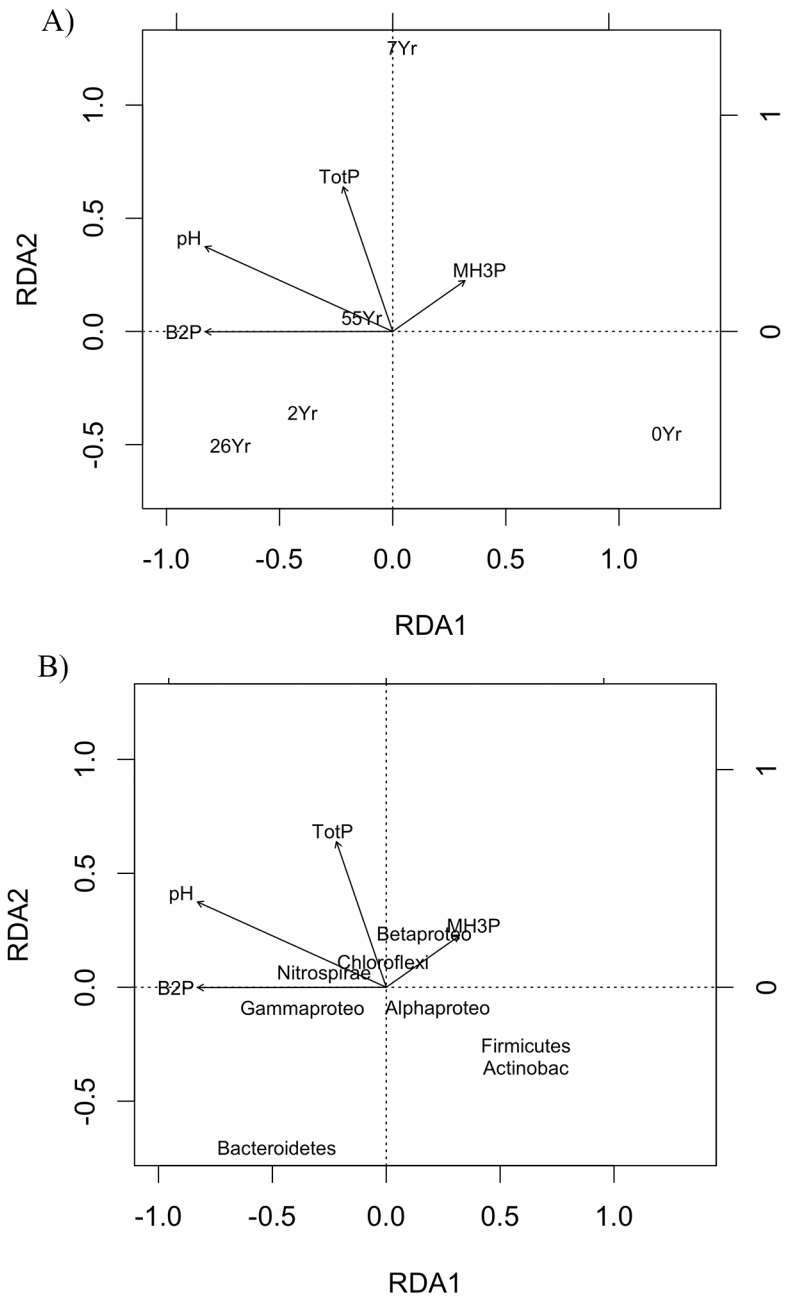

Figure 4.5 Redundancy analysis of: A) sediment characteristics and retored and reference sites, and B) EBPR phyla and sediment characteristics. Abbreviations include: TotP, total sediment P; MH3P, MehlichIII-P; B2P, Bray 2-P; and pH. EBPR phyla include Betaproteo, Betaproteobacteria; Gammaproteo, Gammaproteobacteria; Alphaproteo, Alphaproteobacteria.

4.4 Discussion

A decrease in Total P was observed in older aged restoration sites, and Bray-2 fractions increased with age, suggesting that transformations of P forms accompanied restoration. Bray 2-P concentrations had increased (~ 50 mg kg^{-1}) by 2-years post-restoration, with levels nearing reference concentrations between 7 and 26 years. Bray 2-P extracts include easily exchangeable, acid-soluble P forms such as inorganic orthophosphate bound to salts and cations (Bray and Kurtz, 1945) and is often considered an indicator of active microbial activity (Reddy and DeLaune, 2008). The increase of Bray 2-P suggests P removal and transformation is occurring between 7 and 26-years after tidal re-connection.

The Mehlich III-P method is a multi-element extraction developed as a test to determine the most effective concentration of P in soils for optimum plant growth (45–50 mg kg^{-1}) in both acidic and basic soils. Mehlich III-P primarily extracts inorganic P bound to cations such as Fe, Al, Ca, and Mg and is the standard soil test for P concentrations in the southern U.S. (Mehlich, 1984; Richardson and Reddy, 2013). Mehlich III-P concentrations were below levels required to support plant growth at the 2-year site; in contrast, Mehlich III concentrations at the 7, 26, and 55-year sites, as well as the reference sites (newly restored and pasture) were sufficiently high to support new plant growth. Plant recovery in terms of species composition and productivity (cover) occurred between 3-6 years post-restoration (Kidd, 2017).

There was a pool of residual P in sediments that was highly resistant to extraction, which also means that it should be highly resistant to removal and/or breakdown in the environment (Reddy and DeLaune, 2008). Residual P was relatively low at the reference sites (0-year and natural) and at the 26 and 55-year sites; however, concentrations were much higher at the 2 and

7-year sites. The large concentration of Total P in the 2 and 7-year sites could be due to slowly decaying organic matter (i.e. plant and animal) from legacy use of the formerly diked wetlands as pasture. However, organic matter content at those sites was low (10.4 and 15.5%; respectively) and bulk density was high (0.7 and 0.5; respectively). The changes in soil environments immediately following reclamation caused by an altered flooding regime have dramatic changes on redox potential from oxidized to reducing, and radical alterations of pore water pH from neutral (6–8) to acidic (3), which leads to a large-scale death of plants and changes in microbial fauna (Blackwell et al., 2004). The large, residual pools of P were not present in the 26, 55-year and natural reference site Further study of P forms by P-NMR or other P extraction methods would illuminate whether this resistant P is organic or inorganic and give some information about how it might be transformed, removed, or sequestered.

The greatest abundance of Betaproteobacteria was observed at the 7-year site (12.3%), which was comparable with both reference sites (~11% at both 0-year and natural). The 7-year site also had the greatest concentration of sediment Total P and residual P, indicating the group may be correlated with sediment nutrient concentrations. The Betaproteobacteria include organisms involved in nutrient cycling, including ammonia-oxidizers, denitrifiers, and polyphosphate-accumulating organisms (Mino et al., 1998), and their abundance has been shown to increase in eutrophic aquatic ecosystems (Wobus et al., 2003). Not only have high abundances of Betaproteobacteria been observed in agricultural soils, decreases in their abundances were observed along a gradient of restored wetlands, suggesting they respond to land-use changes (Hartman et al., 2008).

Candidatus Accumulibacter phosphatis (CAP), a member of the Betaproteobacteria, is the most prominent organism responsible for successful removal of P in EBPR plants (Bond et

al., 1995; Hesselmann et al., 1999; Crocetti et al., 2000; Zilles et al., 2002), and its presence suggests that oscillating redox conditions are present in the environment (Oehmen et al., 2007). In one of the few studies surveying for CAP in the environment type I was identified (IB, ID, and IE) in estuary sediments (Peterson et al., 2008) indicating estuaries provide suitable nutrient and redox sensitive requirements.

Through nucleotide sequence analysis (MiSeq), we detected 16S CAP rRNA genes across most of the restored wetland and reference sites, except for the 55-year site. The absence of CAP 16S rRNA genes in the 55-year (natural) site maybe due to increased bacteria phylum observed in natural wetlands. For example, bacteria in natural forest soils are phylum rich compared to agriculture soils, which are species rich, but phylum poor (Roesch et al., 2007). The 55-year (natural) site should be phylum rich, and therefore MiSeq sequencing depth may be to shallow to detect rare taxa as observed in the 2, 7, and 26-year sites (< 1%). Detectable (qPCR) abundances of CAP by the *ppk1* gene however, were observed in 2, 55-year, and the natural reference site at low and low-mid elevation. In addition, the only detectable CAP abundances by *ppk1* at mid elevation were found at the natural reference site. The discrepancy of not observing CAP by MiSeq (16S rRNA) at the 55-year site, but detecting CAP abundances by q-PCR with the *ppk1* gene is due to the increased sensitivity of q-PCR compared to high-throughput sequencing techniques such as MiSeq. Quantitative-PCR for the *ppk1* gene detected at minimal 10^3 copies in diluted DNA extracts, a much more sensitive assay compared to high-throughput community sequencing analysis.

Ordination analysis of the EBPR core phyla also indicated that the 2 and 55-year site were more similar with regards to other taxa when compared in restoration and reference sites. In Chapter 3, CAP sequences recovered from Youngs Bay sediments fell into two major lineages

identified as Type I and Type II subgroups (He et al., 2007). Investigations of CAP population structure in EBPR using fluorescence in-situ hybridization (FISH) probes indicated ecological differences between CAP types; during cycles of high nutrients, Type I dominates and polyphosphate is abundant; later in the process, CAP populations shift to Type II when nutrients are depleted and, consequently, polyphosphate is diminished (Acevedo et al., 2012). CAP Type II phenotypes appear to have a competitive advantage over Type I when nutrients are limiting, and they often outcompete other glucose-accumulating bacteria under conditions where redox conditions fluctuate (Welles et al., 2016). The presence of detectable abundances of CAP in restored wetlands suggests that restored wetlands gain P accumulating functions under the right conditions and as long as they are not outcompeted for carbon sources by other taxa. The presence of CAP in restored sites may indicate a Type II response to low nutrients, while detectable abundances in the 55-year and natural site may reflect a return to more nutrient-limiting conditions. Further work using the *ppk1* gene to assess phylogeny of CAP (Type I or II) in restored wetlands could provide evidence for CAP as an indicator of wetland trophic status and therefore restoration development stage.

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Chapter 5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Significance

Estuaries are biologically productive transition zones that play a vital role in transforming, recycling, and sequestering nutrients prior to entering the coastal ocean (Barbier et al., 2011); these transition zones are important areas of P retention and transformation. My dissertation provides new knowledge about the cycling of phosphorus in the Columbia River estuary through the characterization and quantification of phosphorus forms in wetland sediments using ^{31}Phosphorus – NMR spectroscopy. This technique has led to more knowledge about phosphorus (P) cycling in the environment than almost any other method (Cade-Menun, 2015). However, fewer than 20 publications have used ^{31}P-NMR for studies in wetlands, and even fewer (<5) report findings from estuary wetlands. This study provides an initial description of P forms in lateral bay sediments using ^{31}P-NMR. Knowing which forms are present yields insight into possible pathways for retention/sequestration and transformation and provides a structural framework for investigations of biogeochemical processes, which control the distribution, dynamics and availability of P in soils. However, estuaries are complex, dynamic systems and this work should be viewed as an initial survey, variability within sites was not measured and patterns between sites should be supported by further work.

Candidatus Accumulibacter phosphatis (CAP) is a well-studied bacterium discovered in wastewater treatment plants that removes remove excess P from wastewater prior to re-entry into aquatic ecosystems by accumulating phosphorus as polyphosphate. In contrast to wastewater systems, very little work exists on CAP in a natural environment. The only work on CAP in a natural environment led to the identification of nucleotide sequences (Type I) in freshwater ecosystems located near EBPR wastewater plants (Kunin et al., 2008; Peterson et al., 2008). The

results presented in this dissertation are the first to characterize CAP (Type I and II) in a natural environment with oscillations in redox conditions, such as an estuary. CAP has the potential to remove and/or flux orthophosphate in a natural oscillating environment, and further may serve as a microbial indicator of ecosystem health.

Microbes have been proposed as indicators of recovery of biogeochemical cycling in wetlands as they respond quickly to changes in physical and chemical conditions in sediments (Sims et al., 2013). The abundance of members of the class Betaproteobacteria (which includes CAP) has been correlated with trophic status, with higher abundances present in agricultural soils. Changes in their abundances have been associated with restoration age gradients, suggesting that they respond to land-use change (Hartman et al., 2008). In this dissertation I have reported detectable abundances of CAP in restored wetlands. The abundance of members of the class Betaproteobacteria (which includes CAP) has been correlated with trophic status, with higher abundances present in agricultural soils, which provides support for the idea that restoration of tidal connections results not only in changes to physical habitat, but also to biogeochemical function of sediments.

More than 75% of tidal wetlands (~14,560 hectares) in the estuary have lost their hydrologic connection to the river as a result of activities aimed at supporting a diverse coastal economy (Simenstad et al., 1990). Youngs Bay, one of the only oligohaline tidal wetlands in the Columbia River estuary (Bottom et al., 2011), is estimated to have lost 97% of historical tidal wetland habitat since the 1900s due to human activities (Thomas, 1983). By tracking the presence and abundance of CAP in restored wetlands, I was able to show that biological P cycling potential changes with land-use changes in wetlands of the lower Columbia River estuary.

5.2 Conclusions

I utilized ^{31}P-NMR to identify and quantify structural forms of P in lateral bay sediments of the Columbia River estuary to set a structural framework for investigation of biogeochemical cycling of P (Chapter 2). A range of P forms were detected across the estuary with inorganic forms (orthophosphate) dominating and organic forms making up ~30% of extracts. Polyphosphate and pyrophosphate were found throughout the lateral bays in some sites contributing up to 10% of extracted P forms. Variability of P forms was low across the salinity gradient; however, phytate and orthophosphate were more abundant at sites with low dissolved oxygen concentrations. Low dissolved inorganic P (DIP) measured across the lateral bays and variable total sediment P concentrations suggest the occurrence of P buffering or mineral precipitation reactions within sediments and perhaps rapid cycling of available orthophosphate. Preliminary data from a few samples in late summer may support the degradation of phytate as a source of DIP observed in late summer months as previous studies have hypothesized sourced from lateral bays (Gilbert et al., 2013).

Based on the discovery of polyphosphates in lateral bay sediments, I set out to identify a novel organism, *Candidatus* Accumulibacter phosphatis (CAP), a polyphosphate-accumulating organism potentially uniquely suited to life in an estuary (Chapter 3). Estuaries are dynamic and biologically productive ecosystems that are characterized by large fluctuations in environmental conditions, offering an ideal setting to evaluate the conceptual model of PAO metabolism (Figure 5.1) in a transitional, redox-sensitive environment. I retrieved nucleotide sequences associated with CAP using both the 16S rRNA and polyphosphate kinase (*ppk1*) genes in a survey of lateral bay sediments in the Columbia River estuary. Sequences associated with both

Types II and I CAP were identified by phylogenetic analysis in all lateral bays except for Baker Bay sediments, which all came from Type I.

Using the *ppk1* gene as a proxy for abundance, northern Baker Bay sediments had the largest relative abundances of CAP ranging from 0.3–3% across seasons, which are similar to levels observed in traditional wastewater treatment plants. Southern lateral bays (Youngs and Cathlamet Bay) by comparison, had lower relative abundances of CAP (0.01–0.32%). Abundances of CAP at BBB in Baker Bay sites were only detectable in spring and summer, while a second site in Baker Bay (BBA) had lower, but detectable levels throughout the year. CAP has the potential to contribute to P cycling in lateral bay sediments of the Columbia River estuary, depending upon season and location ranging from 0.1 to 18 mg kg^{-1} of P (polyphosphate) in sediments, with the greatest influence occurring in brackish sediments at the mouth of the estuary (BBB). The occurrence of CAP throughout estuary wetland sediments indicates they may occur in many environments at the sediment-water interface in which microzones rich in oxygen and nitrate from overlying water and labile organic matter from below would be available, and local hydrodynamic and/or physiology conditions would result in redox fluctuations inducing storage of excess P by polyphosphate under both nutrient replete and nutrient limited conditions.

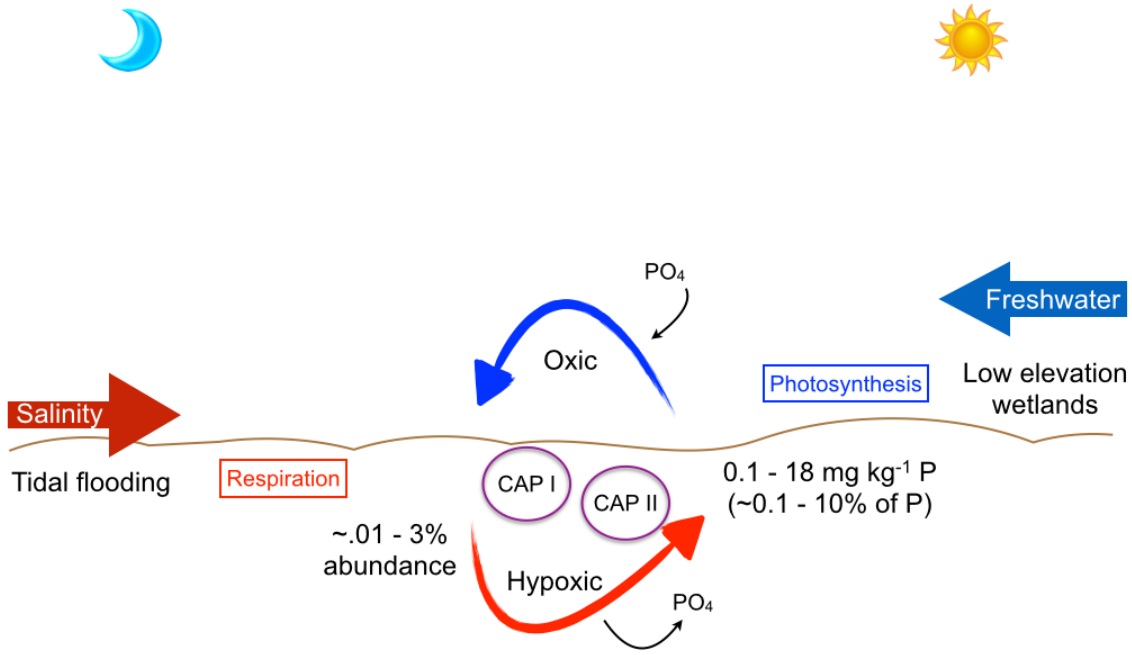

Figure 5.1 A conceptual model of CAP in estuary sediments of the lateral bays of the Columbia River estuary.

In a natural experimental construct in Youngs Bay of the lower estuary I tested the hypothesis that CAP, along with other members of the Betaproteobacteria class, may respond to sediment trophic status and serve as an indicator of land-use change such as wetlands undergoing restoration mitigation. In aged restoration sites total sediment P decreased, while sediment inorganic P increased in aged restoration sites, indicating that legacy sediment P underwent transformation, and removal from sediments post 7-year restoration. Using the EBPR core microbiome as a model of nutrient removal I compared phyla abundances in aged restored wetlands to assess wetlands ability to remove nutrients; Betaproteobacteria was a prominent class across restored wetland sites as well as in wastewater treatment. Quantifiable abundances of CAP occurred in the 2 and 55-year restored and the natural (reference) site, which indicates

that CAP may respond to sediment nutrients. The 2-year site had the highest total P sediment concentrations, while 55-year and the reference site had the lowest levels measured. Phylogenetic differences as observed in EBPR systems indicate that CAP Type I does well in nutrient-replete conditions and Type II in nutrient limiting environments. The CAP phenotypes may respond to low bioavailable nutrients as observed in the 2-year site, and by contrast when nutrients return to normal and/or limiting levels as observed in the 55-year and natural wetland site. A proposed conceptual model for CAP as an indicator of land-use change and/or trophic status of sediments is summarized in Figure 5.2.

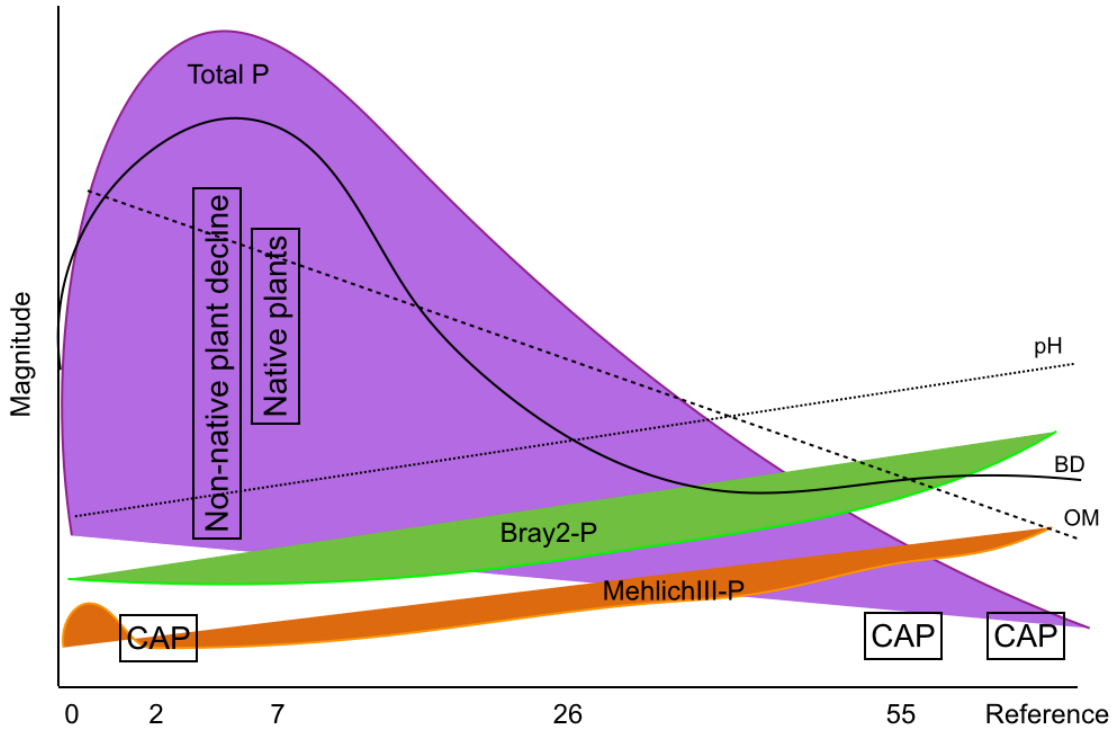

Figure 5.2 Conceptual model for CAP as an indicator of trophic level of soils in wetlands and as an indicator of wetland restoration development.

5.3 Future Directions

This work is one of only a handful of investigations of P cycling in estuary sediments using ^{31}P-NMR, in addition the work described here only characterizes CAP in a natural environment further work is warranted to definitively link CAP to P cycling and provide further evidence as a potential indicator of sediment trophic status and land-use change further questions might include the following:

1. Is there a daily and/or seasonal diversity in P forms in the lateral bays of the estuary? Preliminary data from ^{31}P-NMR analysis of a few lateral bay sediments indicated a difference in organic P (Orthophosphate monoesters and diesters) with season (spring and summer), and diel differences in inorganic P (polyphosphates and pyrophosphates). Further work will help to determine seasonal and daily patterns of P cycling including potential sources and pools.
2. What is the source of the large orthophosphate pool in sediments of the Columbia River estuary? Buffering experiments and further extraction methods for phosphorus with estuary sediments would help determine the source of orthophosphate (i.e. biotic and/or abiotic or both), which would forecast the potential for future eutrophication by excess P in the estuary.
3. What are the CAP phylogenies present in the lateral bays of the estuary? Using the 16S rRNA gene we identified sequences affiliated with both Type I and II, but further phylogeny analysis with the *ppk1* gene would definitively identify CAP types and clades which would provide a link to sediment trophic level in sediments in the lateral bays.

Further, analysis of diel CAP metatranscriptomics would provide information regarding activity of CAP and potentially linking to production of polyphosphates (P-NMR).

4. Can CAP be directly linked to production of polyphosphate in the estuary? Further, can CAP produced polyphosphate be linked to oxyhydroxides and/or mineral precipitation (i.e. Ca) such as observed in diatoms or sulfur bacteria (Schulz and Schulz, 2005; Diaz et al., 2008)? CAP is uncultivable by traditional methods but survives for brief periods of time in bench-top bioreactors and/or field microcosms. Further work could investigate CAP activity in estuary sediments and follow polyphosphate production through diel or tidal cycling.

5.4 References

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