Microbial Phosphorus Cycling and Community Assembly in Wetland Soils and Beyond

by

Wyatt H. Hartman

Department of Environment
Duke University

Date:_______________________
Approved:

Curtis J. Richardson, Supervisor

____________________________________
Emily S. Bernhardt

____________________________________
Daniel D. Richter

____________________________________
Rytas Vilgalys

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Environment in the Graduate School of Duke University

2011
ABSTRACT
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Abstract

Although microbes may strongly influence wetland phosphorus (P) cycling, specific microbial communities and P metabolic processes have not been characterized in wetlands, and microbial P cycling is poorly understood across global ecosystems, especially in soils. The goal of this work is to test the effects of stress and growth factors on microbial communities in wetlands, and on microbial P metabolism and P cycling at ecosystem scales in wetland soils and beyond. I conducted field and laboratory research experiments in wetland soils, which by definition lie along gradients between terrestrial and aquatic ecosystems, and I explicitly compared results in wetlands to adjacent ecosystems to improve inference and impact.

To test relationships between microbial communities, soil stress and resource supply, I compared the distribution and abundance of uncultured bacterial communities to environmental factors across a range of wetland soils including a well-characterized P enrichment gradient, and restoration sequences on organic soils across freshwater wetland types. The strongest predictor of bacterial community composition and diversity was soil pH, which also corresponded with the abundance of some bacterial taxa. Land use and restoration were also strong predictors of bacterial communities, diversity, and the relative abundance of some taxonomic groups. Results from wetland soils in this study were similar to both terrestrial and aquatic ecosystems in the relationship of pH to microbial communities. However, patterns of biogeography I observed in wetlands differed from aquatic systems in their poor relationships to nutrient availability, and from terrestrial ecosystems in the response of microbial diversity to ecosystem restoration.
Accumulation of inorganic polyphosphate (PolyP) is a critical factor in the survival of multiple environmental stresses by bacteria and fungi. This physiological mechanism is best characterized in pure cultures, wastewater, sediments, and I used $^{31}$P-NMR experiments to test whether similar processes influence microbial P cycling in wetland soils. I surveyed PolyP accumulation in soils from different wetland types, and observed PolyP dynamics with flooding and seasonal change in field soils and laboratory microcosms. I found PolyP accumulation only in isolated pocosin peatlands, similar to patterns in the published literature. I observed rapid degradation of PolyP with flooding and anaerobic conditions in soils and microcosms, and I characterized the biological and intracellular origin of PolyP with soil cell lysis treatments and bacterial cultures. While degradation of PolyP with flooding and anaerobic conditions appeared consistent with processes in aquatic sediments, some seasonal patterns were inconsistent, and experimental shifts in aerobic and anaerobic conditions did not result in PolyP accumulation in soil slurry microcosms. Similar to patterns in wetlands, I found prior observations of PolyP accumulation in published $^{31}$P NMR studies of terrestrial habitats were limited to acid organic soils, where PolyP accumulation is thought to be fungal in origin. Fungal accumulation of PolyP may be useful as an alternative model for PolyP accumulation in wetlands, although I did not test for fungal activity or PolyP metabolism.

To evaluate relationships between microbial P metabolism and growth, I compared concentrations of P in soil microbial biomass with the soil metabolic quotient ($q$CO$_2$) by compiling a large-scale dataset of the carbon (C), nitrogen (N) and P contents of soils and microbial biomass, along with C mineralization rates across global wetland and terrestrial ecosystems (358 observations). The ratios of these elements
(stoichiometry) in biomass may reflect nutrient limitation (ecological stoichiometry), or be related to growth rates (Biological Stoichiometry). My results suggest that the growth of microbial biomass pools may be limited by N availability, while microbial metabolism was highly correlated to P availability, which suggests P limitation of microbial metabolism. This pattern may reflect cellular processes described by Biological Stoichiometry, although microbial stoichiometry was only indirectly related to respiration or metabolic rates. I found differences in the N:P ratios of soil microbial biomass among ecosystems and habitats, although high variation within habitats may be related to available inorganic P, season, metabolic states, or P and C rich energy storage compounds. Variation in microbial respiration and metabolic rates with soil pH suggests important influences of microbial communities and their responses to stress on metabolism and P cycling.

My dissertation research represents early contributions to the understanding of microbial communities and specific processes of microbial P metabolism in wetlands, including PolyP accumulation and Biological Stoichiometry, which underpin microbial cycling of P and C. Together, my research findings broadly indicate differences in microbial P metabolism among habitats in wetlands and other ecosystems, which suggests the prevailing paradigm of uniform P cycling by microbes will be inadequate to characterize the role of microbes in wetland P cycling and retention. While I observed some concomitant shifts in microbial communities, PolyP accumulation, and microbial stoichiometry with soil pH, land use, and habitat factors, relationships between specific microbial groups and their P metabolism is beyond the scope of this work, but represents an exciting frontier for future research studies.
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1. Introduction

1.1 Problem statement

Human agricultural activities and land use change have led to dramatic shifts in global phosphorus (P) cycling, with considerable impacts on water quality in downstream ecosystems receiving P inputs from watershed runoff (Bennett et al. 2001, Carpenter 2008, Filippelli 2008, Schindler et al. 2008, Paerl 2009). Wetlands play a critical role in controlling landscape transport of phosphorus (P), and substantial efforts to restore wetland ecosystems have been motivated by their potential to mitigate impacts to aquatic ecosystems that are often P limited (Johnston 1991, Zedler 2003, Carpenter 2008, Richardson 2008, Hoffmann et al. 2009). Remobilization of P in wetland soils with shifts in hydrology presents a particular challenge to wetland management, and although P retention is often considered to be a function of the abiotic sorption potential of soil iron, aluminum, and clay minerals (Richardson 1985, Johnston 1991, Aldous et al. 2005, Aldous et al. 2007), microbes may also strongly influence wetland P dynamics. Microbes dominate P uptake kinetics in wetland soils (Richardson and Marshall 1986, Kellogg and Bridgham 2003, Noe et al. 2003), and large proportion of soil P in wetlands may be held within the biomass of microbes, ranging from 25-50% of total P in wetland mineral soils (Walbridge 1991, Wright et al. 2001, McDowell and Sharpley 2003) to up to 70% of total P in ombrotrophic peat soils (Williams and Silcock 2001).

Despite the significant role of microbes in wetland P cycling, influences of specific microbial P metabolic processes on wetland P cycling are not well understood, and the composition and function of uncultured microbial communities in wetlands has not previously been explored (Gutknecht et al. 2006). Key linkages between microbial community structure and biogeochemical functions are especially understudied in
wetlands (Drenovsky et al. 2008), with previous work largely limited to the study of
culturable microbes involved in specific transformations of C, N, S, and Fe, which likely
do not reflect the structure and functions of natural assemblages (Gutknecht et al. 2006).
Understanding the influences of microbial metabolism on wetland P cycling is
particularly challenging, as the relevant processes are not only poorly explored in
wetlands, but have also received scant attention and review across all global ecosystems.

Microbes play a critical role in controlling phosphorus cycling and retention in
both aquatic and terrestrial ecosystems (Schulz and Schulz 2005, Gadd 2007, Ballantyne
which is coupled with global cycling of carbon, nitrogen, and oxygen (Kump and
recent reviews have synthesized knowledge of specific metabolic processes, taxonomic
differentiation, and biogeochemical significance of microbial turnover of carbon (Conrad
2010), sulfur (Muyzer and Stams 2008, Barton and Fauque 2009), and iron (Weber et al.
2006), phosphorus (P) is commonly overlooked in reviews of microbial element cycling
(Gutknecht et al. 2006, Falkowski et al. 2008). Relationships between microbial
metabolism, taxonomic differentiation and P cycling are poorly understood, and not
well integrated even in marine ecosystems where microbial P cycling has been best
studied (Dyhrman et al. 2007, Paytan and McLaughlin 2007).

Still less is understood about microbial P metabolism in soils, due to a
considerably greater focus on carbon and nitrogen cycling in most terrestrial ecosystem
studies (Bradford et al. 2008, Strickland et al. 2010, Vitousek et al. 2010), despite
widespread co-limitation of primary productivity by N and P across terrestrial, wetland,

1.2 Motivation and scope of inquiry

The primary purpose of the research in this dissertation is to contribute to the basic understanding of controls over microbial communities, P cycling, and metabolism in wetland soils, including effects of wetland restoration. However, there is considerable impetus to evaluate the generality of patterns and processes in wetlands, as Microbial P cycling is poorly understood and in need of synthesis across all ecosystems, and the relevant processes in wetlands may be illuminated by comparisons to adjacent terrestrial and aquatic ecosystems. Comparisons across ecosystems may benefit our understanding of both biogeochemical cycling (Grimm et al. 2003) and microbial ecology (Dinsdale et al. 2008, Angly et al. 2009), as microbial life is distributed across all habitats of the biosphere (Handelsman et al. 2007). Such large-scale comparisons may serve to improve inference in the study of microbial communities, as they encompass global endpoints of environmental gradients in moisture, nutrients, and productivity along which different microbial communities, metabolic processes, and biogeochemical patterns are arrayed. The study of microbial P metabolism and communities in wetlands may conversely aid in the interpretation of global patterns, as wetland ecosystems
encompass environmental gradients spanning terrestrial and aquatic ecosystems, and are hotspots of biogeochemical activity that critically influence transfer of elements from land to waters.

Thus, while the field research and experimental manipulations of microbial P cycling and communities in this dissertation are focused in wetland soils, these findings are explicitly compared to patterns and processes in terrestrial soils, aquatic ecosystems, and cultured organisms throughout the text. To address the need for an empirical basis to compare microbial P cycling across markedly different terrestrial, wetland, and aquatic ecosystems, where possible I have compiled large scale meta-analyses across ecosystems to compliment narrative review of published findings, establishing relevant patterns and processes in wetland soils and beyond.

The individual chapters in this dissertation contribute discovery-based research to explore basic patterns in microbial communities, and effects of P metabolism on phosphorus cycling and other ecosystem functions. Due to the primitive state of prior knowledge and practical limitations on dissertation research, efforts to determine biogeochemical processes and community patterns are tested separately. However, given the broad significance of these processes, considerable effort is made to compare data and results with findings in other ecosystems, and to closely examine mechanisms linking microbial P metabolism to ecosystem structure-function relationships.

1.3 Research goals and objectives

The general purpose of the research presented in this dissertation is to address critical knowledge gaps in scientific understanding of the role of microbial communities in phosphorus cycling, especially in wetland soils where microbial P cycling is of considerable interest for ecosystem management. Although I separately evaluate
patterns in microbial community structure and P metabolism, I approach each from the perspective of life history strategies, in particular comparing relationships of each with \textit{growth} and \textit{stress} responses.

Life history theory postulates the functional traits of organisms and their biogeographic patterns in the environment may be shaped by selection for growth (\textit{r}- vs. \textit{K}- selection) and stress responses (C,S,R strategies) (Pianka 1970, Grime 1977, Parry 1981), and microbial growth and stress responses may be closely coupled with P metabolism. In particular, microbial responses to \textit{stress} are coordinated by accumulation of inorganic polyphosphate (PolyP), which critically shapes global P biogeochemical cycling, and is well studied in wastewater treatment systems and aquatic sediments (Seviour et al. 2003, Brown and Kornberg 2004, Schulz and Schulz 2005, Hupfer and Lewandowski 2008, Seufferheld et al. 2008). Differences in microbial \textit{growth} rates may be coupled with cellular P concentrations as the result of relationships between ribosomal P demand and general metabolism described across heterotrophs by the growth rate hypothesis (GRH) (Elser et al. 1996, Elser et al. 2000b). These metabolic processes may critically determine the influence of microbes over P cycling, and link conditions in the environment (resources, stress) to microbial communities and P turnover at ecosystem scales.

The goals of this dissertation are to 1) to compare patterns in bacterial biogeography in response to \textit{stress} and resource availability (\textit{growth}), and 2) evaluate the biogeochemical significance of microbial P metabolism linked with \textit{stress} (PolyP) and \textit{growth} (GRH) responses. These goals and specific objectives are described below:
GOAL 1: Patterns of microbial communities with resources and stress

Exploratory biogeographic approaches are a critical first step to establish gradient responses of species and infer controls over communities, and suggest functional traits. I postulate microbial community responses to resources and stress in wetlands may be common across wetlands, and potentially with terrestrial and aquatic ecosystems.

Objective 1: Bacterial biogeography across wetland soil gradients

To determine how microbial communities respond to environmental resources and stresses, I compare the biogeography and diversity of bacteria in wetland soils across different wetland types and land uses, spanning a range of environmental conditions and soil pH values. This range of conditions includes samples obtained from a wetland eutrophication gradient caused by agricultural P runoff, and from cropped, restored, and reference wetlands. I compare controls over bacterial communities in wetlands to those in terrestrial and aquatic ecosystems to evaluate the generality of my findings (Chapter 2).

GOAL 2: Scaling of microbial P physiology to ecosystem P biogeochemistry

Ecosystem biogeochemistry is shaped by the physiology of organisms, but microbial P metabolism is poorly understood, especially in soils. Representative microbes are not readily obtained from soils to allow direct testing of physiological processes, but controls over metabolism may be inferred from ecosystem scale variation in biogeochemical processes using metabolic scaling approaches (Enquist et al. 2003, Enquist et al. 2007, Sinsabaugh and Shah 2010). Conversely, the application of scaling
approaches is particularly well suited to assess the biogeochemical implications of metabolic processes, as measurements are obtained at ecosystem not organismal scales.

**Objective 2: Scaling of P metabolism and stress (Polyphosphate)**

To determine the biogeochemical significance of effects of environmental stresses on microbial P cycling, I test the distribution of PolyP across a range of wetland soils encompassing common stress gradients, and evaluate the effects of dynamic stresses with natural flooding events and experimental manipulation of oxygen availability. These results are compared by meta-analysis to patterns in terrestrial and aquatic ecosystems to aid in the interpretation of research findings and the search for common biological and environmental mechanisms (*Chapter 3*).

**Objective 3: Scaling of P physiology and growth (Stoichiometry)**

To address the role of microbes in P biogeochemistry, and potential differences in P metabolism along environmental gradients, I test the ecological and biological stoichiometry of microbial biomass in published data from wetland and terrestrial soils, including relationships with rates of respiration per unit microbial biomass. This global scale data synthesis approach was chosen due to problems with measurements of both microbial element pools and respiration in wetlands, the scarcity of available data in wetlands, and the desire to compare causes and effects of microbial biomass stoichiometry across the extremes of soil conditions across global ecosystem (*Chapter 4*).

In *Chapter 5*, I review the findings of each of these studies, and discuss their implications for understanding microbial P cycling and P retention in wetlands.
2. Environmental and anthropogenic controls over bacterial communities in wetland soils

2.1 Introduction

Soil bacterial communities play a critical role in regulating the cycling, retention, and release of major nutrients and soil carbon in freshwater wetlands, with demonstrably large effects on water quality (Richardson 2008) and global carbon cycling (Roulet 2000). However, little is known about the taxonomic composition of uncultured soil bacteria in freshwater wetlands relative to other ecosystems, despite the disproportionate influence of wetlands in controlling biogeochemical cycling at landscape scales (Richardson 2008). With a single exception in a Sphagnum bog (Dedysh et al. 2006), existing knowledge of bacterial communities in freshwater wetlands has been obtained using DNA fingerprinting (D’Angelo 2005, Angeloni et al. 2006), group specific probes (Kowalchuk et al. 1998, Sizova et al. 2003, Castro et al. 2004), or culture-based methods (Sizova et al. 2003), which either have not identified bacterial taxonomic groups or do not adequately represent the vast diversity of uncultured soil bacteria (Tiedje et al. 1999). Furthermore, the environmental and anthropogenic factors controlling the distribution and abundance of bacterial groups in freshwater wetland soils are unknown.

To predict the effects of ecosystem change on wetland functions, improved understanding of the ecological responses of uncultured bacterial communities to ecosystem alteration is needed to compliment existing knowledge of bacterial functional groups controlling specific biogeochemical processes. The importance of understanding controls over wetland bacterial communities is underscored by the unique nature of wetlands as transitional ecosystems, the role wetland bacteria play in regulating
biogeochemical fluxes across different ecosystem types, and increasing efforts to restore
the functionality of degraded wetlands subjected to land use change (Mitsch and
Gosselink 2007). In this unique study, I demonstrate the spectrum of uncultured
bacterial communities across a range of freshwater wetland types and quantify the
influence of soil chemistry, land use, restoration, and soil nutrient concentrations on
bacterial assemblages.

Freshwater wetlands are transitional gradients between terrestrial and aquatic
ecosystems, and thus may have environmental and anthropogenic controls over
bacterial community structure similar to those of their neighboring ecosystems. Land
use (McCaig et al. 2001, Buckley and Schmidt 2003) and soil chemistry (McCaig et al.
2001, Kennedy et al. 2004) have been shown to control microbial communities in several
terrestrial systems. Ecosystem restoration has also been shown to alter microbial
communities in terrestrial (DeGrood et al. 2005, McKinley et al. 2005) and wetland
systems (D’Angelo 2005), although the specific phylogenetic groups of microbes affected
by restoration have not yet been determined in either of these systems. Eutrophication
and productivity gradients appear to be the primary determinants of microbial
community composition in freshwater aquatic ecosystems (Rajendran et al. 1997, Wobus
et al. 2003). To capture the range of likely controls over uncultured bacterial
communities across freshwater wetland types, I sampled sites representing a range of
soil chemistry and land uses, including reference wetlands, agricultural and restored
wetlands, and sites along a nutrient enrichment gradient.

The sites selected represented a range of land uses encompassing natural,
disturbed, and restored conditions across several freshwater wetland types, including
pocosins (evergreen shrub bogs), riverine and nonriverine swamp forests, and
calcareous fens. I determined the relative abundance of major phylogenetic groups of bacteria present (Fig. 1), and basic soil chemistry (Appendix A, Table 12) at nine sites in the North Carolina (NC) coastal plain (pH 3.5–6.0) and four sites in the Florida Everglades along a well-studied (Richardson 2008) nutrient enrichment gradient (pH 6.5–7.4, soil P concentrations ranging from 1,800 mg kg⁻¹ to 350 mg kg⁻¹). At each of the NC coastal plain wetland complexes I sampled soils from the following three land uses: (i) a wetland that had been converted to row crop agriculture; (ii) a restored wetland where ditches had been filled, tree seedlings had been planted, and natural vegetative recolonization had occurred; and (iii) a reference wetland representing conditions of the undisturbed ecosystem. I compared changes in the relative abundance of bacterial phylogenetic groups to soil chemistry (pH, % carbon, % nitrogen, and % phosphorus) and land use categories.

2.2 Materials and methods

2.2.1 Site descriptions, soil collection and soil analyses

Soil samples were collected in the fall of 2003 from a range of wetland sites along gradients of differing land use history in the North Carolina coastal plain, and along a eutrophication gradient in the Florida Everglades. I sampled three NC coastal plain locations that each had agricultural wetlands, restored wetlands, and reference wetlands in close proximity: Barra Farms, Long Swamp, and Parker Farms.

The Barra Farms site (BF) is part of a 975-ha Carolina bay complex located in Cumberland County, North Carolina (Bruland et al. 2003). Soils at the site have been classified as Croatan mucks (Terric Haplosapristes). Past alterations to the site included clearing and ditching in the 1960s for conversion to agriculture, and intensive farming during the 1970s and 80s. In the fall of 1997, 250 ha of the site were restored to wetland...
by filling ditches and planting woody seedlings. Samples were obtained from existing agricultural soils (BFA), from the 6-year-old restored area (BFS), and from a reference site in a nonriverine swamp forest section of the site that was never converted to agriculture (BFF).

Long Swamp (LS) is a 10-ha site located in Hoke County, North Carolina. The soils at the site have been classified as Johnston loams (Cumulic Humaquepts) and Rains loamy sands (Typic Paleaquults). The site is located in a flat, forested headwater area of LS stream. Past alterations of the site include clearing and ditching for conversion to agriculture, as well as timber harvesting. The site was restored in 1998 by filling in ditches and planting woody seedlings. Soils were collected in restored areas that had been impacted by agriculture (LSA) and a 5-year-old forest clearing (LSC), as well as from a reference forested section of the site that had not been previously cleared (LSF).

Parker Farms (PF) is a 160-ha site located in Beaufort County, North Carolina (Bruland and Richardson 2006). The soils at the site have been classified as Wasda mucks (Histic Humaquepts) and Ponzer mucks (Terric Haplosaprists). This site was originally a nonriverine swamp forest that was cleared, ditched, and converted to agriculture. In 1995 the site was restored by filling ditches and planting with woody seedlings. Samples were collected from a nearby agricultural field with Terric Haplosaprists soils that had just been incorporated into the Pocosin Lakes National Wildlife Refuge (PFA), as well as from the 8-year-old restored area of Parker Farms (PFS), and a reference wetland on the Parker Farms tract (Bruland and Richardson 2006).

The Florida Everglades is part of an ongoing study along a 40-year nutrient-enrichment gradient in the northern part of the subtropical Everglades (26° 15′ N, 80° 23′ W). Surface-water and soil P has been shown to be elevated above natural background
concentrations up to 7 km into the interior of WCA-2A (DeBusk et al. 1994, McCormick et al. 1996). Soils were collected at 1, 3, and 6 km from the D water control structure in WCA-2A, along a well-studied nutrient-enrichment gradient that declines in intensity moving away from the water control structure. Plant communities were dominated by Typha domingensis at 1 and 3 km along the gradient (D1T and D3T, respectively), while at 6 km, samples were collected from areas dominated by Cladium jamaicense (D6C) and from open sloughs colonized by Eleocharis elongata (D6E).

At each sampling location, the top 10 cm of soil was collected from three points within a 5-m radius. Soils were sieved wet and replicate samples were pooled and homogenized. Soil organic matter was determined by loss on ignition, total N was determined by carbon, hydrogen, nitrogen (CHN) analysis, total P was determined by Murphy Riley following a perchloric acid digest (O'Halloran and Cade-Menun 1993), and pH was determined in 1:1 soil:water slurries.

### 2.2.2 Bacterial 16S rDNA sequencing and analysis

Soil DNA was extracted using an Ultra Clean MoBio soil DNA extraction kit (MoBio Labs). Extracted DNA was amplified using bacterial specific 16S rDNA primers BSF 343/15 (TACGGRAGGCAG) and BSR 926/20 (CCGTCAATTYTTTRAGTT), which amplify a ca. 560-bp fragment (Wilmotte et al. 1993). DNA was amplified by PCR with an initial denaturation step of 94 °C for 3 min, followed by 25 cycles of 94 °C for 1 min, 50 °C for 30 s, and 72 °C for 2 min, and a final annealing step at 72 °C for 7 min. Ninety-five clones were obtained from each soil sample by cloning amplified DNA using a TOPO TA cloning kit (Invitrogen Corp.). Individual clone colonies were amplified by PCR using denaturation at 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with a final annealing step at 72 °C for 10 min. Clone
PCR products were purified using a Qiagen PCR purification kit (Qiagen, Inc.). Amplified clone DNA was sequenced using ABI BigDye (Applied Biosystems, Inc.) on an ABI 3700 capillary DNA sequencer. Sequences were deposited in GenBank under accession numbers EF443271–EF444484.

Microbial DNA sequences identified were compared to NCBI Blast (Benson et al. 2004) and RDP sequence classifier databases (Maidak et al. 2001) for identification, with only close matches (> 98%) accepted for identification. Only about 15% of sequences were identified with database matches. Poorly matching sequences (< 65% identity) were screened for chimeric recombination using RDP Chimera Checker (Maidak et al. 2001). OTUs at 97% sequence similarity (Stackebrandt and Goebel 1994) were obtained for each using Sequencher (Gene Codes, Inc.). Phylogenetic identities of unknown sequences were determined by creating a phylogenetic tree of sequences in the clone library (Fig. 1) using parsimony analysis in PAUP (Sinauer Assoc., Inc.). Maximum likelihood analyses were not used because of the large size of the clone library (> 1,300 sequences). Phylogenetic identities of unidentified sequences were assigned at phyla or class levels by comparing clade positions to sequences identified by BLAST and RDP, and known sequences from a database of 218 16S sequences representing major bacterial groups obtained from RDP (Maidak et al. 2001).

2.2.3 Data analysis

Microbial diversity was calculated from both OTU data and phylogenetic data by obtaining Shannon’s index (H’) using EstimateS (Colwell and Coddington 1994). Microbial diversity (H’) was compared to soil C, N, P, and pH using simple linear regression in S-PLUS (Version 6.2, Insightful Software, Inc.). Phylogenetic data on microbial community composition at each site was compared to soil chemical
parameters (C, N, P, and pH) using partial and pure-partial Mantels’ tests (Mantel 1967, Sanderson et al. 1995, King et al. 2004) of Euclidean distance matrices. The Mantel test procedure was carried out in S-PLUS using code developed by S. Goslee (Urban et al. 2002). The significance of the Mantel correlation was assessed by permutation, as the elements of these matrices are not independent (Manly 1997). Significance of the coefficients was estimated by bootstrapping with 1,000 random permutations. Mantel correlation coefficients do not behave like product-moment correlation coefficients, and do not have to be large in absolute value to be statistically significant (Legendre and Fortin 1989). Path diagrams (Leduc et al. 1992) were created as a visual framework for examining the correlations among bacterial community composition, land use, and soil chemistry. Ordination of bacterial communities was performed using principal components analysis (PCA) of the relative abundance of different taxonomic groups compared to soil chemistry data using PC ORD 5 (MjM software design). I also used UNIFRAC (Lozupone and Knight 2005) to compare bacterial communities among sites and land use treatments, and results from UNIFRAC ordination were nearly identical to those obtained by PCA. I used results from PCA ordination based upon the relative abundance of taxonomic groups rather than sequence-based distance from UNIFRAC because 16S rRNA sequence phylogeny does not accurately represent bacterial taxonomy, but rather is useful as a taxonomic marker to be compared to known sequence phylogeny, as in my approach using relative abundance of taxonomic groups determined by phylogenetic relationships to a guide tree of known organisms.
2.3 Results and discussion

2.3.1 Taxonomic composition of bacteria across wetland types

Uncultured bacteria communities obtained from wetland soils represented a number of diverse evolutionary lineages, and I resolved the taxonomic identity of the vast majority of cloned 16S rDNA fragments (Fig. 1).

Figure 1: Phylogenetic tree of 16S rDNA sequences obtained from freshwater wetland soils. Percent of total composition of major clades is given from 95 sequences at each of 13 sites. Minor clades are numbered: 1) Nitrospira-2.6%; 2) unknown - 4.0%; 3) Spirochaetes - 1.3%; 4) Prosectobacter - 2.8%; 5) Planctomyces - 1.0%. Not shown are Gemmamantimonas - 0.7% and Cyanobacteria 0.7%. Clade sizes are not directly proportionate to percent composition due to uneven inclusion of known guide sequences.
The taxonomic composition of soil bacterial assemblages varied greatly between soils of NC coastal plain wetlands and the Florida Everglades, but much less within these two regions (Fig. 2). The bacterial groups present were similar among soils from pocosin bogs, and riverine and nonriverine swamp forests in the NC coastal plain, although the relative abundance of the groups present varied markedly. The composition abundance of dominant bacterial groups was unexpectedly uniform among soils collected along the Everglades nutrient-enrichment gradient, a result contrasting with observed shifts in the diversity of methanogenic Archaea along this gradient (Castro et al. 2004).

Bacterial communities in soils from NC coastal plain wetlands included diverse assemblages of bacterial phylogenetic groups (see Fig. 2), dominated by the Acidobacteria (mean 38.1% of clones), α-proteobacteria (17.4%), and Actinobacteria (9.7%). Other bacterial groups present included the Cytophaga- Flavobacterium-Bacteriodies (CFB) groups and the β-, δ-, and γ- divisions of the phylum Proteobacteria. These bacterial taxa together accounted for an average of 86.6% of the bacterial clones obtained from soils of the North Carolina pocosin bogs, and riverine and nonriverine swamps.

Bacterial communities in soils along the Everglades nutrient enrichment transect (see Fig. 2) were dominated by green nonsulfur bacteria (GNSB, mean 38.1% of clones), δ- proteobacteria (14.6%), and α- proteobacteria (12.2%). Other bacterial groups present included β- and γ- proteobacteria, Nitrospira, CFB groups, Acidobacteria, Spirochaetes, and an unknown bacterial clade. These bacterial groups accounted for 89.1% of the clones obtained from soils along the Everglades nutrient enrichment gradient.
Figure 2: Taxonomic composition of bacterial communities across different freshwater wetland ecosystems and land use types. Taxonomic composition was determined by a phylogenetic tree of 95 clones of bacterial 16S rDNA from each site (Fig. 1). Site abbreviations are color coded by land use: nutrient enriched Everglades sites are brown, agricultural wetlands are red, restored wetlands are blue, and reference wetlands are green. Sites abbreviations are described in detail in Materials and Methods.

Most of the bacterial groups present in the freshwater wetlands I studied are widely distributed in surveys of uncultured microbial communities across terrestrial and aquatic ecosystems, although the composition of these groups varies across ecosystems (Appendix A, Table 13). The abundances of bacterial groups in the Everglades sites diverged the most from other ecosystems. Bacterial assemblages in the pocosin bogs I sampled were similar to those found in a *Sphagnum* bog (Dedysh et al. 2006). I used
Mantel’s tests to determine the independent influences of soil pH, land use, and nutrient concentrations on the distribution, abundance and diversity of bacterial taxonomic groups across several freshwater wetland types.

2.3.2 Controls over bacterial communities by soils and land use

Bacterial community composition and diversity responded most strongly to soil pH across all of the wetland sites surveyed. Bacterial communities were highly correlated with soil pH \((r = 0.853)\), even after accounting for the effects of wetland type, land use and restoration, and all other soil chemical variables using pure-partial Mantel’s tests \((r = 0.747)\) (Fig. 3). Soil pH also predicted diversity of bacterial phyla and “species” (97% sequence similarity operational taxonomic units or OTUs) across all wetland sites (Fig. 4 A and B). Effects of environmental pH on bacterial community composition and diversity have been recently noted in aquatic (Lindstrom et al. 2005) and terrestrial ecosystems (Fierer and Jackson 2006), respectively, although the present work is unique in linking pH with sequence-based changes in bacterial communities. Observed shifts in bacterial communities with pH do not appear to be methodological artifacts, as soil DNA-extraction efficiency does not vary with pH (Zhou et al. 1996). Fittingly, I observed a strong increase in the abundance of Acidobacteria with lower pH (Fig. 4C), a relationship also found across terrestrial soils (Eichorst et al. 2007). The abundances of the Actinobacteria and α-proteobacteria had curvilinear relationships with soil pH (Fig. 4D), suggesting pH optima for these taxa.
Figure 3: Mantel path analysis linking taxonomic composition of microbial communities to soil chemistry, land use, and wetland type. (A) All wetland types surveyed, land use categories are: Everglades water conservation area (WCA), agriculture, restored, and reference. (B) North Carolina coastal plain wetlands were analyzed separately to determine effects of wetland restoration. Solid lines are partial Mantel correlation coefficients, while dashed lines are pure-partial Mantel correlation coefficients, conditional on all other variables. Where Mantel correlations are significant, line width is proportional to the correlation coefficient, and p-values are in parentheses.
Figure 4: Effects of soil pH on bacterial diversity and the relative abundances of select bacterial taxa. Effects of pH on diversity are assessed by (A) Shannon’s index (H’) of phylogenetic groups, and (B) “species” level operational taxonomic units (OTUs). Soil pH also determines the abundance of some bacterial taxa, including (C) Acidobacteria, and (D) Actinobacteria, and α-proteobacteria.

Land use also significantly influenced bacterial community composition across wetland types. Bacterial assemblages clearly differed between wetland soils from the NC coastal plain and the Florida Everglades, and among coastal plain wetlands based on land use (Fig. 5). Land use predicted bacterial community composition across all of the wetland sites studied, even after accounting for wetland type and soil chemistry using pure-partial Mantel’s tests (see Fig. 3A). Land-use change in upland systems has been shown to influence microbial community composition across disturbance gradients, ranging from agricultural fields to fallow grasslands to undisturbed
grasslands (McCaig et al. 2001, Buckley and Schmidt 2003), and among different cultivation practices in agricultural fields (Girvan et al. 2003).

Figure 5: Principal components ordination of bacterial community composition shows clustering by land use in wetland soils. Axis 1 explains 52.6% of variance, while Axis 2 describes an additional 19.2% of variance among samples. Factor loadings are shown with blue vectors for bacterial taxonomic groups with > 20% loading. Bacterial group abbreviations (clockwise from top) are Acid: Acidobacteria; δ-pr: δ-proteobacteria; GNSB: Green Non Sulfur Bacteria; Nitr: Nitrospira; Spir: Spirochaetes; β-pr: β-proteobacteria; CFB: Cytophaga-Flavobacterium-Bacteriodies; Pros: Prosectobacter; α-pr: α-proteobacteria; Acti: Actinobacteria. Site abbreviations are described in detail in Materials and Methods.

To determine the effects of wetland restoration on bacterial assemblages, I separately analyzed bacteria only in NC coastal plain soils, where restored sites could be compared with agricultural and reference wetlands within the same wetland type. Bacterial community composition was strongly related to wetland restoration category ($r = 0.713$), even after accounting for wetland type and soil chemistry using pure-partial Mantel’s tests ($r = 0.593$) (see Fig. 3B). Bacterial diversity at both species and phyla levels was negatively correlated with wetland restoration, with significant differences among restoration categories at all NC coastal plain sites in Shannon’s index ($H'$)-based
OTU accumulation ($P = 0.006$) and phylogenetic tree categories ($P = 0.001$) (data in Appendix A, Table 14). Wetland restoration also strongly influenced the normalized ratio of *Proteobacteria* to *Acidobacteria* (Fig. 6A), which is believed to reflect soil trophic status (Smit et al. 2001), and resulted in decreased abundance of the $\beta$-proteobacteria relative to agricultural soils (Fig. 6B).

Figure 6: Abundance of bacterial taxonomic groups varies with land use across wetland soils. Italicized letters indicate statistically different groups determined by Tukey’s multiple comparisons. (A) Land use altered the normalized ratio of all *Proteobacteria* to *Acidobacteria* ($P < 0.001$). (B) The abundance of $\beta$-proteobacteria was greater in agricultural wetlands than in other land uses ($P < 0.001$). Statistical grouping of sites was the same for the normalized ratio of $\beta$-proteobacteria to *Acidobacteria* as for the ratio of all *Proteobacteria* to *Acidobacteria* ($P < 0.001$, data in Appendix A, Table 14). Land use categories follow those in Fig. 5.

Bacterial diversity of restored wetlands was intermediate between higher diversity agricultural soils and lower diversity reference wetlands at all of the NC coastal plains sites (Fig. 7), a result opposite of that found in restoration of terrestrial ecosystems, where reference soils have the most diverse bacterial communities (DeGrood et al. 2005, McKinley et al. 2005). However, wetland soils in this study were restored from agricultural fields rather than spoils (DeGrood et al. 2005), and unlike more neutral soils (McKinley et al. 2005), were likely limed as well as fertilized. Liming has also been shown to affect microbial communities in acidic grassland soils, although by decreasing rather than increasing diversity (Kennedy et al. 2004). Wetland
restoration generally represents a return to less fertile soil conditions, characterized by partial recovery of acidity and anoxia in soils following the cessation of liming and fertilization, and increased flooding (Bruland et al. 2003), which may limit the diversity of bacteria by increasing metabolic stresses. Suitably, the lower bacterial diversity in reference wetland soils appears to be related to increased dominance of the Acidobacteria in less disturbed wetlands (see Figs. 2 and 5).

![Graph](image)

**Figure 7**: Soil bacterial diversity shifts with land use and restoration across NC wetland types. Collector’s curves present the number of unique bacterial “species” (defined at 97%) obtained from a given site, called operational taxonomic units (OTUs). Restoration land use categories are Agriculture (red), Restored (blue), Reference wetlands (green). Wetland types are (A) pocosin bogs, (B) riverine swamp forests, (C) non-riverine swamp forests. Sites abbreviations are described in detail in Materials and Methods.

I found some correspondences between soil nutrient concentrations and bacterial communities of wetland soils. Soil nitrogen and phosphorus concentrations were correlated with bacterial community composition across all wetland types (see Fig. 3A). However, soil nutrient concentrations did not predict bacterial community composition in wetland soils of the NC coastal plain (see Fig. 3B), and there was little difference in bacterial community composition along the Florida Everglades nutrient enrichment transect (see Figs. 2 and 5).

Weaker relationships between nutrients and bacterial communities I observed at
local scales may suggest regional scale relationships are the result of high nutrient concentrations and distinct bacterial communities in Everglades soils (see Fig. 5). Although microbial communities reflect trophic status in aquatic ecosystems (Rajendran et al. 1997, Wobus et al. 2003), the response of microbial communities in wetland soils may be less pronounced as a result of the predominance of soil-bound nutrients in wetlands (Richardson 2008), as microbial communities often do not correspond to soil nutrient status in terrestrial soils (McCaig et al. 2001, Smit et al. 2001). Stronger relationships between bacterial communities and nutrients in wetlands may also result from analysis of available nutrient pools instead of total nutrient concentrations in future studies.

2.4 Conclusions

My findings demonstrate responses of bacterial communities to environmental and anthropogenic gradients in wetland soils, and I emphasize a comparative approach with terrestrial and aquatic ecosystems. This approach to linking biogeography with ecosystem change is complimentary with studies that seek to determine bacterial functional groups (Zak et al. 2006). Although specific bacterial groups have been linked to biogeochemical cycles in wetlands [e.g. (Kowalchuk et al. 1998, Sizova et al. 2003, Castro et al. 2004)], structure-function relationships vary in degree and kind with biogeochemical process, and element cycling may be affected by previously unknown organisms (Wagner 2002). I emphasize that understanding controls over the distribution and abundance of uncultured bacterial communities is a required first step in determining structure-function relationships that compliment attempts to delineate functional guilds (Naeem and Wright 2003). My approach also addresses the lack of prior knowledge of the composition and controls over uncultured bacterial communities
in freshwater wetlands, and the impact of environmental change on these ecosystems, which may alter both bacterial community structure and function.

The results of this study reveal shifts in the composition of whole bacterial communities, and the abundance of specific taxonomic groups with environmental gradients that may reflect changes in biogeochemical cycling. Soil pH broadly altered the composition and diversity of bacteria in wetland soils, and affected specific taxa, including the *Acidobacteria*, *Actinobacteria*, and *α-proteobacteria*. Soil pH also alters bacterial growth and biogeochemical process rates in mixed peat-bog cultures (Sizova et al. 2003) and controls degradation of lignocellulose in wetlands (Benner et al. 1985). The effect of pH on decomposition might be mediated by shifts in bacterial composition with pH, as the acidophillic *Acidobacteria* are oligotrophs characterized by slow growth rates and metabolism of more refractory carbon substrates characteristic of peat soils (Eichorst et al. 2007). Analogously, I observed a greater abundance of the *β-proteobacteria* in agricultural soils (see Fig. 6B), and shifts in their abundance along restoration gradients (see Fig. 5), suggesting an important response to land-use change. The *β-proteobacteria* increase in abundance with eutrophication in aquatic ecosystems (Wobus et al. 2003), and are capable of ammonium oxidation (Kowalchuk et al. 1998, Wagner 2002), denitrification, and polyphosphate accumulation (Wagner 2002), indicating an important role of this group in nutrient cycling in eutrophic ecosystems. Changes in the abundance of bacterial groups may be readily indexed, as I found wetland restoration altered the normalized ratio of *Proteobacteria* to *Acidobacteria* (see Fig. 6A), which has been suggested as a broad indicator of trophic status across a range of terrestrial soils (Smit et al. 2001).
I discovered that the composition, relative abundance, and diversity of bacterial groups in wetland soils were determined by soil pH, land use, and restoration. While relationships between soil pH and bacterial communities were consistent with those in aquatic (Lindstrom et al. 2005) and terrestrial ecosystems (Fierer and Jackson 2006), wetland restoration resulted in decreased bacterial diversity, a finding opposite of results obtained in terrestrial soils (DeGrood et al. 2005, McKinley et al. 2005). Relationships between nutrient concentrations and wetland bacterial communities were less robust than those in aquatic systems, but not unlike results in terrestrial systems. Further cross-system comparisons of bacterial communities and environmental gradients may reveal emergent properties across ecosystems, like those linking terrestrial and aquatic biogeochemistry (Grimm et al. 2003). My findings may also have more immediate implications, as I demonstrate bacterial indicators that may be applied to wetland restoration and management, like those suggested for terrestrial (Harris 2003) and aquatic ecosystems (Paerl et al. 2006).
3. Distribution and dynamics of microbial polyphosphate accumulation in wetland soils compared to aquatic and terrestrial ecosystems: a $^{31}$P-NMR study

3.1 Introduction

Wetlands play a critical role in controlling landscape transport of phosphorus (P), and substantial efforts to restore wetland ecosystems have been motivated by the potential for wetlands to improve downstream water quality, often in P limited aquatic ecosystems (Johnston 1991, Zedler 2003, Carpenter 2008, Richardson 2008, Hoffmann et al. 2009). The major mechanisms controlling long term P retention in wetlands are generally thought to reflect abiotic processes, including physical trapping of suspended sediments rich in P, or adsorption of inorganic P to aluminum and iron in soil clay minerals and humic ligands (Richardson 1985, Reddy et al. 1999, Darke and Walbridge 2000, Hoffmann et al. 2009). However, remobilization of soil P may pose a significant challenge for management of long term P retention in wetlands, with commonly observed releases of inorganic P following flooding and development of soil anoxia (Reddy et al. 1999, Aldous et al. 2005, Aldous et al. 2007, Zak et al. 2008, Ardon et al. 2010) resulting from biogeochemical processes that arise primarily from the metabolism of soil microbes.

Although flood-induced releases of inorganic P in wetland soils are often ascribed to anaerobic reduction of iron by microbes, resulting in desorption of Fe-bound P (Aldous et al. 2005, Aldous et al. 2007, Zak et al. 2008, Ardon et al. 2010), flood induced turnover of P within the soil microbial biomass may play an important, if understudied role in wetland P release (Wright and Lockaby 2001, McDowell and Sharpley 2003, Aldous et al. 2007). Microbes dominate uptake of phosphorus added to wetland soils
(Richardson and Marshall 1986, Walbridge 1991, Kellogg and Bridgham 2003, Noe et al. 2003), and may comprise a large proportion of soil total P pools, ranging from 25-50% of total P in wetland mineral soils (Walbridge 1991, Wright et al. 2001, McDowell and Sharpley 2003) to up to 70% of total P in ombrotrophic peat soils (Williams and Silcock 2001). Flooding induced changes in microbial biomass phosphorus pools and stoichiometry in wetland soils (McLatchey and Reddy 1998, Brake et al. 1999, Wright and Lockaby 2001, Li et al. 2006) suggest a potential role of microbial cell death or alteration of P metabolism in wetland P release, but microbial P turnover has not been well studied in wetlands, and specific process of microbial P metabolism have not been previously considered (Gutknecht et al. 2006).

However, the role of microbial metabolism in phosphorus cycling and anaerobic P release has been of considerable interest in aquatic ecosystems, where sediment P dynamics have recently been linked to microbial metabolism of inorganic polyphosphate (Hupfer et al. 2008), a long chain of orthophosphate monomers accumulated in intracellular bodies that were among the first subcellular features indentified in microbes (Seufferheld et al. 2008, Rao et al. 2009). Microbial polyphosphate (PolyP) accumulation occurs in both freshwater and marine sediments, and the anaerobic degradation of microbial PolyP has been directly implicated in release of inorganic P from sediments (Hupfer et al. 2004, Sannigrahi 2005, Schulz and Schulz 2005, Hupfer and Lewandowski 2008, Ahlgren et al. 2011). Microbial turnover of PolyP has been also been linked to diagenesis of stable mineral P forms (apatites) in marine sediments, which critically influence global P cycling and productivity (Schulz and Schulz 2005, Diaz et al. 2008).
Early interest in microbial PolyP accumulation in aquatic sediments was motivated by the role of PolyP accumulation in P removal in wastewater treatment (Hupfer et al. 2008). Microbial polyphosphate accumulation is widely harnessed to reduce inorganic P concentrations in effluents of well studied Enhanced Biological Phosphorus Removal (EBPR) municipal wastewater treatment systems, which can remove up to 40 mg P/L from wastewater by precipitating PolyP accumulating bacteria in sludge (Mino 2000, Seviour et al. 2003, Mino and Satoh 2006, Majed et al. 2009). As in aquatic sediments, microbial accumulation of PolyP in EBPR wastewater systems occurs under aerobic, but not anaerobic conditions.

Although polyphosphate has also been observed in wetland soils in a limited number of studies (Bedrock et al. 1994, Sundareshwar et al. 2009), little effort has been made to explore the distribution of PolyP across wetland types, or characterize processes contributing to accumulation or turnover of PolyP in wetland soils. While wetland soils differ considerably from aquatic sediments and wastewater systems, PolyP accumulation has been observed in bacteria from acetate amended sediment slurries of wetland soils (Khoshmanesh et al. 2002), and I posit that PolyP accumulation in wetland soils reflects dynamic microbial P metabolism.

Polyphosphate accumulation may occur across a broad range of bacterial (Liu et al. 2001, Mudaly et al. 2001, Kawaharasaki et al. 2002, Zilles et al. 2002) and fungal taxa (Makarov et al. 2005, Bünemann et al. 2008b), and PolyP accumulation is critical to microbial survival of a wide range of environmental stresses (Brown and Kornberg 2004, Seufferheld et al. 2008). In *E. coli*, polyphosphate is directly linked to central gene regulation of stationary phase (dormant state) metabolism, which coordinates responses to numerous stresses including oxidating conditions, low pH, salinity, and starvation.

Survival of environmental stresses is a critical requirement of microbial life in wetland soils, which present a wide range of extreme environments including anaerobic conditions and fluctuating oxygen availability, extremes of high and low pH and nutrient availability across wetland types, and salinity challenges in coastal marshes (Mitsch and Gosselink 2007). The central role of PolyP in microbial stress responses suggests the distribution of PolyP among wetland types may vary based on differences in environmental stresses characteristic of particular wetland habitats. Patterns of PolyP cycling with oxygen availability in aquatic sediments and wastewater treatment systems also suggest that PolyP accumulation in wetland soils may vary with flooding and oxygen availability.

To explore the distribution of PolyP across wetland soils, I analyzed samples from a range of organic and mineral soils of the North Carolina piedmont and coastal plain using $^{31}\text{P}$-NMR spectroscopy to quantify the relative abundance of PolyP in soil extracts (Cade-Menun 2005, Turner et al. 2005), similar to methods commonly employed to detect PolyP accumulation in aquatic sediments (Hupfer et al. 2008). I sampled wetlands encompassing a watershed gradient including isolated headwater wetlands,
riverine floodplains, and brackish and salt marshes, spanning a range of soil fertility, organic matter, pH and salinity. I more closely studied the dynamics of PolyP accumulation in a pocosin wetland complex where PolyP had previously been detected (Sundareshwar et al. 2009). I evaluated effects of rainfall and flooding events, and seasonal change on PolyP in soils in a 50 year old tall pocosin forest with native vegetation, an 8 year old restored wetland, and agricultural field on drained pocosin soil, all directly adjacent to one another in a wetland mitigation bank where hydrology, soils, water quality (Bruland et al. 2003), and soil bacterial communities (Hartman et al. 2008) have previously been characterized. At these pocosin sites, I attempted to characterize the biological origin of soil PolyP, and tested the effects of flooding and oxygen availability on Poly P accumulation and turnover in soil microcosm experiments. Finally, I compared the observed patterns in the distribution and dynamics of PolyP in wetlands to observations of PolyP in published $^{31}$P-NMR studies in wetlands, aquatic ecosystems, and terrestrial soils to better interpret my observational and experimental findings.

### 3.2 Materials and Methods

#### 3.2.1 Soil sampling and characterization

I obtained soil samples from a range of wetland types and land uses, representing extremes in soil pH, nutrient status, organic matter content, and salinity. Wetlands sampled included agricultural, restored, and reference land uses in pocosin peatlands, a floodplain swamp, and brackish and saline marshes. Pocosin wetlands at the Barra Farms wetland mitigation bank were chosen as the primary site for study of seasonal variation and responses to rainfall and natural flooding events in May (23 and 30) and November (3 and 14) of 2009, as PolyP had previously been observed in these
soils (Sundareshwar et al. 2009). To expand the range of wetland types sampled, I also conducted a one-time survey of other wetland types (short pocosin, floodplain forest, brackish and salt marsh) on June 8, 2009.

Analyses of environmental samples using 31P-NMR seldom analyze replicate samples, and I chose sample plots at each site carefully to obtain soils under stands of vegetation characteristic of each habitat type. Plots at the Barra Farms sites were chosen by geo-reference to previous studies (Hartman et al. 2008), while plots in the wetland survey sites were selected from low lying areas where the water table was 5 - 10 cm from the surface, to capture both aerobic and anaerobic soil conditions.

From each plot, I collected three replicate samples of the top 10 cm of soil A horizons, within a 3 m plot radius. Replicates samples were sieved, homogenized and pooled within each plot for subsequent analyses by 31P NMR and a basic suite of soil chemical parameters. Soil organic matter was determined by loss on ignition, total N was determined by carbon, hydrogen, nitrogen (CHN) analysis, total P was determined by Murphy Riley following a perchloric acid digest (O’Halloran and Cade-Menun 1993), and pH was determined in 1:1 soil:water slurries, with three subsamples of each soil sample tested for each analyte. Data from this characterization of soil chemistry is given for each soils sampled from the field in Table 1 following description of study sites.

3.2.2 Study sites

3.2.2.1 Barra Farms wetland mitigation bank

The Barra Farms site (34° 55’ N, 78° 42’ W) is part of a 975-ha Carolina bay complex located in Cumberland County, North Carolina (Bruland et al. 2003, Sundareshwar et al. 2009). Soils at the site have been classified as Croatan Mucks (Terric Haplosapristis). Past alterations to the site included clearing and ditching in the 1960s.
for conversion to agriculture, and intensive farming during the 1970s and 80s. In the fall of 1997, 250 ha of the site were restored to wetlands by filling ditches and planting woody seedlings. Samples were obtained from existing agricultural soils (BFA), from the restored area (BFS), and from a reference site in a nonriverine swamp forest that was never converted to agriculture (BFF). Agricultural soils sampled at Barra Farms are used to produce corn (Zea mays) row crops. The restored wetland at Barra Farms is dominated by Bald Cypress (Taxodium distichum), and Atlantic White Cedar (Chamaecyparis thyoides), with invasive species (Phragmites australis and Microstegium vimineum) overtaking planted species in open areas, as described by (Bruland et al. 2003). The reference site is a tall pocosin forest logged ca. 50 yrs ago, dominated by Pond Pine (Pinus serotina), Loblolly Bay (Gordonia lasianthus), and Umbrella Magnolia (Magnolia tripetala).

3.2.2 Pocosin Lakes National Wildlife Refuge

Soil samples were also obtained from a protected reference short pocosin wetland located in the Croatan National Forest (35° 41’ N, 76° 41’ W) in Carteret County, North Carolina. Soils at the site have been classified as Pungo Mucks (Typic Haplosaprist). Short pocosin vegetation at the site includes Fetterbush (Lyonia lucida), Inkberry (Ilex glabra), Honeycups (Zenobia pulverulenta), Loblolly Bay (Gordonia lasianthus), and Pond Pine (Pinus serotina).

3.2.2.3 Neuse River Floodplain

Soils were collected from the low-lying active floodplain of the Neuse River, just south of the town of Kinston in Lenoir County, North Carolina (35° 15’ N, 77° 37’ W). Soils at the site have been classified as Wehadkee Fine Sandy Loams (Fluvaquentic Endoaquepts). These poorly drained mineral soils were obtained under a stand of flood
tolerant Bald Cypress trees (*Taxodium distichum*), and samples were collected near the vegetated margin of a backwater depressional area.

Table 1: Soil chemistry and P extraction data for soils collected from the field for analysis by *31*P-NMR. Data are given for soil pH, and total pools of soil carbon (C), nitrogen (N), and phosphorus (P), along with total P in NaOH extracts used for solution *31*P-NMR (P extr) and the % of soil P in NaOH extracts (%P extr). Units for C, N, P, and P extr are given in mg of each analyte per kilogram soil. Asterisks next to sampling dates indicate soils collected at the Barra Farms pocosin bogs under flooded conditions (**), while soils from all sites sampled in the wetland survey were obtained where the water table was approximately 5 cm from the surface (*).

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<td>6.78</td>
<td>0.256</td>
<td>0.192</td>
<td>74.9</td>
</tr>
<tr>
<td></td>
<td>30 May **</td>
<td>5.15</td>
<td>281.7</td>
<td>6.49</td>
<td>0.227</td>
<td>0.109</td>
<td>47.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 Nov</td>
<td>4.73</td>
<td>328.2</td>
<td>6.99</td>
<td>0.209</td>
<td>0.270</td>
<td>129.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 Nov **</td>
<td>5.05</td>
<td>372.5</td>
<td>9.24</td>
<td>0.893</td>
<td>0.333</td>
<td>37.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BFF</td>
<td>23 May</td>
<td>3.67</td>
<td>297.2</td>
<td>9.16</td>
<td>0.418</td>
<td>0.363</td>
<td>86.9</td>
</tr>
<tr>
<td></td>
<td>3 Nov</td>
<td>3.78</td>
<td>276.0</td>
<td>7.10</td>
<td>0.224</td>
<td>0.150</td>
<td>67.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 Nov</td>
<td>3.54</td>
<td>284.0</td>
<td>8.89</td>
<td>0.437</td>
<td>0.089</td>
<td>20.3</td>
<td></td>
</tr>
<tr>
<td>Salt marsh</td>
<td>7 Jun *</td>
<td>3.91</td>
<td>105.9</td>
<td>6.82</td>
<td>2.238</td>
<td>0.255</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td>Brackish marsh</td>
<td>7 Jun *</td>
<td>4.33</td>
<td>254.7</td>
<td>15.31</td>
<td>0.508</td>
<td>0.474</td>
<td>93.4</td>
<td></td>
</tr>
<tr>
<td>Floodplain</td>
<td>7 Jun *</td>
<td>5.10</td>
<td>13.1</td>
<td>0.85</td>
<td>0.711</td>
<td>0.343</td>
<td>48.2</td>
<td></td>
</tr>
<tr>
<td>Pocosin reference</td>
<td>15 Jun *</td>
<td>3.55</td>
<td>412.1</td>
<td>11.67</td>
<td>0.807</td>
<td>0.438</td>
<td>54.3</td>
<td></td>
</tr>
</tbody>
</table>

### 3.2.2.4 Hadnot Creek brackish marsh

Soils were collected from a brackish marsh in the Croatan Gamelands section of the Croatan National Forest along Hadnot creek, near its confluence with the White Oak River estuary (34° 45’ N, 77° 6’ W) in Carteret County, North Carolina. Soils have been
identified as Lafitte Mucks (Typic Haplosapristis). Samples were collected from hemic peats in the low marsh under a dense monotypic stand of Black Needlerush (*Junucus roemerianus*), indicative of mesohaline conditions where riverine floodplains are tidally influenced.

### 3.2.2.5 Cedar Point National Park

Soils were collected from salt marsh soils at Cedar Point National Park in the Croatan National Forest near the mouth of the White Oak River estuary (34° 41’ N, 77° 5’ W) in Carteret County, North Carolina. These soils have been classified as Hobucken Mucks (Typic Hydraquents). Soils were collected in the low marsh under a dense monotypic stand of short form Smooth Cordgrass (*Spartina alterniflora*), indicating high salinity conditions and regular tidal flushing of marsh soils.

### 3.2.3 Soil extraction and $^{31}$P NMR analysis

While different permutations of extracting procedures for $^{31}$P NMR analysis were explicitly tested, all soil NMR preparations were modified from a basic procedure (Cade-Menun 1996) considered effective for soils and other environmental samples (Cade-Menun 2005, Turner et al. 2005). Two replicates of each composite soil sample were extracted within 24 hours of collection, with 1.5 g of soil (dry-weight equivalent) shaken at 150 rpm with 30 mL 0.25 M NaOH + 0.05 M Na-EDTA for four hours, rather than the original 16 hours (Cade-Menun 1996). The reduced shaking time procedure (McDowell et al. 2007b) was chosen to minimize the potential for alkaline hydrolysis of PolyP in NaOH extracting solutions (Turner et al. 2003d, Cade-Menun et al. 2006).

In a previous study of PolyP accumulation in lake sediments, PolyP was obtained from some sediments extracted with 0.25 M NaOH, but not detected in 0.25 M NaOH + 0.05 M Na-EDTA extracts of identical sediments (Ahlgren et al. 2007). To
ensure PolyP was extracted from soil, I separately analyzed initial soil samples from each wetland site obtained in May or June using both standard NaOH-EDTA extracts and NaOH extracts alone. NaOH was selected as the extractant of choice for subsequent analysis of samples collected in the field and manipulated (flooded with forced aerobic or anaerobic conditions) in the lab due to slightly greater recovery of PolyP in the reference site at Barra Farms where PolyP was highest, although differences in P speciation among extracts were small in all soils sampled (data presented in Results and Discussion), and no drastic changes in PolyP extraction were observed in any soils sampled.

All shaken soil extracts were centrifuged for 10 minutes at 1200 g, and 2 mL of supernatant from each extract were set aside for determination of total P using a perchloric acid digest followed by the Murphy-Riley method for analysis of inorganic P (O’Halloran and Cade-Menun 1993). The remaining unfiltered supernatant from soil extracts (~27 mL) was immediately frozen and lyophilized, and lyophilized extracts were stored at -80° C until further analysis by 31P NMR. Hydrolysis of PolyP is a concern in alkaline extracts, and may particularly occur during lyophilization of extracts used to concentrate samples for analysis 31P-NMR. (Cade-Menun et al. 2006). To ensure PolyP was not degraded during lyophilization, I tested effects of neutralization of extracts prior to lyophilization (using 1 M HCl) compared to the standard extraction procedure, using both NaOH and NaOH + EDTA extracts of the Barra Farms reference site where PolyP was highest. I found only minute effects of solution pH on PolyP hydrolysis during lyophilization, with differences in PolyP recovery of 2.5% in NaOH + EDTA extracts, and 0.9% in NaOH extracts (Appendix B, Fig 22, Table 15). Subsequent extracts were not neutralized prior to lyophilization due to the poor buffer capacity in
NaOH extracting solutions used in later experiments, and the miniscule effects of neutralization.

The two replicate lyophilized extracts were combined into a single reconstituted sample in order to increase the concentration of phosphorus in NMR tubes to improve signal strength, and decrease NMR run times needed to achieve satisfactory signal to noise ratios where soil P concentrations were low. The two lyophilized, replicate samples of each soil composite were combined and pulverized prior to being reconstituted in 0.8 mL D$_2$O, 0.4 mL 10 M NaOH, and 1.4 mL deionized water, although the volume of reconstituted extracts was adjusted as needed where the amount of lyophilized material was too great to allow for complete dissolution. The pH of reconstituted extracts was checked and adjusted to pH 13 with 1 M NaOH (up to 0.25 mL) as necessary. Reconstituted extracts were allowed to dissolve for 30 minutes with occasional vortexing, prior to centrifugation for 10 min at 1400 g. The remaining supernatant (ca. 2.5 mL) was transferred to 10 mm NMR tubes for immediate analysis.

Solution $^{31}$P NMR spectra were obtained using a Varian Inova 500 MHz spectrometer with a 10 mm probe (Varian Inc., Palo Alto). The NMR parameters were: 67° pulse, 2.0 s acquisition time, 0.3 second pulse delay at 25° C. Due to high variability in phosphorus concentrations in reconstituted soil extracts, individual NMR runs were periodically monitored to determine optimal run times needed to obtain resolution of potential PolyP peaks from background noise, usually ranging from 4 – 24 hours (8,000-24,000 scans). Identification of phosphorus forms in extracts was determined by their chemical shift (ppm) relative to an external standard of H$_3$PO$_4$, which was assigned a standardized value of 6 ppm. Peak assignments and compound identification are were determined following (Turner et al. 2003d, Cade-Menun 2005), and are shown in Fig. 8.
Peak areas were calculated by integrating spectra processed with 10 Hz line-broadening using Varian vnmrj NMR software.

Figure 8: Compound identification for soil solution $^{31}$P NMR spectra. Peak assignments are phosphonates (20 ppm), orthophosphate (6 ppm), P monoesters (4 ppm), P lipids (2 ppm) and DNA-P (0 ppm), pyrophosphate (-4 ppm), and polyphosphate (-20 ppm). Compound ppm shifts are from (Turner et al. 2003d, Cade-Menun 2005).

3.2.4 Biological characterization of PolyP in pocosin soils

To characterize the biological origin of soil PolyP, I tested shifts in PolyP with soil sample processing treatments designed to lyse or kill microbial cells, and cultured cells from soil innocula. For these experiments I used soils from the Barra Farms reference site where the greatest PolyP accumulation was observed. I compared PolyP pools in standard (control) extracts to soil samples that were sonicated in extracting solutions (1 minute, 20 Khz at 100 watts in an icebath) to ensure complete cell lysis and the rapid release of PolyP in living tissues (Turner 2008). I also air-dried soils prior to extraction (McDowell et al. 2006, Turner 2008) to observe the effects of more gradual death of soil microbes on PolyP determined in subsequent $^{31}$P-NMR analyses.
Bacterial cultures were obtained using a low nutrient defined medium shown to obtain difficult to culture members of the *Acidobacteria* and *Verrucomicrobia* (Stevenson et al. 2004) that are broadly representative of bacterial communities in wetland soils (Stevenson et al. 2004), and media were inoculated with 1% soil by weight obtained from the Barra Farms reference tall pocosin. However, cultures were grown in liquid phase media rather than on agar plates (Stevenson et al. 2004) to allow for cytometric staining for PolyP. Five replicate cultures with 25 mL of media were incubated in 250 mL filter cap culture flasks in a sterilized positive pressure hood that remained sealed, with airflow only used during sampling. Cultures were monitored by microscopy for PolyP accumulation by Neisser staining (Hupfer et al. 2008) at 1, 3, 5, 7, 14, 21 and 30 days, and Neisser positive cultures were checked with DAPI staining of PolyP (Hupfer et al. 2008).

### 3.2.5 Simulated flooding and anoxia in soil microcosms

To provide an initial assessment of the effects of flooding and anoxia on the accumulation of PolyP, I created a simple set of flooded soil microcosms using soils from the Barra Farms wetlands complex representing a range of land use conditions (BFA, BFS, BFF), obtained on May 23, 2009. Microcosms consisted of 1.5 g of soil (same as soil NMR extracts), submerged with 5 mL of DI H₂O for 3 days (to avoid drastic changes in microbial communities), with three replicates of each land use type subjected to each of two flooding treatments. Flooding treatments were Aerobic: flooded soils bubbled with pressurized air through spinal tap needles; and Anaerobic: flooded soils incubated in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake MI, USA). Soil microcosms were extracted directly using concentrated NaOH-EDTA extracting solution to account for dilution by flooding water and given a final concentration of 0.25 M.
Replicate samples from each treatment were combined for analysis by solution $^{31}$P NMR using sample preparation protocols as previously described.

### 3.2.6 Manipulation of oxygen availability in soil slurry microcosms

To more definitively determine the effects of oxygen availability and cycling on microbial PolyP accumulation in wetland soils, I tested soils from the three land use treatments at the Barra Farms wetland complex (BFA, BFS, BFF), where PolyP accumulation was observed previously (Sundareshwar et al. 2009). Sieved soil samples were used for soil slurry microcosms, which were created within 48 hours of soil collection. Soil slurries (630 mL DI H$_2$O, 70 g soil dw. equivalent) were placed in tightly sealed brown glass 1 L jars with one-way vent valves, and bubbled with pressurized air or N$_2$ gas. To ensure uniform distribution of these gasses, slurries were stirred continuously using Teflon coated magnetic stir bars, and commercially available redox probes (Yokagawa, Tokyo) were used to continuously monitor oxidative potential (Eh) with an automated data logger system, similar to the experimental apparatus described by (Yu et al. 2007). The same composite soil samples used for slurry microcosms were extracted immediately following collection to determine initial conditions in field soils (collected Nov 3, 2009). Initial field fresh soil samples were compared to samples of experimental soil slurries obtained at the end of experimental treatment phases.

I simultaneously conducted two experiments to compare the effects of opposite shifts from aerobic to anaerobic conditions (as in sediment PolyP release), and from anaerobic to aerobic conditions (as in EBPR wastewater treatment) on PolyP cycling in soil slurry microcosms. For each of these experiments the initial treatment phase lasted 7 days, upon which samples were obtained and the composition of gas was switched. The second phase of the experiment was run for only 3 days. In each experiment, I used
three experimental replicates of soil slurries from BFF and BFS sites, but only two replicates from agricultural soils (BFA) due to limitation of the number of microcosm chambers, and low levels of PolyP previously detected at the BFA site (Sundareshwar et al. 2009).

Samples were extracted from homogenized soil slurries through a tygon tubing sampling port with a sealed one-way valve using sterile syringes, and two 27 mL replicate samples were obtained from each experimental unit at each time period. To account for variability among experimental replicates, I selected samples from the two experimental units of each soil in each treatment with the greatest consistency in redox potential (Eh). Soil slurries were extracted using a 3 mL of a 10 x concentrated extracting solution of 0.25 M NaOH to account for dilution by water in slurries. Duplicate extracts from each experimental unit were pooled, and the pooled samples from two selected replicates of each treatment in each soil were combined for analysis by solution $^{31}$P NMR using sample preparation methods described previously.

### 3.2.7 Global distribution of PolyP in soils and sediments

In order to contextualize experimental results in wetland soils, I compiled a literature review and data-synthesis of the global distribution of PolyP in soils and sediments determined by $^{31}$P-NMR, including marine, freshwater, riverine, wetland, and terrestrial habitats. Relevant publications were obtained by screening papers citing two important methodological developments describing a widely used procedure for extraction of organic P for $^{31}$P NMR (Cade-Menun 1996) and the assignment of $^{31}$P NMR peaks to various compounds (Turner et al. 2003d). I recorded data on the percent frequency of occurrence of PolyP, pyrophosphate (PyroP), and phosphonates (Phn) with
>1% of total peak area in $^{31}$P NMR spectra, and classified studies by ecosystem type and habitat factors (Appendix B, Table 16).

### 3.3 Results and Discussion

#### 3.3.1 Distribution of PolyP in wetland soils

To determine the distribution and abundance of polyphosphate in wetland soils, I obtained $^{31}$P-NMR spectra from soils extracts collected from a range of wetland types and land use histories spanning a landscape hydrologic gradient, including isolated headwater wetlands, riverine floodplains, and brackish and salt marshes. However, polyphosphate was found only in pocosin soils, where PolyP pools (-20 ppm) accounted for up to 11% of extracted P (Fig. 9, Table 2). The relative abundance of long chain PolyP in soils collected early in the growing season at the Barra Farms pocosin complex clearly differed with land use history (Fig. 9a) with a greater relative abundance of PolyP in reference tall pocosin soils (11.2%) than in soil from the 8 year old restored wetland (6.7%), and only trace amounts of PolyP (0.7%) were found in agricultural soils (Fig. 9a, Table 2). Polyphosphate was also present in relatively large amounts (8.6 %) in reference short pocosin soils obtained from the Pocosin Lakes site (Fig. 9b, Table 2).

Polyphosphate was not detected by $^{31}$P NMR analysis of soil extracts from forested floodplain mineral soils, or brackish and salt marsh soils, although trace quantities of pyrophosphate (-4 ppm) were detected in salt marsh soils (Fig. 9b, Table 3). To address potential effects of differences in P sorption across wetland types on PolyP recovery, soils were extracted with both NaOH and NaOH + EDTA (Table 3). Although inclusion of the metal chelator EDTA may impair recovery of PolyP in some aquatic sediments (Ahlgren et al. 2007), no effect of EDTA on PolyP recovery was observed in floodplain or marsh soils (Table 3). The relative abundance of nucleic acid P (0 ppm) in
Figure 9: Distribution of P forms in $^{31}$P-NMR spectra of NaOH extracts of wetland soils collected in 2009: a) samples obtained from the Barra Farms wetland complex in late May, including flooding of the restored site following a moderate rain event; b) samples from a survey of NC wetland types including a salinity gradient of periodically inundated wetland soils; c) soils obtained from the Barra Farms complex in early November, and d) in mid-November following several days of heavy rainfall, flooding BFA and BFS sites, but not the BFF tall pocosin. Abbreviations of Barra Farms land use treatments are: BFA (Agriculture), BFS (Restored), BFF (Reference).
Table 2: Relative abundance of phosphorus forms in $^{31}$P NMR spectra of NaOH soil extracts from different land use categories at the Barra Farms wetland complex, with samples collected in May 2009. Site abbreviations and NMR spectra are presented in Fig. 9a. Phosphorus forms obtained by NMR were: phosphonates (Phn, -20 ppm); inorganic orthophosphate ($P_i$, 6 ppm); P monoesters (P-mono, 4 ppm); P lipids (PL, 2 ppm) and nucleic acids (DNA-P, 0 ppm); pyrophosphate (PyroP, -4 ppm), and polyphosphate (PolyP, -20 ppm).

<table>
<thead>
<tr>
<th>Date</th>
<th>Wetland Site</th>
<th>% Phn</th>
<th>% $P_i$</th>
<th>% P-mono</th>
<th>% PL</th>
<th>% DNA</th>
<th>% PyroP</th>
<th>% PolyP</th>
</tr>
</thead>
<tbody>
<tr>
<td>23 May</td>
<td>BFA</td>
<td>-</td>
<td>73.0</td>
<td>20.6</td>
<td>1.6</td>
<td>3.7</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>23 May</td>
<td>BFS</td>
<td>-</td>
<td>32.3</td>
<td>30.1</td>
<td>1.8</td>
<td>27.6</td>
<td>1.5</td>
<td>6.7</td>
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<tr>
<td>30 May</td>
<td>BFS flood</td>
<td>2.5</td>
<td>52.6</td>
<td>21.5</td>
<td>0.6</td>
<td>17.7</td>
<td>5.1</td>
<td>-</td>
</tr>
<tr>
<td>23 May</td>
<td>BFF</td>
<td>3.7</td>
<td>19.0</td>
<td>38.3</td>
<td>9.8</td>
<td>17.4</td>
<td>0.6</td>
<td>11.2</td>
</tr>
</tbody>
</table>

coastal marsh and agricultural soils was lower than in pocosin and restored wetland soils, and was lowest in floodplain soils (Fig. 9), which may suggest the lack of PolyP in these soils could be associated with lower microbial biomass or activity.

Table 3: Relative abundance of phosphorus forms in $^{31}$P NMR spectra of NaOH and NaOH + EDTA soil extracts from a range of wetland types in the North Carolina coastal plain. NMR spectra obtained from soils extracted with 0.5 M NaOH are presented in Fig. 9b. Additional NMR spectra were obtained from the same homogenized soil samples extracted with 0.25 M NaOH and 0.05 M Na-EDTA are presented here, but not in Fig. 9 due to broadly similar results to extraction using NaOH only. Abbreviations of P forms are given in Table 1.

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Wetland type</th>
<th>% Phn</th>
<th>% $P_i$</th>
<th>% P-mono</th>
<th>% PL</th>
<th>% DNA</th>
<th>% PyroP</th>
<th>% PolyP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td>Salt marsh</td>
<td>-</td>
<td>41.1</td>
<td>36.5</td>
<td>9.6</td>
<td>11.0</td>
<td>1.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Brackish marsh</td>
<td>-</td>
<td>37.8</td>
<td>37.1</td>
<td>11.3</td>
<td>12.6</td>
<td>1.2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Floodplain</td>
<td>-</td>
<td>87.8</td>
<td>10.2</td>
<td>-</td>
<td>2.0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Pocosin</td>
<td>5.2</td>
<td>24.8</td>
<td>26.7</td>
<td>12.1</td>
<td>19.4</td>
<td>3.3</td>
<td>8.6</td>
</tr>
<tr>
<td>NaOH</td>
<td>Salt marsh</td>
<td>-</td>
<td>51.7</td>
<td>38.3</td>
<td>5.2</td>
<td>15.1</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td>+EDTA</td>
<td>Brackish marsh</td>
<td>-</td>
<td>56.3</td>
<td>42.9</td>
<td>13.0</td>
<td>9.5</td>
<td>1.2</td>
<td>-</td>
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<tr>
<td></td>
<td>Floodplain</td>
<td>-</td>
<td>87.6</td>
<td>10.3</td>
<td>1.0</td>
<td>1.1</td>
<td>0.0</td>
<td>-</td>
</tr>
</tbody>
</table>
Results from the survey of soil P speciation across contrasting wetland types may suggest that PolyP accumulation is not a common feature of all wetlands, is instead unique to isolated acidic peatlands like the pocosins at the Barra Farms complex (Fig. 9), where soil pH values were 3.5-5.3 (Table 1). Review of published $^{31}$P NMR studies also indicates the relative infrequency of observations of PolyP in wetland soils, with PolyP detected in only 11 NMR spectra from three other studies, out of 119 $^{31}$P-NMR spectra of wetland soil extracts published in 13 studies (Appendix B, Table 16). Other published observations of large accumulation of PolyP in wetlands have also been limited to hydrologically isolated peat soils (Appendix B, Table 16), including an earlier study of the same pocosin sites in the Barra Farms mitigation bank (Sundareshwar et al. 2009), a reseeded Scottish blanket peat bog, where no pH data was provided (Bedrock et al. 1994), and a deep marsh soil (pH 4.3) in the Everglades Agricultural Area (Cheesman et al. 2010). However, only trace levels of PolyP were detected in isolated prairie pothole wetlands with higher soil pH (5.8-6.8) values (Sundareshwar et al. 2009), suggesting that low soil pH may be an important factor in PolyP accumulation in isolated wetland soils.

While low soil pH may appear to be a contributing factor in the distribution of PolyP in wetlands, I found little support for PolyP as a marker of general stress factors like oxygen fluctuation or salinity in wetlands, with no PolyP detected in floodplains or coastal marshes in the present study or the published literature, although relatively few studies have tested P speciation by $^{31}$P-NMR in these wetland types (Appendix B, Table 16).

**3.3.2 Effects of season and flooding on PolyP in pocosin soils**

I observed shifts in soil phosphorus forms with seasonal senescence of vegetation and flooding in the field at the Barra Farms pocosin wetland complex. Large pools of
PolyP in Barra Farms reference tall pocosin (BFF) and restored wetland soils (BFS) observed in May were greatly diminished in early November samples (Fig. 9a,c Tables 2,4). However, a relatively large peak of pyrophosphate (PyroP, -4 ppm) was present in soils collected from the reference tall pocosin (BFF) in November, but not early in the growing season in May (Fig 9a,c, Tables 2,4), which may indicate some conversion of PolyP to pyrophosphate (PyroP, P$_2$O$_6$) with seasonal change. Soils obtained at the end of the growing season in early November (Fig. 9c, Table 4) appeared to have less nucleic acid P (0 ppm) and P-lipids (2 ppm) than soils obtained early in the growing season in May (Fig. 9a, Table 2), suggesting a seasonal decline in microbial biomass and activity.

Shifts in soil phosphorus speciation were also observed with flooding events in the field in both May and November following significant rain events. Flooding at the restored wetland site (BFS) in May resulted in reduced relative abundance of PolyP and nucleic acid P, and increased abundance of PyroP (Fig. 9a, Table 2). Conversely, rain events in November resulted in a large increase in the relative abundance of long chain PolyP at the Barra Farms reference site (BFF) where soils were not submerged, and the

Table 4: Effects of heavy rainfall and flooding on the relative abundance of phosphorus forms in soils across different land use types at the Barra Farms wetland complex, determined by $^{31}$P-NMR in NaOH soil extracts. NMR spectra are presented in Fig. 9 c-d. Barra Farms agricultural (BFA) and restored soils (BFS) were submerged under standing water on Nov. 14, while reference tall pocosin soils (BFF) were not flooded. Abbreviations of P forms are given in Table 1.

<table>
<thead>
<tr>
<th>Date</th>
<th>Soil</th>
<th>% Phn</th>
<th>% P$_i$</th>
<th>% P-mono</th>
<th>% PL</th>
<th>% DNA</th>
<th>% PyroP</th>
<th>% PolyP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nov. 3</td>
<td>BFA</td>
<td>-</td>
<td>63.1</td>
<td>35.2</td>
<td>-</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BFS</td>
<td>-</td>
<td>28.4</td>
<td>55.2</td>
<td>3.5</td>
<td>11.4</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>BFF</td>
<td>4.7</td>
<td>22.8</td>
<td>41.6</td>
<td>6.9</td>
<td>13.8</td>
<td>4.5</td>
<td>5.7</td>
</tr>
<tr>
<td>Nov. 14</td>
<td>BFA</td>
<td>0.0</td>
<td>26.7</td>
<td>48.5</td>
<td>8.6</td>
<td>12.7</td>
<td>2.5</td>
<td>1.1</td>
</tr>
<tr>
<td>(Post-</td>
<td>BFS</td>
<td>0.4</td>
<td>74.1</td>
<td>17.1</td>
<td>2.3</td>
<td>4.1</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>flood)</td>
<td>BFF</td>
<td>5.9</td>
<td>24.0</td>
<td>34.3</td>
<td>11.3</td>
<td>12.0</td>
<td>3.7</td>
<td>8.7</td>
</tr>
</tbody>
</table>
appearance of trace amounts of PolyP in submerged soils in the agricultural (BFA) and restored wetland (BFS) site, with mixed effects on nucleic acid P and P lipids (Fig. 9c-d, Table 4).

Dynamics of polyphosphate in soils of the Barra Farms pocosin complex with flooding and season suggest that both PolyP and PyroP are actively cycled in soil, in contrast to existing claims that these condensed inorganic P forms accumulate over time in wetlands as the result of their low biological availability (Sundareshwar et al. 2001, Sundareshwar et al. 2009). Degradation of PolyP with flooding was observed in only one week in the Barra Farms restored site in May (Fig 9 a, Table 2), and accumulation of PolyP with rain and flooding was observed to occur in only 11 days in November across land use types (Fig 9c, d, Table 4). These results not only suggest that soil PolyP may be actively cycled, but rapid accumulation of PolyP also suggests that this cycling is due to the activity of soil microbes. An association between PolyP accumulation and microbial activity is not only suggested by PolyP turnover with flooding and rain events, but also by seasonal patterns, with degradation of PolyP in the Barra Farms reference site at the end of the growing season (Fig 9a,c, Tables 2,4). Where PolyP degradation was observed with flooding (Fig. 9a) or seasonal senescence (Fig 9a,c), some accumulation of PyroP was observed, suggesting PyroP in wetland soil may be a product of PolyP hydrolysis.

3.3.3 Biological characterization of PolyP in pocosin soils

To characterize the processes driving polyphosphate accumulation and turnover, I conducted soil lysis and culture experiments on soils from the Barra Farms reference tall pocosin (BFF) to investigate whether PolyP cycling in wetlands is of a biological origin, as suggested by dynamics in the field. I found that compared to standard soil extraction procedures, sonication of soils in extracting solutions prior to $^{31}$P NMR
analysis resulted in increased recovery of PolyP (Fig 10, Table 5). Ultrasonication is used to rupture living microbial cells and release their contents in extractions of both pure cultures and soils (Tate et al. 1988, Ault-Riche et al. 1998) and the greater recovery of PolyP (and nucleic acid P) I found in sonicated extracts likely indicates some soil PolyP is held specifically within living microbial cells.

![Figure 10: Effects of changes in extraction methods and sample handling designed to destroy microbial cells on recovery of soil PolyP (~20 ppm) from Barra Farms reference tall pocosin (BFF), assessed by $^{31}$P-NMR. Samples were obtained on May 23, 2009, and lethal changes to sample processing included sonication of soils in extracting solution, and air-drying of soils prior to extraction.](image)

Air-drying of soils should also significantly contribute to cell lysis, albeit more slowly than the instantaneous burst and release in sonicated extracts. Importantly, PolyP was destroyed in soils that were air dried for one week, while PyroP was detected in air-dried soils (Fig 10, Table 5). Dessicated soils may not be conducive to microbial degradation processes, suggesting that destruction of PolyP with air-drying may occur within PolyP accumulating cells, due to residual action of the polyphosphate kinase enzyme (ppk), which reversibly polymerizes and degrades PolyP, producing some PyroP during PolyP degradation (Kornberg et al. 1999, Rao et al. 2009). Degradation of
PolyP and large increases in PyroP have also been recently observed where microbes have been poisoned with formalin in aquatic sediments (Ahlgren et al. 2011).

Table 5: Effects of changes to sample handling designed to destroy microbial cells on the relative abundance of P forms in soil extracts from Barra Farms reference tall pocosin (BFF), determined by $^{31}$P NMR. Data accompany spectra displayed in Figure 10. Abbreviations of P forms are given in Table 1.

<table>
<thead>
<tr>
<th>Extraction Treatment</th>
<th>% Phn</th>
<th>% P</th>
<th>% P-monophosphate</th>
<th>% PL</th>
<th>% DNA</th>
<th>% PyroP</th>
<th>% PolyP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH + EDTA</td>
<td>4.8</td>
<td>19.6</td>
<td>39.8</td>
<td>9.8</td>
<td>16.7</td>
<td>1.3</td>
<td>8.1</td>
</tr>
<tr>
<td>NaOH only</td>
<td>3.7</td>
<td>19.0</td>
<td>38.3</td>
<td>9.8</td>
<td>17.4</td>
<td>0.6</td>
<td>11.2</td>
</tr>
<tr>
<td>Sonicated in NaOH</td>
<td>3.6</td>
<td>16.5</td>
<td>33.0</td>
<td>11.8</td>
<td>18.7</td>
<td>0.1</td>
<td>16.2</td>
</tr>
<tr>
<td>Air Dried, NaOH</td>
<td>-</td>
<td>23.8</td>
<td>45.6</td>
<td>5.3</td>
<td>17.7</td>
<td>7.6</td>
<td>-</td>
</tr>
</tbody>
</table>

To determine whether PolyP accumulation occurred within microbial cells, I tested low-nutrient cultures inoculated with soils from the Barra Farms reference tall pocosin (BFF). The low nutrient medium I used to obtain microbial cultures has been shown to obtain difficult to culture bacterial groups including Acidobacteria and Verrucomicrobia (Stevenson et al. 2004), which are abundant in these pocosin soils (Hartman et al. 2008). I found evidence of bacterial PolyP accumulation in cultured cells, with independent indications of PolyP by both Neisser and DAPI stains (Hupfer et al. 2008) shown in Figure 11. Although culture experiments clearly showed PolyP accumulation within microbial cells, these results may not closely reflect biological processes in soils in the field. Accumulation of PolyP is widespread among numerous groups of bacteria and fungi, and PolyP accumulation is commonly observed in static cultures in the stationary growth phase when nutrients have been depleted (Kornberg et al. 1999). To observe how accumulation and degradation of polyphosphate vary with
conditions more representative of those in natural soils, I experimentally manipulated oxygen availability in soil microcosms using pocosin soils from Barra Farms.

Figure 11: Cytochemical staining of PolyP in cultured bacteria (indicated by arrows) obtained from soil innocula from the Barra Farms reference wetland (BFF). A) Nessier staining indicates the presence of cellular PolyP with dark blue-purple inclusions (volutin or metachromatic granules); B) DAPI staining indicates the presence of cellular PolyP by yellow fluorescence (525 nm), while DAPI stained DNA shows blue fluorescence (460 nm). Both figures are shown at 1000 X magnification.

Bacteria were cultured from soil innocula at Barra Farms using a low-nutrient medium (Stevenson et al. 2004) that may obtain bacterial groups similar to uncultured communities in wetland soils (Hartman et al. 2008).

3.3.4 Dynamics of PolyP in pocosin soil microcosms

To better assess the effects of flooding and oxygen availability on polyphosphate accumulation and degradation in soils of Barra Farms pocosin wetlands, I altered oxygen availability in settled soil microcosms and well mixed soil slurry microcosm experiments. Microcosms with settled, homogenized soils were flooded and either bubbled with oxygen or evacuated and incubated in an anaerobic chamber for three days prior to extraction for analysis by $^{31}$P-NMR.
Phosphorus forms in aerobic incubations (Fig. 12, Table 6) of soils from reference tall pocosins (BFF) were largely similar to results from field fresh soils from which they were obtained (Fig. 9a, Table 2), while PolyP was considerably degraded under anaerobic conditions in BFF soil mesocosms (Fig. 12b, Table 6) relative to field moist soils.

![Figure 12: Results of manipulation of oxygen availability on phosphorus speciation in flooded soil microcosms. Three replicate homogenized soil samples (1.5 g dwt. equiv.) were submerged (5 mL DI H₂O added to soils in 50 mL tubes) and either a) bubbled with house air through spinal tap needles, or b) incubated in an anaerobic chamber. Soils were obtained on May 23, 2009, and NMR spectra for field fresh (control) soils are displayed in Fig. 9a.]

More subtle variation in P speciation occurred with aerobic and anaerobic treatments in agricultural soils (BFA), with increases in PyroP (-4 ppm) in both treatments, and trace amounts of PolyP (-20 ppm) appearing in anaerobic treatments (Fig. 12a, Table 6). Soils from restored wetlands (BFS) appeared similar in both treatments, with small amounts of PolyP in field moist soils (Fig. 9a, Table 2) disappearing in three day flooded soil microcosm incubations (Fig. 12a, Table 6).
Table 6: Effects of manipulation of oxygen availability in submerged soil microcosms from the Barra Farms wetland complex on the relative abundance of soil P forms determined by $^{31}$P-NMR in NaOH extracts. NMR spectra are presented in Fig. 12. NMR spectra for source (control) soils, collected in the field on May 23, 2009 are presented in Fig. 9a, and quantitation of spectra for source soils is presented in Table 3. Abbreviations of P forms are given in Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soil</th>
<th>% Phn</th>
<th>% $P_i$</th>
<th>% $P_{-mono}$</th>
<th>% PL</th>
<th>% DNA</th>
<th>% PyroP</th>
<th>% PolyP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air bubbles</td>
<td>BFA</td>
<td>1.2</td>
<td>70.3</td>
<td>20.2</td>
<td>2.9</td>
<td>3.6</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>BFS</td>
<td>-</td>
<td>29.8</td>
<td>46.9</td>
<td>9.5</td>
<td>13.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BFF</td>
<td>4.0</td>
<td>21.9</td>
<td>34.6</td>
<td>13.2</td>
<td>15.2</td>
<td>1.6</td>
<td>9.3</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>BFA</td>
<td>0.8</td>
<td>70.3</td>
<td>18.2</td>
<td>4.5</td>
<td>3.7</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>BFS</td>
<td>-</td>
<td>36.0</td>
<td>44.8</td>
<td>5.9</td>
<td>11.9</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BFF</td>
<td>-</td>
<td>30.4</td>
<td>38.9</td>
<td>3.0</td>
<td>20.9</td>
<td>-</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Degradation of PolyP in anaerobic, settled soil microcosms from the Barra Farms reference wetland (BFF) appears broadly congruent with patterns of anaerobic PolyP release by microbes in freshwater and marine sediments, and may also be related to the flood induced release of PolyP at the restored pocosin site (BFS) observed in May (Fig 9a). However, rainfall and flooding presented nearly opposite effects on soil PolyP in soils collected from pocosin sites in November (Fig. 9c,d), and I conducted a more controlled experiment to determine the effects of opposing shifts to anaerobic or aerobic conditions to better assess effects of oxygen availability on soil PolyP dynamics.

To assess the effects of dynamic shifts in oxygen availability on PolyP accumulation and degradation, I incubated soils in well mixed soil slurry microcosms, with continuous measurement of oxidation-reduction potential (ORP) to monitor development of anaerobic conditions. Soils used for this experiment were obtained on Nov. 3, and $^{31}$P NMR spectra from field fresh soils are shown in Figure 9c (Table 3). I conducted two simultaneous experiments characterized the effects of shifting from aerobic to anaerobic conditions (Fig. 13, Table 7), like conditions leading to sediment P
Figure 13: Results of shifting soil slurry microcosms to anaerobic conditions on the distribution of phosphorus forms in Barr farms soils. Shifts in oxidation-reduction potential (ORP) during the experiment and sampling times are shown in a). NMR spectra from pooled samples obtained from aerobic slurries are presented in b), while spectra from samples obtained following shifts to anaerobic conditions are shown in c). Soils used in experimental slurries were obtained on Nov. 3, 2009, and spectra for field fresh (initial) soils are shown in Fig. 9c.
Figure 14: Results of shifting soil slurry microcosms from anaerobic to aerobic conditions on the distribution of phosphorus forms in Barra farms soils. Shifts in oxidation-reduction potential (ORP) during the experiment and sampling times are shown in a). Only one microcosm chamber was sampled for BFA treatments, due to problems with the second replicate (shown in lighter color), likely due to clogging of bubbling tube by soil particles. NMR spectra from pooled samples obtained from anaerobic slurries are presented in b), while spectra from samples obtained following shifts to aerobic conditions are shown in c). Soils used in experimental slurries were obtained on Nov. 3, 2009, and spectra for field fresh (initial) soils are shown in Fig. 9c.
Table 7: Effects of dynamic changes in oxygen availability on the relative abundance of phosphorus forms in $^{31}$P-NMR spectra obtained from NaOH extracts of soil slurry microcosm experiments on Barra Farms soils. Accompanying NMR spectra for shifts to anaerobic conditions are shown in Fig. 13, and results of shifts from anaerobic to aerobic conditions are presented in Fig. 14. Abbreviations of P forms are given in Table 1.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sample</th>
<th>Soil</th>
<th>Eh</th>
<th>% Phn</th>
<th>% P$_i$</th>
<th>% P-mono</th>
<th>% PL</th>
<th>% DNA</th>
<th>% PyroP</th>
<th>% PolyP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air: 7 d,</td>
<td>T$_1$, Air</td>
<td>BFA</td>
<td>375.7</td>
<td>0.4</td>
<td>62.2</td>
<td>33.7</td>
<td>0.4</td>
<td>0.9</td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>N$_2$: 3 d</td>
<td>BFS</td>
<td>246.3</td>
<td>-</td>
<td>35.8</td>
<td>51.6</td>
<td>3.2</td>
<td>9.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BFF</td>
<td>230.3</td>
<td>-</td>
<td>57.1</td>
<td>38.6</td>
<td>-</td>
<td>-</td>
<td>4.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>T$_2$, N$_2$</td>
<td>BFA</td>
<td>-262.7</td>
<td>-</td>
<td>71.9</td>
<td>27.5</td>
<td>-</td>
<td>0.4</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BFS</td>
<td>-344.4</td>
<td>-</td>
<td>33.7</td>
<td>41.1</td>
<td>6.6</td>
<td>13.2</td>
<td>5.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BFF</td>
<td>270.3</td>
<td>-</td>
<td>64.2</td>
<td>27.9</td>
<td>-</td>
<td>-</td>
<td>8.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N$_2$: 7 d,</td>
<td>T$_1$, N$_2$</td>
<td>BFA</td>
<td>-279.1</td>
<td>-</td>
<td>83.0</td>
<td>17.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Air: 3 d</td>
<td>BFS</td>
<td>14.3</td>
<td>-</td>
<td>37.6</td>
<td>37.6</td>
<td>6.9</td>
<td>10.6</td>
<td>7.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BFF</td>
<td>276.1</td>
<td>1.1</td>
<td>46.2</td>
<td>38.5</td>
<td>2.3</td>
<td>12.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>T$_2$, Air</td>
<td>BFA</td>
<td>142.7</td>
<td>-</td>
<td>64.3</td>
<td>31.1</td>
<td>1.3</td>
<td>1.1</td>
<td>2.2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BFS</td>
<td>292.8</td>
<td>-</td>
<td>50.9</td>
<td>47.0</td>
<td>-</td>
<td>2.1</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BFF</td>
<td>460.5</td>
<td>1.2</td>
<td>32.3</td>
<td>39.7</td>
<td>6.8</td>
<td>6.8</td>
<td>13.1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

release, and the opposite shift from anaerobic to aerobic conditions (Fig. 14, Table 7), like conditions driving PolyP accumulation in EBPR wastewater treatment systems.

Although I expected accumulation and degradation of PolyP with dynamic shifts in oxygen availability, PolyP was not observed in any experimental treatment of soil slurry microcosms (Figs. 13-14). However, small amounts of PyroP (~ 4 ppm) were observed under aerobic conditions in the initial incubation phase of slurry microcosms from agricultural soils (BFA, Fig. 13a), and following a shift from anaerobic to aerobic conditions in microcosms from both agricultural (BFA) and reference wetland (BFF) soils (Fig. 14b).
Although soils used in slurry microcosm incubations were collected at the end of the growing season when PolyP was not observed in any of the pocosin soils (Fig. 9c), several factors suggest that the condition of these soils at the time of collection does not explain the lack of PolyP in microcosm experiments. Large accumulation of PolyP at the reference wetland (BFF), and smaller accumulations of PolyP in both agricultural and restored soils were observed in the field following heavy rains only 11 days after microcosm slurry soils were collected (Fig. 9d). Moreover, while nucleic acid P pools (DNA-P, 0 ppm) were lower at the time of collection than during the growing season (Fig 9a,c), possibly indicating reduced microbial biomass or activity, these pools were active in soils in the field in November following rainfall events (Fig. 9d), but drastically reduced in microcosm slurries obtained from these soils (Figs. 13-14). These results appear to indicate that the extreme saturation or continuous mixing of soil slurries was destructive to microbes present in pocosin soils.

3.3.5 Comparison of results to known processes of PolyP metabolism

I hypothesized that microbial PolyP accumulation would vary with wetland type due to the critical role of PolyP in microbial metabolic response to a number of environmental stresses including pH, oxygen availability, and salinity (Kornberg et al. 1999, Brown and Kornberg 2004, Seufferheld et al. 2008), that vary among wetland types (Mitsch and Gosselink 2007). However, the distribution of PolyP accumulation in wetlands soils observed in this study provides limited support for this hypothesis. Independent observations and tests indicated that PolyP is a dynamic pool in wetland soils, likely held within the biomass of microbes. However, large accumulations of PolyP were not observed widely across wetlands, and appeared restricted to pocosin soils, suggesting salinity is not an important factor in soil PolyP accumulation, and
fluctuating water tables alone may not drive accumulation of PolyP. While PolyP was not present in all acidic soils I tested, pocosin soils where PolyP was found had acidic (3.5-5.1) pH values, and the largest PolyP accumulations were found in the most acidic (3.5-3.6) soils at reference tall and short pocosin sites (Table 1).

I also predicted PolyP in wetland soils would accumulate under aerobic conditions, and be degraded under anaerobic conditions, based on PolyP dynamics in aquatic sediments and wastewater treatment systems (Sannigrahi 2005, Hupfer and Lewandowski 2008, Majed et al. 2009, Ahlgren et al. 2011). Some observations of this study present similar patterns of aerobic PolyP accumulation and anaerobic breakdown of PolyP in wetlands. Large accumulations of PolyP were only observed in soils that were not flooded (Fig. 9a,b,d), and degradation of PolyP occurred with flooding in the field (Fig. 9a, BFS). A similar pattern of PolyP degradation was observed in flooded soil microcosms under anaerobic conditions (Fig. 12b, BFF), while bubbling of flooded soils with oxygen did not result in PolyP degradation (Fig. 12b, BFF). However, dynamic accumulation and degradation of PolyP with oxygen availability was not observed in soil slurry microcosms, where no PolyP was detected (Fig 13-14).

Some observations of this study were not consistent with stress or oxygen induced PolyP metabolic processes characterized in cultured bacteria, aquatic sediments, and sewage treatment, particularly seasonal patterns observed in the field. Polyphosphate found in reference and restored pocosin soils during the growing season in May were absent in initial samples in November, but PolyP accumulation was observed in pocosin soils following heavy rains in November, especially in the reference tall pocosin that was not flooded (Fig 9a,c,d). These seasonal and hydrologic patterns suggest wetland PolyP accumulation is related to periods of microbial activity (growing
season, rainfall stimulation) rather than reflecting dormancy as described by stress-induced PolyP accumulation in the stationary phase (Kornberg et al. 1999). Patterns of PolyP accumulation with flooding in November (Fig 9c,d) also do not closely conform to the model of aerobic PolyP accumulation and anaerobic degradation from bacteria in sediments and wastewaters.

The distribution and dynamics of polyphosphate accumulation suggest PolyP metabolism in wetlands may differ from processes characterized in bacterial cultures, sediments, and wastewaters. Although PolyP is seldom discussed in cross ecosystem reviews of $^{31}$P-NMR studies in upland soils (Cade-Menun 2005, Turner et al. 2005) I found PolyP in 51 published $^{31}$P-NMR spectra from 8 studies spanning a range of terrestrial habitats and northern peat-based soils (Fig. 15, Appendix B, Table 16). Terrestrial soil PolyP pools appeared to be greatest in acidic, organic surface horizons, including old growth cedar-hemlock forests (4-20% PolyP, pH 2.9-3.8) in Vancouver, Canada (Cade-Menun et al. 2000a, b), boreal birch forests and tundras, closely aligned with peatland soil characteristics (1-15% PolyP, pH 3.8-4.1) in Norway (Turner et al. 2004), and an acidic grassland soil (5% PolyP, pH 3.7) in Northern England (Turner et al. 2003b). Trace levels of PolyP have also been detected in moderately acidic (pH 4.7-5.6) grassland soils (Turner et al. 2003c), and moderately acidic (pH 4.5-6.4) grassland and forest soils along chronosequences spanning 130,000 years of soil development (McDowell et al. 2007a).

The presence of PolyP in terrestrial soils has been ascribed to fungal activity (Turner et al. 2004, Makarov et al. 2005), and PolyP accumulation by fungi is known to be associated with P uptake and transport to mychorrhizal symbionts (Makarov et al. 2002b, Ezawa et al. 2004, Makarov et al. 2005, Koukol et al. 2008, Hijikata et al. 2010).
The relationship between terrestrial PolyP accumulation and acid organic soils in terrestrial soils I found in published $^{31}$P NMR data, and the well known affinity of fungi

Figure 15: Global distribution of polyphosphate, pyrophosphate, and phosphonates in sediments and soils. Figure expresses the percent frequency of $^{31}$P-NMR spectra obtained detecting phosphorus species at >1% of total peak areas, but does not represent signal intensity. Figure shows distribution of among habitats of a) polyphosphate—PolyP, b) pyrophosphate—PyroP, and c) phosphonates, and the total number of spectra obtained for each habitat is provided in parentheses. Asterisks (*) indicate number of samples obtained from wetland soils in the present study, included in the sample numbers for each wetland habitat. Deep Sediments are > 5 cm, Surface Sediments are 0-5 cm depths, and Trap data are from sediment traps to capture settling particulate matter. Data are from Table 16.
for low pH and low nutrient soils (Baath and Anderson 2003, Gadd 2007, Rousk et al. 2010a) may further suggest that PolyP accumulation in terrestrial soils is related to the activity of fungi (Makarov et al. 2005). Although fungal activity is considered less often in wetlands, peatland soils support > 500 fungal taxa (Thormann 2006), which can comprise up to 55-99% of soil microbial biomass in northern peatlands (Golovchenko et al. 2007) and 24-40% of soil microbial activity (Winsborough and Basiliko 2010).

The apparent limitation of PolyP accumulation to hydrologically isolated wetlands with acidic peat soils appears largely congruent with patterns of fungal PolyP accumulation in acidic terrestrial soils, which could suggest PolyP accumulation in wetlands is similarly related to fungal activity. While I found PolyP accumulation in cultured bacterial cells obtained from pocosin soil inocula, the dynamics of PolyP I observed in pocosin soils were not entirely consistent with metabolic processes identified in bacterial cultures and aquatic sediments. Although I did not directly observe fungal PolyP accumulation in wetlands, I suggest this mechanism could plausibly account for patterns in distribution and dynamics of PolyP in wetlands I observed, including seasonal patterns in PolyP in field soils and the lack of PolyP in well mixed soil slurry microcosms, given previous observations of seasonal and hydrologic variation of mycorrhizal fungi in wetland soils (Turner and Friese 1998, Bohrer et al. 2004).

3.4 Conclusions

The role of microbial metabolism in wetland cycling and P release is poorly understood, and I assessed the distribution and dynamics of polyphosphate in wetland soils to evaluate whether microbes might cycle PolyP in wetlands analogously to better studied processes in aquatic sediments, EBPR wastewater treatment systems, and
cultured microbes. I anticipated PolyP accumulation would vary among wetland types based on the critical role of PolyP in response to oxygen, salinity, and pH stress in cultured microbes. However, I did not detect PolyP in frequently inundated floodplain and coastal marsh soils where composite soil samples included the interface between aerobic and anaerobic conditions, and expected differences in PolyP accumulation with salinity of coastal marshes were not observed. I only found PolyP accumulation in hyrdologically isolated pocosins, where PolyP pools were greatest in the native forest, and appear to partially recover from trace levels in converted agricultural fields with wetland restoration, as observed in a previous study (Sundareshwar et al. 2009).

Existing studies of wetland soils using $^{31}$P-NMR also suggest soil PolyP may be limited to isolated wetlands with acid organic soils, and I more intensively characterized PolyP accumulation and turnover across land uses in the pocosin complex. I found soil polyphosphate in these pocosin soils is actively turned over with flooding and seasonal change, and appears to be held within the microbial biomass. I found PolyP accumulation in pocosin soils where soils were not flooded, and observed the rapid degradation of PolyP with flooding, saturation, and anaerobic conditions, analogous to the aerobic accumulation of PolyP and anaerobic degradation of PolyP in aquatic sediments and EBPR wastewater treatment systems. However, experimental shifts from anaerobic to aerobic conditions like those in EBPR systems did not generate PolyP in soil slurry microcosms, where PolyP was destroyed even under oxic conditions.

Seasonal patterns of PolyP degradation at the end of the growing season in reference and pocosin soils suggest that PolyP accumulation is associated with microbial activity, rather than a state of dormancy predicted by stress-induced mechanisms of PolyP accumulation in cultured bacteria. Polyphosphate accumulation by fungi in
upland soils could provide an alternate model for PolyP metabolism in wetland soils, although I did not test for the presence of soil fungi or their PolyP accumulation, and further work is needed to evaluate the potential role of fungi in PolyP accumulation in wetlands.

I also observed dynamic shifts in P speciation that suggest some polyphosphate may be converted to pyrophosphate with seasonal change, flooding, or dessication (Fig. 9-10), likely reflecting cell death and degradation of PolyP to PyroP by the polyphosphate kinase (ppk) enzyme (Kornberg et al. 1999). Similar conversion of PolyP to PyroP has recently been observed following cell poisoning in lake sediments (Ahlgren et al. 2011), and these results could indicate that the wide distribution of PyroP across soils and sediments (Fig. 15) reflects PolyP turnover by microorganisms.

While my results show dynamic cycling of polyphosphate by microbes in wetland soils, considerable work remains to be done to understand the nature of this process and its importance for wetland P cycling and flood induced P release. My findings suggest that microbial PolyP cycling may contribute to flood-induced P release in some wetlands, with dynamic PolyP pools in pocosin soils accounting for up to 11% of extracted P, similar to the proportion of PolyP obtained from freshwater and marine sediments (Hupfer et al. 2004, Diaz et al. 2008). Although my findings suggest PolyP accumulation may be limited to acid organic soils of isolated headwater wetlands, this pattern might conversely reflect a particular efficacy of microbial P uptake in these wetlands. This function appears to have been partially remediated by wetland restoration at the Barra Farms mitigation complex, and could be related to lowered outflow P concentrations from the restored wetland or shifts in microbial communities observed with restoration in these same sites (Bruland et al. 2003, Hartman et al. 2008).
4. Differential nutrient limitation of soil microbial biomass and metabolic quotients ($q\text{CO}_2$): Is there a biological stoichiometry of soil microbes?

4.1 Introduction

Variation in microbial metabolism poses a critical uncertainty in current understanding of carbon (C) cycling in terrestrial and aquatic ecosystems (López-Urrutia and Morán 2007, Allison et al. 2010), and improved understanding of microbial mediation of C and nutrient cycling is needed to predict ecosystem responses to human alteration of land use, climate, and nutrient availability (Bradford et al. 2008, Chapin et al. 2009). Turnover of C in terrestrial soils may depend closely on availability of major nutrients like nitrogen (N) and phosphorus (P) (Bradford et al. 2008), and ratios of these elements relative to microbial demand strongly influences C and nutrient mineralization during decomposition (Manzoni and Porporato 2009, Manzoni et al. 2010).

The relative demand of soil microbes for C, N, and P are broadly to occur in fixed ratios, as expressed by global patterns in the stoichiometry of enzyme activities and soil microbial biomass pools (Cleveland and Liptzin 2007, Sinsabaugh et al. 2009). However, the C:N:P stoichiometry of microbes varies considerably among ecosystems, habitats, and taxonomic groups (Bratbak 1985, Gundersen et al. 2002, Makino et al. 2003, Makino and Cotner 2004, Cleveland and Liptzin 2007), and multiple element limitation models predict an important sensitivity of microbial C use efficiency to variation in microbial stoichiometry (Manzoni et al. 2010). While relatively little is known about relationships between microbial stoichiometry and metabolism, variation in their stoichiometry may be coupled with differences in growth rates, like other heterotrophs (Makino et al. 2003).
Metabolic relationships between carbon and nutrient cycling in heterotrophs can be understood as a function of the biochemical composition of the cellular machinery. Particularly, the Growth Rate Hypothesis (GRH) describes a relationship between cellular growth rate and P concentration that results from the requirement of growing cells for P-rich ribosomes to produce new proteins (Elser et al. 1996, Elser et al. 2000b). Relationships between organismal stoichiometry and growth rate described by the GRH, also known as Biological Stoichiometry, appear consistently across heterotrophs spanning several orders of magnitude in size, and are importantly linked to trophic status of heterotrophs, autotrophs, and whole ecosystems (Elser et al. 1996, Elser et al. 2000a, Elser et al. 2003, Güsewell et al. 2003, Arrigo 2005, Agren 2008, Sterner et al. 2008, Elser et al. 2010, Reich et al. 2010). While the relationships between stoichiometry and metabolism described by the GRH are essentially untested in soils, the GRH would imply soil microbial metabolism may be particularly sensitive to the availability of P.

The availability of soil P may be particularly important for the growth and metabolism of microbes, with important implications for soil C cycling. On average, the relative biomass P demand of soil microbes (C:N:P = 60:7:1) is considerably greater than relative P supply in soils (C:N:P = 186:13:1), and plant litter (C:N:P = 3144:45:1) inputs (Cleveland and Liptzin 2007). Moreover, the global stoichiometry of enzyme activities (C:N:P =1:1:1) (Sinsabaugh et al. 2009) implies microbial allocation to P uptake is considerably greater than that of C and N when compared to the requirements for the growth of biomass. While the role of P availability in ecosystem C turnover is understudied relative to N in soils, recent findings indicate many terrestrial ecosystems may be co-limited by N and P, and that P availability may influence soil C cycling even

The present study explores supporting evidence for metabolic mechanisms relating the effects of soil P on soil C cycling, and compares the effects of P availability on the biomass, stoichiometry, and metabolic rates of microbes, across the widest possible range of global conditions in terrestrial and wetland soils. Soil C turnover by microbes depends on both the biomass of microbes and their metabolic rates, and I explore the effects of P availability on microbial biomass and metabolism separately, as differences in the C use efficiency (biomass growth vs. respiration per unit biomass) may arise from nutrient limitation or shifts in microbial stoichiometry (Manzoni et al. 2010). Variation in the stoichiometry of soil microbial biomass is of particular interest to the present work due to the potential relationship between biomass P stoichiometry and metabolic rates described by the Growth Rate Hypothesis (Elser et al. 1996, Elser et al. 2000b).

To explore the potential mediation of microbial metabolism by P availability in soils, I compiled a cross-ecosystem dataset of soil and microbial C, N, and P pools, and soil C mineralization rates measured simultaneously in the literature, spanning global variation in climate, land use, and vegetation in terrestrial and wetland ecosystems. I modeled relationships among measurements of soil resource availability (C, N, and P pools and ratios, inorganic P availability), microbial biomass, microbial stoichiometry, C mineralization rates, and rates of microbial metabolism, and accounted for the additional influences of soil pH, climate, land use and vegetation. Soil C mineralization data were obtained only from studies with controlled incubations rather than field measurements to eliminate root respiration and normalize temperature and moisture
conditions, and I calculated the microbial metabolic quotient ($q_{\text{CO}_2}$) as the rate of C mineralization per unit of microbial biomass C to index variation in the metabolic rates of soil microbial communities (Anderson and Domsch 1990, Anderson and Domsch 1993). While exploratory in nature, this approach allows a first assessment of basic relationships between soil microbial metabolism and P availability across a range of conditions encompassing global extremes in productivity and decomposition.

### 4.2 Materials and Methods

#### 4.2.1. Data compilation

To determine the influence of resource availability on microbial growth and metabolism in soils, I compiled data on soil and microbial stoichiometry and respiration, and metabolism from existing published studies, focusing exclusively on studies including measurements of microbial C,N, and P due to the central role of microbial stoichiometry in my research hypotheses. Data from many relevant studies were compiled in a recent data synthesis of the stoichiometry of the microbial biomass in terrestrial soils (Cleveland and Liptzin 2007). While my approach to literature review and data collection was broadly similar to that of (Cleveland and Liptzin 2007), and data was re-used where appropriate, I used different search methods to obtain a greater number of publications encompassing a broader range of soil habitats (including wetlands and crops), and explicitly categorized the major habitats, vegetation types, and land uses from which samples were obtained. Importantly, I collected additional data parameters not included in the previous study (Cleveland and Liptzin 2007), particularly measurements of soil C mineralization and $q_{\text{CO}_2}$ along with measurements of available inorganic P and soil pH where data was available in studies simultaneously measuring microbial biomass C,N, and P pools.
4.2.1.1 Literature review

To efficiently obtain publications with complete microbial biomass C, N, and P data, all literature searches included the term “microbial biomass phosphorus” (including quotes), as studies of microbial biomass P (MBP) often include data on microbial C and N pools, while more prevalent studies of microbial C:N stoichiometry often do not analyze microbial P (Cleveland and Liptzin 2007). While citation based literature searches have been used to obtain publications citing common methods for determining microbial P pools (Cleveland and Liptzin 2007), I used a general search for “microbial biomass phosphorus” to obtain publications that might cite methods in the authors’ earlier work rather than the original methods papers.

To obtain a dataset with the most comprehensive coverage of global terrestrial ecosystems, I conducted a stratified literature search, with general searches followed by searches for data in specific ecosystem types. All literature searches contained the text “microbial biomass phosphorus,” with additional search strings describing terrestrial biomes and land uses along with wetland habitats. A general search was conducted to obtain the most likely results by searching for the string: ““microbial biomass phosphorus” C/P soil.” Ecosystem specific searches were more relaxed to allow closer examination of work in under-sampled habitats. These searches included “microbial biomass phosphorus” combined with “tropical”, “subtropical”, “coniferous”, “deciduous”, “savannah”, “pasture”, “crop”, “desert”, “boreal”, and “tundra”. Even more specific searches were applied to obtain data from wetland habitats, including searches for “microbial biomass phosphorus” with “wetland”, “peatland”, “bog”, “fen”, “salt marsh”, “estuary”, “floodplain”, “paddy”, and “palustrine”. Each of these habitat specific searches were repeated with the additional terms “CO2” and “qCO2,”
(generating nested, redundant results) to assess the availability of studies simultaneously measuring MBP and soil C cycling.

Literature searches were conducted with Google Scholar for its open access to those who may wish to repeat the approach, and citations from search results were compiled in bulk using the free Zotero plug-in (www.Zotero.org) for the Firefox browser described by (Hull et al. 2008). From the comprehensive search results database, redundant citations were removed and publications were screened based on the inclusion of data on microbial biomass P and other microbial biomass pools.

### 4.2.1.2 Data collection and processing

I obtained 99 publications with relevant data, although fewer (66) were included in the final dataset due to severe ecosystem disturbance or unusual samples, data presented in graphical formats only, use of non-standard methods, or incomplete data preventing the calculation of standard units. Data was obtained only from publications measuring microbial biomass C, N, and P concentrations using standard chloroform fumigation-extraction methods (0.5 M KCl to extract biomass C and N, and 0.5 M NaHCO$_3$ to extract biomass P), following (Cleveland and Liptzin 2007), as these are the most widely used and accepted methods for determining C and N concentrations in soil microbial biomass (Brookes 2001). To obtain coverage of the greatest range of ecosystem productivity and decomposition, I collected data from as many soils as possible, including samples from altered ecosystems reflecting human disturbance (e.g. crops, pastures and grazed savannas) that were excluded from the previous study (Cleveland and Liptzin 2007). Publications used as data sources are listed in Appendix C, Table 17.

Soils and soil datasets were cross-classified by climate, land use, vegetation, and depth horizon. While I broadly attempted to include the greatest number of samples,
some soils were omitted to due poor fit with classification schemes, such as relatively unusual vegetation (e.g. agroforestry monotypes). Climate categories were assigned based upon author descriptions of climatic variables, and studies were assigned to Tropical, Subtropical, Savanna, Desert, Temperate, Boreal, and Tundra categories. Seasonally rainy, mid-latitude climates were classified as Subtropical (monsoonal, annual precipitation $\geq 1000$ cm) or Savanna ($< 1000$) if not adequately described. Land use and vegetation categories included Wetlands (Organic and Mineral Soils), Forest soils (Tropical, Coniferous, Deciduous, and Boreal), Forest O horizons (Forest Floor and Litter), Pastures, Crops, Tundra, and Desert. Most grasslands (excluding tundra heaths) were classified as pastures, as the vast majority of soils included grazing animals. However, fertilized pastures were excluded due to extremely high variability of soil and microbial factors and the difficulty of accounting for differences in fertilization treatments across studies. Soils were also classified by horizon, including forest litter and humic horizons where available, as well as by depth, although soil samples collected $> 10$ cm from the surface were excluded in the present analysis.

My final dataset includes measurements from 355 soils published in 66 studies (Appendix C, Table 20), a substantial increase from data obtained by citations of MBP methods alone (Cleveland and Liptzin 2007) (186 soils, 48 studies). Like the previous study (Cleveland and Liptzin 2007), the unit of observation for each study depended upon the units of data presented in the original publications, which in some cases were treatment means rather than individual samples. To standardize data and allow for comparison of elemental stoichiometry, all measurements of microbial biomass C, N, and P were converted into units of mmol/kg soil. Where data presented were not calculated using conversions for extraction efficiency, standard correction coefficients
(0.45 for microbial C and N, 0.40 for microbial P) were applied (Brookes 2001). Data were also collected for total pools of soil C, N, and P, and converted to mmol/kg soil.

Data on soil pH, inorganic P, and C mineralization rates were obtained from the final 66 publications with microbial biomass C, N, and P data where available. Soil pH data was collected where available, as soil pH is known to strongly affect the community composition of soil microbes (Hartman et al. 2008, Lauber et al. 2009).

Extractable inorganic phosphorus data was obtained from studies using the Olsen extraction (0.5 M NaHCO$_3$), and data was converted to mmol/kg soil (Olsen et al. 1954). Olsen P is thought to reflect soil phosphorus availability (Olsen et al. 1954), and is necessarily obtained for the measurement of microbial biomass P (Brookes et al. 1982), but not always reported in publications. Data on extractable C and N were relatively rare compared to extractable P, and were not included in subsequent analyses.

Soil C mineralization data were only collected from studies that determined respiration rates using incubation of soils and litter with standardized moisture in jars using the average hourly respiration over a ten hour period (Anderson and Domsch 1990), and data were converted to obtain an average rate of C mineralization expressed as $\mu$mol CO$_2$-C/h/g soil. Metabolic rates of soil microbial communities were determined by calculating the metabolic quotient $q_{CO_2}$ (Anderson and Domsch 1993), by dividing C mineralization rates (per gram of soil) by microbial biomass (per gram of soil), to yield $q_{CO_2}$ values expressed in mmol CO$_2$-C/mol MBC/h. Respiration and microbial biomass data obtained using substrate addition methods (e.g. substrate induced respiration- (Anderson and Domsch 1985)) were not considered as these methods do not represent the respiration of natural soil carbon, and may not be comparable with soil incubation measurements. Importantly, all soil C mineralization
measurement data analyzed was obtained concurrently with measurements of microbial biomass C, N, and P pools and soil data presented in the same published studies.

4.2.2. Statistical methods

I explored relationships between climate, vegetation and soils, and the growth, stoichiometry, and metabolism of microbes, with all statistical analyses conducted using the free, open source R statistics software (www.r-project.org). Nutrient concentrations in the environment are often distributed log-normally (Qian 2009), and measurements of elements in soils were log$_{10}$ transformed to improve normality, and facilitate fitting of size-dependent allometric models commonly used to determine stoichiometric relationships (Cleveland and Liptzin 2007, Sterner et al. 2008, Reich et al. 2010).

To assess the importance of differences among ecosystems in determining soil chemistry and microbial physiology, variation in the stoichiometry of soils and microbes, along with soil respiration and microbial metabolic quotients were tested among climatic regions and vegetation types using one-way ANOVA. Pairwise differences among ecosystem types were determined using Tukey’s tests, and general linear models (GLM) were used to determine the proportion of variance in soils, microbes, and metabolism explained by climate and vegetation categories.

Stoichiometric relationships in soils and microbes were determined using a size-dependent approach, which describes allometric relationships based on the power function $y = a x^b$ (Cleveland and Liptzin 2007, Sterner et al. 2008, Reich et al. 2010). Log transformation of this power relationship yields the linear function $\log y = a + b (\log x)$, allowing the use of linear regressions of log$_{10}$ transformed data to determine stoichiometric relationships among nutrients in soils and microbes. Similar models were constructed for each pairwise combination of soil, microbe, and respiration variables. To
aid in visualization of multiple covariant relationships, I created a corrleogram based on Pearson’s correlations using the CORRGRAM package in R (Friendly 2002).

Multiple regression models were compared with different combinations of factors in the environment, soils, and microbes. Only select parameters of interest for the growth, stoichiometry, and respiration of microbes were modeled, including microbial biomass C, microbial biomass C:P and N:P, soil respiration (CO₂) and qCO₂. Stepwise model selection procedures did not informatively discriminate among models, due to the large number of missing data points for different parameters among studies in the dataset. While information-theoretic approaches based on Akaike’s information criteria (AIC) penalize model likelihood based on the number of parameters, large gaps in data for some parameters reduced the model sum of squares more dramatically than the AIC penalty for the additional parameters, so that automated model selection by AIC methods always choose full sets of variables with the least number of observations.

Instead, multiple regression models were tested manually, using both forward and reverse selection to obtain candidate models, and a branching approach was used to test further interactions of significant variables until no further significant models could be obtained. Due to the presence of significant interaction terms among variables, and differences in sample size among parameters confounding information criteria like the AIC, I did not select a single “best model.” Instead, an illustrative set of candidate models are presented with their criteria to show the simultaneous significance of different sets of ecosystem and soil predictor variables, and their inter-relationships. This approach acknowledges that there may be several possible models of these ecosystem processes (Whittingham et al. 2006, Lukacs et al. 2007), which are not well enough understood to justify a priori models needed for SEM (Sutton-Grier et al. 2009).
4.3 Results and Discussion

4.3.1 Global variation in soil resource availability (C:N:P)

Variation in the relative availability of P in soils is poorly characterized due to the emphasis of many decomposition models and ecosystem studies solely on soil N and C:N ratios in soils (Hessen et al. 2004, Manzoni and Porporato 2009), especially compared to well characterized differences in N:P ratios of vegetation and leaf litter at global scales (Gusewell 2004, Hedin 2004, McGroddy et al. 2004, Elser et al. 2010, Reich et al. 2010). I found that soil C:N ratios varied primarily among leaf litter layers and organic soils from wetlands and tundras, but not consistently among soils of other ecosystems or with accumulation of soil C (Fig. 16a). Unlike soil C:N, soil C:P varied considerably among land use and vegetation types ($R^2 = 0.631$), and was closely related to soil C (Fig 16b), showing dilution of soil P relative to C with increasing soil carbon.

Soil N:P ratios were also coupled with soil C accumulation (Fig 16c), and closely tied to with variation among land use and vegetation types ($R^2 = 0.557$) but not climate ($R^2 = 0.196$, Appendix E, Table 23). Patterns of soil N:P stoichiometry (Appendix D, Table 21) broadly matched nutrient limitation of primary productivity in soils, with P limitation (soil N:P > 16:1) of tropical forests (Cleveland et al. 2002, Cleveland et al. 2004) and organic wetland soils (Bedford et al. 1999), and some boreal forests (Giesler et al. 2002), and N limitation (N:P < 16:1) of temperate forests, grasslands, and crops in temperate and tropical savannah climates (Vitousek and Howarth 1991). As soil C:N ratios are largely stable across many soils (Fig. 16a), variation in soil N:P may be largely shaped by variation in C:P ratios, which depend strongly on soil C (Fig. 16b). Thus, major differences in nutrient availability (soil C:N:P) across land use and vegetation types appear to arise from dilution of soil P concentrations by soil C accumulation.
Figure 16: Scaling of soil a) C:N, b) C:P, and c) N:P ratios with soil carbon accumulation. Soil stoichiometry and C are log transformed for normality. Dashed lines indicate regression fits, and solid lines indicate the Redfield (1958) ratios (C:N:P = 106:16:1). Soil C:N (a) was fit separately for organic soil horizons and forest litter, and all other soils.
4.3.2 Soil resources and microbial biomass pools

Patterns of soil resource availability and microbial biomass carbon pools suggest the biomass of soil microbes is primarily limited by N availability. Microbial biomass C pools were closely coupled with soil C and N pools, but not with to soil P (Fig 17). The non-linear relationship between microbial C and soil C (Fig. 17a, allometric slope < 1), indicates the proportion of soil C as microbial biomass (MBC/C) declines with increasing C, suggesting some nutrient limitation of microbial growth where soil C (and thus C:N:P) are high. While both soil C and N are closely coupled to MBC, considerably greater C:N ratios of soils than microbes (C:N = 18.8 and 10.7, respectively, Appendix B, Table 21-22) suggest that N primarily limits the growth of microbial biomass in soils, as predicted by a stoichiometric model of microbial C and N (Schimel and Weintraub 2003).

Weak effects of P on microbial growth in soils may be suggested by multiple regression models linking P availability (C:P and Pi) to microbial biomass (Table 8), but these do not appear to be primary drivers of MBC pools. While inorganic P concentrations improved the fit of models of MBC, my strongest models did not include P as a factor due to missing data (Table 8). The model explaining the most variance in MBC included soil C:P, N, and climate (Table 8, Model 8). However, positive correlations between MBC and soil C:P and N:P ratios ($R^2 = 0.485$ and 0.446 respectively) arise in part due to autocorrelation with soil C (Fig. 16), and these relationships suggest MBC actually increases in proportion to soil P dilution. Considerable differences in MBC relative to soil P, but not soil C or N (Fig. 17), may suggest that soil microbial communities may have evolved mechanisms to cope with large variation in the relative availability of P across soil habitats.
Figure 17: Variation of soil microbial biomass C (MBC) with a) soil carbon (C), b) soil nitrogen (N), and c) soil phosphorus (P) contents. Dashed lines are regression fits. Outliers from the general relationship between MBC and soil C in a), including floodplain mineral soils (Schilling 2005) (shown in inset in panel a, with 1:1 line) and arctic tundra (Johnasson 2002) were removed prior to fitting regressions. Relationships between MBC and soil P were also fit for all soils, and tested separately for only forest and pasture soils (elliptical region with fit line, panel c).
Table 8: Summary of selected results from multiple regression modeling of soil microbial biomass (MBC). Only models with all predictors simultaneously significant are shown. To account for differences in the availability of data for different soil parameters (missing data for one parameter results in discarded observations in regression), I computed the test statistic \( \% \text{Var} = R^2 \times n \text{ samples in model} / n \text{ total samples} \). Models including \( P_i \) (Models 9-13) had low \( \% \text{Var} \) due to missing data, and the greatest \( \% \text{Var} \) was described by model 8, which had an AIC close to that of models including \( P_i \). Linear regression results for individual predictors are given in Appendix E, Table 23.

<table>
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<tr>
<th>#</th>
<th>Model</th>
<th>( R^2 )</th>
<th>AIC</th>
<th>df</th>
<th>% Var</th>
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<td>8</td>
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<td>9</td>
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4.3.3 Variation in stoichiometry of soil microbes

The C:N:P stoichiometry of the microbial biomass appeared relatively homeostatic compared to large variation in the stoichiometry of soils across habitats (Fig. 18), and was not closely related to the C:N:P stoichiometry of soils (Appendix E, Table 23), as previously observed by (Cleveland and Liptzin 2007). The C:N ratios of the soil microbial biomass were particularly tightly constrained, while greater variation in the C:P and N:P ratios of soil microbes (Fig. 18) may be linked to functional variation.
Figure 18: Scaling of pools of carbon (C), nitrogen (N), and phosphorus (P) in soils and soil microbial biomass across terrestrial and wetland ecosystems. Relationships in plots show variation in a) C:N ratios b) C:P ratios c) N:P ratios of soils, microbial biomass, and combined data. Log transformed data are plotted to express size dependent relationships (dashed lines are regression fits) in comparison to the Redfield (1958) ratios (solid black lines). Point colors follow legends in other figures.
Considerably greater variation in C:P and N:P ratios than C:N ratios are also commonly observed in the stoichiometry of plants, plankton, and seston (Heldal et al. 2003, McGroddy et al. 2004, Sterner et al. 2008), and this variation is linked with differences among ecosystem types (Sterner et al. 2008) and functional traits (Reich and Oleksyn 2004, Arrigo 2005, Westoby and Wright 2006, Elser et al. 2010).

While the C:N:P stoichiometry of the microbial biomass is considered to be relatively constant across terrestrial soils (Cleveland and Liptzin 2007), my results show differences in microbial N:P ratios with land use and vegetation type (Fig. 19). Shifts in microbial stoichiometry have previously been observed with land use change in forests and grasslands (Yeates and Saggar 1998, Ross et al. 1999, Chen et al. 2000, 2004, Chen et al. 2008), and I found land use and vegetation type were the strongest single predictor of

![Figure 19: Variation in N:P stoichiometry of soil microbial biomass with land use and vegetation. Letters on x-axis above the plot show group differences among vegetation types (using Tukey’s tests), and number of samples for each vegetation type are given on the lower x-axis. Solid horizontal line is the Redfield (1958) ratio N:P = 16:1, dashed line is average microbial N:P (6.9) reported by Cleveland and Lipzin (2007). Overall variance described by vegetation was determined using a general linear model, which was highly significant (p < 0.001).](image)
microbial N:P ratios, while other predictors alone, including soil stoichiometry were quite weak. Observed variation in microbial N:P across habitats may be related to changes in the relative availability of soil P. Although microbial and soil stoichiometry were not closely linked, soil C:P and N:P ratios were significant factors in all models of microbial N:P, as was inorganic P despite the large amount of missing P data (Table 9, models 3-8). Relationships between climate and microbial N:P (Table 9) may also be liked to P availability though differences in soil weathering (Cleveland et al. 2002, Cleveland et al. 2004, Hedin 2004).

My results may also suggest weak size-dependent relationships of microbial N:P pools. Although support for the non-linear scaling of microbial C:P and N:P ratios was not strong (Fig. 18 b,c, allometric slopes < 1), microbial biomass C was a significant predictor in all models of microbial N:P (Table 9), suggesting a size-dependence of microbial N:P ratios. Clear size patterns of size dependent variation in the N:P ratios of plants, algae, seston, and animals (Elser et al. 1996, Heldal et al. 2003, McGroddy et al.

<table>
<thead>
<tr>
<th>#</th>
<th>Model</th>
<th>R²</th>
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<td>5</td>
<td>mN:P ~ NP+MBC+Pi+Climate</td>
<td>0.491</td>
<td>-2</td>
<td>93</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
<td>mN:P ~ CP+MBC+Pi+Climate</td>
<td>0.528</td>
<td>-9</td>
<td>93</td>
<td>26</td>
</tr>
<tr>
<td>7</td>
<td>mN:P ~ CP+MBC+Pi+Vegetation</td>
<td>0.555</td>
<td>-11</td>
<td>88</td>
<td>26</td>
</tr>
<tr>
<td>8</td>
<td><strong>mN:P ~ CP+MBC+Pi+Climate+CP*Climate</strong></td>
<td><strong>0.570</strong></td>
<td><strong>-15</strong></td>
<td><strong>89</strong></td>
<td><strong>27</strong></td>
</tr>
</tbody>
</table>


4.3.4 Factors shaping the microbial metabolic quotient (qCO₂)
While carbon mineralization rates in soil incubations were closely linked to pools of microbial biomass C across habitats (Fig. 20a), the primary interest of this study lies in
variation of the metabolic rates of soil microbes, which I assessed by the residuals of the relationship of MBC and CO$_2$ (Table 10), and analysis of the metabolic quotient $q$CO$_2$ (CO$_2$:MBC) in soils (Anderson and Domsch 1990, Anderson and Domsch 1993). In particular, I hypothesized that microbial metabolic rates may be linked to microbial P stoichiometry, as described in other heterotrophs by the Growth Rate Hypothesis.

In Figure 20: Soil C mineralization rates (CO$_2$) vary with a) microbial biomass C (MBC) and b) soil pH. C mineralization rates were measured in glass jar incubations in studies with concurrent measurements of microbial biomass C,N, and P. Dashed line in a) is the regression fit, and solid line is the 1:1 line.

Inorganic available P (P$_i$) contributed to the best fitting multiple regression models of soil C mineralization (Table 10, Models 15-19), and was the strongest single predictor of microbial metabolism ($q$CO$_2$) in soils (Fig. 21a). The fit between available P and $q$CO$_2$ is quite similar to the fit of inorganic P availability and substrate induced respiration recently observed across land use categories (Strickland et al. 2010). This finding may suggest that the effects of soil P availability on soil C mineralization and
Table 10: Multivariate models of soil C mineralization rates (CO$_2$) as a function of ecosystem, soil and microbial factors. Only models with all predictors simultaneously significant are shown. The test statistic %Var accounts for differences in the availability of data for different soil parameters (described in Table 8), and models including Pi had low %Var due to missing data. Model 9 had the greatest %Var, although adding pH (Model 14) also resulted in a large %Var with a much smaller AIC. Linear regression results for individual predictors are given in Appendix E, Table 23.

<table>
<thead>
<tr>
<th>#</th>
<th>Model</th>
<th>$R^2$</th>
<th>AIC</th>
<th>df</th>
<th>%Var</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CO2 ~ MBC</td>
<td>0.697</td>
<td>91.7</td>
<td>91</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>CO2 ~ MBC + pH</td>
<td>0.729</td>
<td>66.8</td>
<td>70</td>
<td>56</td>
</tr>
<tr>
<td>3</td>
<td>CO2 ~ MBC + CP</td>
<td>0.735</td>
<td>72.1</td>
<td>86</td>
<td>69</td>
</tr>
<tr>
<td>4</td>
<td>CO2 ~ MBC + mCP</td>
<td>0.743</td>
<td>77.5</td>
<td>91</td>
<td>74</td>
</tr>
<tr>
<td>5</td>
<td>CO2 ~ MBC + mCP + CP + Climate</td>
<td>0.778</td>
<td>60.5</td>
<td>86</td>
<td>74</td>
</tr>
<tr>
<td>6</td>
<td>CO2 ~ MBC + mCP + CP + MBC*CP</td>
<td>0.804</td>
<td>47.6</td>
<td>86</td>
<td>76</td>
</tr>
<tr>
<td>7</td>
<td>CO2 ~ MBC + mCP + Vegetation</td>
<td>0.806</td>
<td>60.6</td>
<td>91</td>
<td>81</td>
</tr>
<tr>
<td>8</td>
<td>CO2 ~ MBC + mCP + CP + Climate + MBC*CP</td>
<td>0.819</td>
<td>43.7</td>
<td>86</td>
<td>77</td>
</tr>
<tr>
<td>9</td>
<td>CO2 ~ MBC + mCP + Vegetation + Climate</td>
<td>0.828</td>
<td>51.2</td>
<td>91</td>
<td>83</td>
</tr>
<tr>
<td>10</td>
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<td>39.5</td>
<td>70</td>
<td>64</td>
</tr>
<tr>
<td>11</td>
<td>CO2 ~ MBC + mCP + CP + MBC*CP + pH</td>
<td>0.836</td>
<td>27.9</td>
<td>65</td>
<td>60</td>
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<tr>
<td>12</td>
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<td>0.864</td>
<td>26.4</td>
<td>70</td>
<td>66</td>
</tr>
<tr>
<td>13</td>
<td>CO2 ~ MBC + mCP + Vegetation + pH + Lat</td>
<td>0.879</td>
<td>20.1</td>
<td>69</td>
<td>67</td>
</tr>
<tr>
<td>14</td>
<td>CO2 ~ MBC + mCP + Vegetation + Climate + pH</td>
<td>0.882</td>
<td>18.6</td>
<td>70</td>
<td>68</td>
</tr>
<tr>
<td>15</td>
<td>CO2 ~ pH + NP + Pi + mNP</td>
<td>0.885</td>
<td>9.5</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>16</td>
<td>CO2 ~ pH + CP + Pi + MBC</td>
<td>0.897</td>
<td>-0.5</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>17</td>
<td>CO2 ~ pH + NP + Pi + MBC</td>
<td>0.902</td>
<td>-1.6</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>18</td>
<td>CO2 ~ pH + NP + MBC + Pi + MBC*Pi</td>
<td>0.935</td>
<td>-10.7</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>19</td>
<td>CO2 ~ pH + CP + MBC + Pi + MBC*Pi</td>
<td>0.935</td>
<td>-10.8</td>
<td>23</td>
<td>24</td>
</tr>
</tbody>
</table>

decomposition recently observed by others (Bradford et al. 2008, Manzoni et al. 2010, Strickland et al. 2010) result primarily from shifts in microbial metabolic rates with P availability, especially as soil microbial pools appear limited by N but not by P.

However, it is unclear if P$_i$ is linked to metabolism directly (e.g. coupling of cellular C
Figure 21: Metabolic quotients ($q_{\text{CO}_2}$) of soil incubations vary with a) available inorganic P and b) soil pH. $q_{\text{CO}_2}$ was calculated as the mol/mol ratio of C mineralization rates measured in glass jar incubations per unit microbial biomass C obtained from the same soils, with units of mmol CO$_2$-C/h/mol MBC-C/g soil.

uptake and phosphorylation by membrane phosphotransferases (Gorke and Stulke 2008), or moderated through the influence of $P_i$ on microbial stoichiometry (Table 9).

Although soil inorganic P was the factor most closely linked with microbial metabolic quotients (Fig. 21a), and required for the best fitting models of soil C mineralization (Table 10, Models 15-19), the models explaining the most variation in CO$_2$ and $q_{\text{CO}_2}$ did not include $P_i$ due to large amounts of missing data (Tables 10 and 11). Instead, the strongest models of microbial metabolism ($q_{\text{CO}_2}$) across all soils included pH, microbial C:P, soil stoichiometry, and vegetation and climate (Table 11).

Soil pH strongly affected both soil C mineralization (Fig. 19b) and metabolic rates ($q_{\text{CO}_2}$) across soils (Fig 21b), with non-linear increases in respiration and metabolism occurring below pH 5 - 5.5. Variation in soil $q_{\text{CO}_2}$ with soil pH has been described in previous studies as the result of increased of maintenance respiration required for
metabolic responses to pH stress (Anderson and Domsch 1993, Blagodatskaya and Anderson 1998). However, shifts in microbial metabolism with pH may also be related to shifts in soil microbial communities, as both fungal:bacterial ratios (Rousk et al. 2009, Rousk et al. 2010b) and the taxonomic composition of soil bacteria vary with pH across a wide range of soils, and like \( q\text{CO}_2 \) may shift about a soil pH value near 5.5 (Hartman et al. 2008, da C Jesus et al. 2009, Jones et al. 2009, Lauber et al. 2009, Chu et al. 2010).

Importantly, soil respiration rates appear linked with the relative abundance of some bacterial taxonomic groups, suggesting \( r \)- vs. \( K \)- selection of microbial life strategies based on differences in growth rates (Fierer et al. 2007).

**Table 11:** Multivariate models of the microbial metabolic quotient, \( q\text{CO}_2 \), as functions of ecosystem, soil, and microbial factors. Only models with all predictors simultaneously significant are shown. The test statistic %Var accounts for differences in the availability of data for different soil parameters (described in Table 8). Model 9 had the greatest %Var and lowest AIC. Linear regression results for individual predictors are given in Appendix E, Table 23.

<table>
<thead>
<tr>
<th>#</th>
<th>Model</th>
<th>( R^2 )</th>
<th>AIC</th>
<th>df</th>
<th>%Var</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( q\text{CO}_2 \sim m\text{CP}+\text{CP} )</td>
<td>0.243</td>
<td>64.0</td>
<td>86</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>( q\text{CO}_2 \sim m\text{CP}+p\text{H}+\text{Lat} )</td>
<td>0.422</td>
<td>47.4</td>
<td>69</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>( q\text{CO}_2 \sim p\text{H} + C + \text{Pi} + \text{CP} )</td>
<td>0.531</td>
<td>-1.1</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>( q\text{CO}_2 \sim m\text{CP}+\text{CP}+m\text{CP}^*\text{CP}+p\text{H} )</td>
<td>0.540</td>
<td>37.9</td>
<td>65</td>
<td>39</td>
</tr>
<tr>
<td>5</td>
<td>( q\text{CO}_2 \sim p\text{H}+m\text{CP}+\text{Vegetation} )</td>
<td>0.610</td>
<td>26.4</td>
<td>70</td>
<td>47</td>
</tr>
<tr>
<td>6</td>
<td>( q\text{CO}_2 \sim p\text{H}+m\text{CP}+\text{Vegetation} + \text{Climate} )</td>
<td>0.664</td>
<td>22.8</td>
<td>70</td>
<td>51</td>
</tr>
<tr>
<td>7</td>
<td>( q\text{CO}_2 \sim p\text{H}+m\text{CP}+\text{NP}+\text{Vegetation} )</td>
<td>0.705</td>
<td>5.9</td>
<td>65</td>
<td>50</td>
</tr>
<tr>
<td>8</td>
<td>( q\text{CO}_2 \sim m\text{CP}+\text{CP}+p\text{H}+\text{Vegetation}+\text{Climate} )</td>
<td>0.765</td>
<td>-9.4</td>
<td>65</td>
<td>55</td>
</tr>
<tr>
<td>9</td>
<td>( q\text{CO}_2 \sim m\text{CP}+\text{NP}+p\text{H}+\text{Vegetation}+\text{Climate} )</td>
<td><strong>0.777</strong></td>
<td><strong>-11.1</strong></td>
<td><strong>65</strong></td>
<td><strong>56</strong></td>
</tr>
</tbody>
</table>

Microbial C:P ratios were a factor in the most robust models of soil C mineralization (\( \text{CO}_2 \)) and metabolic quotient (\( q\text{CO}_2 \)) of soil microbes (Tables 10-11), which may suggest some link between P concentrations and metabolism like the relationships described by the Growth Rate Hypothesis. Previous studies have shown
coincident shifts in microbial stoichiometry and metabolic quotients with land use change (Sparling et al. 1994, Ross et al. 1999, Chen et al. 2000, Chen et al. 2008), although the present work is the first to my knowledge to consider relationships between microbial stoichiometry and metabolism in soils. However, the direct relationship between microbial C:P stoichiometry and $q_{\text{CO}_2}$ was weak ($R^2 = -0.107$, Appendix E, Table 23), and microbial C:P only predicted soil $q_{\text{CO}_2}$ strongly in combination with additional factors including soil stoichiometry, pH, vegetation and climate (Table 11). The influence of these additional factors suggests relationships between the metabolism and P concentration of microbes predicted by the GRH by may vary with effects of soil resources (C:N:P), stress (pH), carbon quality (land use and vegetation), and environment (e.g. climate).

Relationships between microbial stoichiometry and metabolic quotients in soils may be obscured by problems with the scale of measurements, and by biological factors affecting the metabolism and stoichiometry of microbes. Relationships between microbial stoichiometry and metabolism in soils may be dampened by imprecise measurements of whole communities of microbes, and the range of soil microbial biomass pools may not clearly reflect the range of biomass of individual cells. Moreover, ecological factors likely contribute to poor support for the growth rate hypothesis in soil microbes, as shifts in stoichiometry may be associated with shifts in communities in aquatic systems (Makino et al. 2003, Makino and Cotner 2004), and community composition changes with both land use and pH in terrestrial and wetland soils (Hartman et al. 2008, da C Jesus et al. 2009, Jones et al. 2009, Lauber et al. 2009, Chu et al. 2010). Finally, the accumulation of large intracellular stores of C, N, and P in response to stress and dormancy may contribute to shifts in the stoichiometry of
microbes that are associated with metabolic states other than growth (Brown and Kornberg 2004, Kadouri et al. 2005, Schimel et al. 2007, Seufferheld et al. 2008), and these storage compounds may complicate relationships between microbial P stoichiometry and growth even in simple bacterial cultures (Makino and Cotner 2004).

### 4.4 Conclusions

The most striking finding of this exploratory study is the apparent limitation of microbial biomass pools by soil N, and metabolic rates by inorganic P availability. While somewhat counterintuitive, this pattern essentially matches the description of cellular biochemistry given by the Growth Rate Hypothesis (GRH), with biomass growth dependent on the availability of N for the production of N-rich proteins, but with the rate of growth dependent on the availability of P for protein synthesis in P-rich ribosomes (Elser et al. 1996, Elser et al. 2000b). My findings of N limitation of biomass, but P limitation of microbial metabolic quotients in soils are also supported in the dynamic responses of bacteria in pure culture, which stop growing in response to N starvation, but grow at a reduced rate when starved for P (Peterson et al. 2005).

Although differential limitation of biomass growth by N, and metabolic rate by P implies co-limitation of C mineralization by N and P, the relative availability of these elements may affect the C use efficiency (ratio of C assimilated to C respired) of microbes, especially with variation in microbial stoichiometry (Manzoni et al. 2010). My results suggest that because microbial biomass growth is linked to C and N, but not P, microbes face considerable differences in relative P availability across habitats, and the increase of MBC with soil C:P suggests microbial P demand may shift in response to progressive P limitation. Reduced microbial P demand with P limitation may be
accommodated by shifts in microbial N:P ratios I observed across habitat types, which were also linked to P availability and size of the microbial biomass.

While the relationships between P stoichiometry and growth described by Biological Stoichiometry Theory (Elser et al. 1996, Elser et al. 2000b, Elser et al. 2010) would suggest a functional significance of the observed differences in microbial stoichiometry, I found only weakly suggestive support for size-dependence of microbial N:P ratios in soils, and primarily latent relationships between soil C:P ratios and metabolic quotients that might support the Growth Rate Hypothesis in soils. Although the strongest models of $q_{\text{CO2}}$ in soils included microbial C:P ratios, soil resource availability (C:P and N:P) and pH, along with climate and vegetation also contributed to variation in the metabolic rates of soil microbes. I suggest these factors may be related to shifts in the community composition of soil microbes and accumulation of storage compounds related to dormant metabolic states.

Considerable evidence suggests that size dependent relationships in organismal N:P ratios are closely related to growth rate (i.e. Metabolic Stoichiometry), and underlie differentiation of life strategies and functional traits in heterotrophs and autotrophs (Elser et al. 1996, Elser et al. 2000b, Heldal et al. 2003, McGroddy et al. 2004, Reich and Oleksyn 2004, Wright et al. 2004, Arrigo 2005, Westoby and Wright 2006, Elser and Hamilton 2007, Elser et al. 2010, Reich et al. 2010). Although the application of this framework to the soil microbial biomass in bulk is complicated by community scale measurements and the potential role of consumers (Elser et al. 2000a, Mulder and Elser 2009), metabolic relationships have been shown previously at community and ecosystem scales (Enquist et al. 2003, Sinsabaugh and Shah 2010).
While the present study shows only suggestive evidence for Biological Stoichiometry in soil microbes, these relationships present established, testable biochemical mechanisms that may improve understanding of microbial metabolism in C cycling models (López-Urrutia and Morán 2007, Allison et al. 2010, Manzoni et al. 2010). Shifts in metabolism, stoichiometry, and community composition of soil microbes with soil pH, land use, and season observed in separate studies may imply metabolic stoichiometry shapes differences in life strategies of microbes, as in other organisms. While essentially untested, relationships between life strategies, metabolism, and stoichiometry may also provide a needed framework to link microbial community composition with physiological processes and biogeochemical cycling, at the frontier of our understanding of the role of microbial communities in ecosystem functioning (Allison and Martiny 2008, Raes and Bork 2008, DeLong 2009).
5. Conclusions

5.1 Summary of research findings

The goal of this dissertation is to improve understanding of the role of microbial communities in phosphorus cycling, which is of particular interest for application to wetland and water quality management, but remains a critical gap in scientific knowledge across all ecosystems. To approach this poorly explored subject, I used the basic ecological theory that growth and stress responses link community structure and function to address community, physiological, and biogeochemical perspectives on microbial P cycling. I evaluated the responses of wetland microbial communities to stress and growth factors, and assessed the effects of microbial P metabolism related to stress (PolyP accumulation) and growth (Biological Stoichiometry) on ecosystem P biogeochemistry in wetland soils and other ecosystems.

To evaluate how environmental resources and stresses shape the taxonomic composition of microbial communities (Chapter 2), I determined the effects of soil chemistry, land use and restoration, and wetland type on the biogeography and diversity of bacteria in wetland soils. Soil bacterial communities did not vary with a substantial P enrichment gradient in the Florida Everglades, but broadly varied with soil pH across all wetlands. Land use and wetland restoration also had clear effects on bacterial community composition and diversity across wetlands of the N.C. coastal plains, while communities did not vary significantly among wetland types or with soil C,N, and P contents. Controls over bacterial communities in wetland soils share some similarity with those in other ecosystems, such as the broad influence of pH across aquatic, wetland, and terrestrial ecosystems, and effects of land use on microbes in terrestrial soils. However, responses of microbes to wetland restoration differed from
effects of restoration of terrestrial soils, as wetland restoration appears to promote increasing metabolic stress through low nutrient availability and pH. Response of particular microbes associated with both land use, and growth and stress responses suggest the potential to develop microbial indicators for wetland restoration, based upon their ecological strategies.

The patterns in bacterial community composition I found in wetland soils may reflect not only variation in communities with pH and land use, but more generally the habitat preference of bacterial groups (Philippot et al. 2010) related to stress and growth conditions. I found patterns in the biogeography of bacterial taxa indicating pH optima for some bacterial groups, and shifts in the relative abundances of bacterial groups with land use change (Chapter 2). These patterns may roughly reflect ecological strategies of bacteria, with more oligotrophic groups (like Acidobacteria) showing habitat preference for low pH, nutrient poor reference peatlands, and copiotrophic bacterial groups (e.g. β-proteobacteria) more abundant in higher pH, relatively eutrophic agricultural fields on former wetlands. Similar patterns of bacterial community composition with pH and land use in terrestrial soils (Fierer and Jackson 2006, da C Jesus et al. 2009, Lauber et al. 2009) have been informatively described as reflecting the ecological (r- vs. K-) strategies of bacteria, and the relative abundances of these groups may be related to soil C mineralization rates (Fierer et al. 2007).

To assess the biogeochemical significance of stress responses involving microbial P metabolism (Chapter 3), I determined the distribution of phosphorus species and dynamics of PolyP in wetland soils with field observations and experimental tests using $^{31}$P-NMR, and compared these findings to existing results in aquatic and terrestrial ecosystems. While I tested soils from a range of wetland types including salt marshes,
brackish marshes, and riverine swamps, PolyP accumulation was limited to acidic peat soils of isolated wetlands, a finding confirmed by my survey of published $^{31}$P-NMR studies in wetlands. The dynamics of PolyP I observed with flooding and season in the field, and with experimental manipulations, suggest that PolyP occurs in living microbial biomass, and I showed PolyP in soil is likely held in microbial cells, with some wetland bacteria capable of PolyP accumulation. I found some evidence of PolyP degradation with flooding and anaerobic conditions in wetlands like patterns observed in aquatic sediments and wastewater, and repeatedly observed PyroP accumulation following loss of PolyP. However, some shifts in soil PolyP with season and rainfall were inconsistent with processes in culture, sediment, or wastewater, and PolyP accumulation was not induced in soil slurry microcosms. I noted the distribution of PolyP accumulation in upland soils in the literature was, like wetlands, restricted to acid organic soils, and I suggest fungal PolyP accumulation in terrestrial soils may be useful as an alternate model of PolyP metabolism in wetlands, although I did not test this mechanism.

Finally, to evaluate potential relationships between microbial P cycling and metabolism (Chapter 4), I compared existing data on the C:N:P stoichiometry of soils and microbes to soil respiration and metabolic quotients ($q_{CO_2}$) across a large number of wetland and terrestrial soils representing major climatic regions, vegetation types, and land use categories. I found some variation in the N:P stoichiometry of the microbial biomass among ecosystems, and these differences could reflect size-dependent relationships in N:P ratios like those in plants and animals. Microbial stoichiometry was weakly but significantly related to soil respiration or metabolic rates, as might be expected by extending the Growth Rate Hypothesis (GRH) to describe the metabolism of
microbial communities. However, soil inorganic P availability was the strongest predictor of metabolic quotient, indicating an important role for P in microbial metabolism and soil C cycling, even in ecosystems generally thought to be N limited. My strongest results suggest that while microbial biomass pools appear to be limited by soil N, microbial metabolism appears to be limited by soil P availability, consistent with the biochemical mechanisms of cellular growth described by the growth rate hypothesis.

5.2 Integration and implications of research findings

The central goal of the research presented in this dissertation is to improve understanding of both microbial communities and microbial P metabolism in wetlands. However, due to the poor prior state of knowledge and characterization of relevant processes, my research tested microbial communities and P metabolism separately, and I was not able to directly test how microbial communities may be related to P cycling. Exploration of relationships between microbial communities and P cycling implies functional differentiation of microbial P metabolism, in contrast to the conventional view that all microbes use P in the same manner (Cleveland and Liptzin 2007, Sinsabaugh et al. 2009).

Although the central role of P in microbial genetics and energetics may suggest to some that all microbes use P similarly, others have emphasized that these precise factors may contribute to differentiation of microbial life (Elser et al. 2000b, Weider et al. 2005, Jeyasingh and Weider 2007, Souza et al. 2008, Hessen et al. 2010). Yet our understanding of linkages between microbial communities and P cycling is in its infancy, even in aquatic systems where microbial P cycling has been best studied (Makino et al. 2003, Dyhrman et al. 2007). In a coarse sense, the research presented in this dissertation contributes to the dissenting view that microbes may differ with respect
of their use of P, as I found habitat scale patterns reflecting differences in both PolyP accumulation and in the stoichiometry of microbes in wetlands and across other ecosystem types, broadly implying some differentiation of microbial P metabolism.

Prior to the present work, uncultured microbial communities in wetland soils were essentially unknown, and specific physiological processes of microbial P metabolism had not been explored. The different facets of my dissertation research represent early contributions to the understanding of bacterial communities and specific processes of P metabolism in wetlands, including polyphosphate accumulation and Biological Stoichiometry, linking shifts in microbial P metabolism to the cycling of P and C in soils in wetlands and other ecosystems.

To address critical knowledge gaps in knowledge of microbial communities and their P metabolism, I used life history theory as an organizing principle, emphasizing importance of growth and stress responses as an established mechanism linking community structure and function in ecology. I found broad evidence that stress and growth factors shaped both microbial ecology and P metabolism, with important shifts in each with pH stress and differences among habitats in wetland soils and in other ecosystems. These concomitant responses of microbial communities, PolyP accumulation, and microbial stoichiometry and metabolic rates could suggest some functional relationships between microbial community structure and P metabolism. Although others have speculated on the functional differentiation of microbial P metabolism, this subject represents an emerging frontier of our understanding of microbial ecology across all ecosystems (Makino et al. 2003, Makino and Cotner 2004, Weider et al. 2005, Dyhrman et al. 2007, Jeyasingh and Weider 2007).
My findings suggest further development of molecular and metabolic perspective on microbial P cycling will be required to better characterize the role of microbes in wetland P biogeochemistry. My research indicates that simple models of wetland microbial P cycling (e.g. biomass linearly tied to soil C, with fixed stoichiometry) may be inadequate to capture variation in microbial P metabolism, which may be related to community structure and function, and ultimately to the phosphorus biogeochemistry of wetlands. More detailed study of the importance of microbes in wetland P cycling, and the functional differentiation of microbial P metabolism presents a substantial set of challenges that will form the basis of my future research career, and I hope to inspire others to join me in the further pursuit of these important and interesting questions.
Appendix A: Supporting data for Chapter 2

Table 12: Soil chemistry data obtained for each wetland study site, including pH, total carbon, total nitrogen, and total phosphorus. Sites abbreviations are described in detail in Materials and Methods. WCA land use is water control area in the Everglades.

<table>
<thead>
<tr>
<th>Site</th>
<th>Land Use</th>
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<th>Percent Carbon</th>
<th>Percent Total N</th>
<th>Percent Total P</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1T</td>
<td>WCA</td>
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<td>39.4</td>
<td>2.78</td>
<td>1.58</td>
</tr>
<tr>
<td>D3T</td>
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<td>45.7</td>
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<td>1.71</td>
</tr>
<tr>
<td>D6C</td>
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<td>6.47</td>
<td>46.8</td>
<td>3.22</td>
<td>0.52</td>
</tr>
<tr>
<td>D6E</td>
<td>WCA</td>
<td>6.94</td>
<td>46.8</td>
<td>3.46</td>
<td>0.45</td>
</tr>
<tr>
<td>BFA</td>
<td>Agriculture</td>
<td>5.97</td>
<td>13.5</td>
<td>0.50</td>
<td>0.13</td>
</tr>
<tr>
<td>BFS</td>
<td>Restored</td>
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Table 13: Relative abundance of bacterial groups across different ecosystems. References are: 1 (Liles et al. 2003); 2 (Zhou et al. 2003); 3 (Chow et al. 2002); 4 (Dedysh et al. 2006); 5 (Wobus et al. 2003); 6 (Stout and Nusslein 2005); 7 (Horner-Devine et al. 2003).

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LEGEND % Relative abundance: > 15 % 5 -10 % < 5 % 0%
Table 14: Bacterial diversity and relative abundance of select bacterial groups across wetland soils. H’ tree is the Shannon’s index of bacterial diversity computed from the relative abundance of bacterial groups, determined by analysis of a phylogenetic tree assigning bacterial taxonomy to each sequence (see Figs. 1 and 2). H’ OTU is Shannon’s index of bacterial diversity computed from the number of 97% similarity operational taxonomic unit OTUs (see Fig. 7). The proportion of the Acidobacteria, all Proteobacteria, and the β-proteobacteria are provided along with the normalized ratio all Proteobacteria to Acidobacteria.

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<th>% Proteobacteria</th>
<th>% β-proteo</th>
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Appendix B: Supporting information for Chapter 3

Figure 22: Effects of neutralizing soil extracts prior to lyophilization on PolyP recovery determined by \(^{31}\)P-NMR spectroscopy, using different soil extracting solutions. Extracting solutions used and pH of neutralizing treatments accompany each NMR spectrum. Soils extracted were obtained from a homogenized composite sample from the reference tall pocosin at Barra Farms (BFF) on May 23, 2009. The relative abundances of different P forms in these NMR spectra are provided in Table 15.

Table 15: Effects of neutralization of soil extracts prior to lyophilization on the relative abundance of P forms in soil extracts, determined by \(^{31}\)P NMR. Data accompany spectra displayed in Fig. 21. Phosphorus forms obtained by NMR were: phosphonates (Phn, -20 ppm); inorganic orthophosphate (P\(_i\), 6 ppm); P monoesters (P-mono, 4 ppm); P lipids (PL, 2 ppm) and nucleic acids (DNA-P, 0 ppm); pyrophosphate (PyroP, -4 ppm), and polyphosphate (PolyP, -20 ppm).

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99
Table 16: References used for the global comparison of patterns in PolyP in sediments and soils (Fig. 15). References are original $^{31}$P-NMR studies citing key papers for NMR methods (Cade-Menun 1996) and peak assignments (Turner et al. 2003d). Raw data collected is provided here: for the number of NMR spectra in each study (n spectra); and the number of spectra with >1% of total peak area for different P forms.

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<tr>
<td></td>
<td></td>
<td>(McDowell and Cade-Menun 2006)</td>
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<td></td>
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</tr>
<tr>
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<td></td>
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<td>26</td>
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<td>9</td>
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<td></td>
<td></td>
<td>(Turner et al. 2007b)</td>
<td>9</td>
<td>7</td>
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Appendix C: Metadata and dataset for Chapter 4

Table 17: Publications from which data on soil microbial stoichiometry was obtained. Publications are sorted by climate and land use categories, then alphabetically.

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<th>Climate</th>
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<th>Reference</th>
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<th>n. obvs.</th>
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<td>(Cleveland et al. 2004)</td>
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<td>(Pandey and Srivastava 2009)</td>
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<td></td>
<td>4</td>
<td>(Yavitt et al. 1993)</td>
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<td>5</td>
<td>(Gangcay et al. 2001)</td>
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<td></td>
<td>6</td>
<td>(Monkiedje et al. 2006)</td>
<td>Crop</td>
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</tr>
<tr>
<td></td>
<td>7</td>
<td>(Singh and Singh 1995)</td>
<td>Crop</td>
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<tr>
<td></td>
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<td>Total</td>
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<td>(Chen and He 2004)</td>
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<td>(Wang and Wang 2008)</td>
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<td>(Arunachalam and Arunachalam 2000)</td>
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<td></td>
<td>14</td>
<td>(Devi and Yadava 2006)</td>
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<td></td>
<td>21</td>
<td>(Srivastava and Singh 1988)</td>
<td>Crop</td>
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<td>(Schilling and Lockaby 2005)</td>
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Total 90
Table 17, continued (p. 2 of 3):

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<td>31</td>
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<tr>
<td></td>
<td>32</td>
<td>(Khan and Joergensen 2006)</td>
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<tr>
<td></td>
<td>33</td>
<td>(Srivastava and Lal 1994)</td>
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<td>34</td>
<td>(Agbenin and Adeniyi 2005)</td>
<td>Pasture</td>
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<td></td>
<td>37</td>
<td>(Sarig et al. 1996)</td>
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<td>49</td>
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<td></td>
<td>53</td>
<td>(Kopáček et al. 2004)</td>
<td>Heath</td>
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<td></td>
<td>55</td>
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Table 17, continued (p. 3 of 3):

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<th>Reference</th>
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<th>n. obvs.</th>
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<td>57</td>
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<td></td>
<td>58</td>
<td>(Ross et al. 1999)</td>
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<td></td>
<td>59</td>
<td>(Saggar et al. 1998)</td>
<td>Coniferous Forest, Pasture</td>
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<tr>
<td></td>
<td>60</td>
<td>(Santrucková et al. 2004)</td>
<td>Coniferous Forest</td>
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<tr>
<td></td>
<td>61</td>
<td>(Sparling et al. 1994)</td>
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<td>63</td>
<td>(Lagerström et al. 2009)</td>
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<td></td>
<td>64</td>
<td>(Larsen et al. 2007)</td>
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<td></td>
<td>65</td>
<td>(Schmidt et al. 2002)</td>
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<td></td>
<td>66</td>
<td>(Jonasson et al. 1996)</td>
<td>Tundra</td>
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<td></td>
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<td><strong>Total</strong></td>
<td><strong>24</strong></td>
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<td><strong>Total soils</strong></td>
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Table 18: Codes for climate categories used to describe soils in the full microbial stoichiometry data set (Table 20).

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<td>Boreal</td>
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<td>Tundra</td>
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Table 19: Codes used to describe land use and vegetation classification of soils in the full microbial stoichiometry data set (Table 20).

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<td>4</td>
<td>Dry Scrub</td>
</tr>
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<td>Crop</td>
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<tr>
<td>6</td>
<td>Pasture</td>
</tr>
<tr>
<td>7</td>
<td>Tundra Heath</td>
</tr>
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<td>8</td>
<td>Boreal Forest</td>
</tr>
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<td>9</td>
<td>Wetland, Mineral Soil</td>
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<td>10</td>
<td>Wetland, Organic Soil</td>
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<tr>
<td>11</td>
<td>Forest Floor, Coniferous</td>
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<td>12</td>
<td>Forest Floor, Deciduous</td>
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<td>13</td>
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<td>14</td>
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</table>
Table 20: Data set obtained for soil and microbial stoichiometry across ecosystems, classified by climate (Clim.) and land use (L.U.). Codes for climate and land use categories are given in Table 17 and Table 18, respectively. All soil chemical and microbial pools are expressed as mmol / kg soil. The metabolic quotient $qCO_2$ is expressed as (µmol CO$_2$ / kg soil / h) / (mmol MBC / kg soil).

<table>
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<th>L.U.</th>
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<th>C</th>
<th>N</th>
<th>P</th>
<th>Pi</th>
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<th>MBN</th>
<th>MBP</th>
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# Appendix D: Supplemental summary data for Chapter 4

Table 21: Complete summary data for soil stoichiometry, classified by land use, vegetation, and climate. Letters indicate significantly differences in among vegetation classes by Tukey's multiple comparisons.

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119
Table 22: Complete summary data for soil microbial biomass stoichiometry, classified by land use, vegetation, and climate. Letters indicate significantly differences in among vegetation classes by Tukey’s multiple comparisons.

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Table 22, continued (p. 2 of 2):

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**Note:**
- Values indicate significant differences within columns.
- a, b, c represent significant differences between regions.

**Source:** Natural Resources Conservation Service.
Appendix E: Pairwise regression results for Chapter 4

Table 23: Results of pairwise linear regressions of all measured parameters. Relationships with categorical predictors (Climate, Vegetation) were assessed using generalized linear models. Table is presented in sections to accommodate large format. Pi:P is the ratio of inorganic (Olsen) P to soil total P, and Cm:C is the ratio of MBC:Soil C. These relationships are shown graphically in the correlogram that follows in Fig. 23.

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Legend:
- *Spurious
- "": p > 0.05
- *": p < 0.05
- **": p < 0.01
- ***": p < 0.001
Table 23, continued (p. 2 of 2): mC:N, mC:P, and mN:P are the C:N, C:P, and N:P ratios in microbial biomass. Pm:P is the ratio of microbial P to total P, and Pm:Pi is the ratio of microbial P to inorganic P.

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<tr>
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<td><strong>0.055</strong>*</td>
<td>0.021</td>
<td>0.313</td>
<td>0.338</td>
<td><strong>0.697</strong></td>
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<tr>
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<tr>
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<td><strong>0.144</strong>*</td>
<td>0.677*</td>
<td>0.491*</td>
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<tr>
<td>Pi</td>
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<td>-</td>
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<td>0.174</td>
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<td>C:N</td>
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<td><strong>0.034</strong>*</td>
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<td>-0.324*</td>
<td>0.032*</td>
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<td><strong>-0.107</strong>*</td>
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<tr>
<td>mN:P</td>
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<tr>
<td>Pm:P</td>
<td>*Spurious</td>
<td>0.483*</td>
<td>-</td>
<td><strong>0.296</strong></td>
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Figure 23: Correlogram for visualization of pairwise relationships among all variables measured and computed for metaanalysis. Color intensity is proportionate to Pearson correlations. Blue color represents positive correlations and red indicates negative correlations. Ordering of variables follows Table 22 above.
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size on phosphorus changes in a soil cultivated intermittently: analysis by 31P

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Biography

Wyatt H. Hartman was born in 1979 in Park Ridge, Illinois. His early interests in science predated even his junior high school science fair project, which received a runner-up prize at the regional fair. In high school he participated in ecological research on butterflies, conducted an independent study of fungi in local woodlands and wetlands, visited area biomedical laboratories through an exchange program, and was co-president of his high school Ecology Club. He found passionate and experienced mentors at the interface of science and environmental management as an undergraduate at Cornell University, where he worked as an assistant in three laboratories and conducted three independent research projects, graduating in 2001 with a B.S. in Resource Ecology from the Department of Natural Resources.

Prior to his arrival at Duke in 2002, Wyatt worked for the New York City Department of Water Quality, and the Pacific Estuarine Research Laboratory, completing a research tour of ecosystems including grasslands, alpine tundra, forests, wetlands, streams, lakes, and estuaries. His undergraduate thesis work was chosen for the Best Student Poster award by the Society of Wetland Scientists in 2003, and in 2004 he was awarded a Graduate Research Fellowship by the National Science Foundation to pursue independent dissertation research on polyphosphate accumulation in wetland microbial communities. Wyatt also received a Student Research Grant from the Society of Wetland Scientists in 2005, and was recognized with an award for the Best Student Presentation at the International Symposium on Wetland Biogeochemistry in 2007. His work on bacterial communities of wetland soils was selected for the Dean’s Award for Outstanding Student Manuscript by the faculty of the Nicholas School of the
Environment in 2009, based on his 2008 work with Dr. Richardson and Dr. Vilgalys, “Environmental and anthropogenic controls over bacterial communities in wetland soils,” which was published in the Proceedings of the National Academy of Science.

Wyatt has served as an ad-hoc reviewer for the journals Biogeochemistry and Microbial Ecology, and hopes to soon publish work from the remaining chapters of his dissertation. He has given guest lectures in Environmental Microbiology, Wetlands Ecology and Management, and Environmental Data Analysis, and has served as a teaching assistant for five graduate level classes on wetlands, statistics, and aspects of environmental management. His teaching experience also has included an Introduction to College Teaching course at Duke, and volunteer teaching to share hands on exploration of the sensory experiences of different wetland soils with 2-5th grade students at C.C. Spaulding School in Durham. He hopes to continue to incorporate service-oriented primary school science teaching activities to share the enthusiasm for discovery given to him by his teachers, and plans to supplement field and lab exercises (such as Winogradsky columns) used to illustrate the diversity of soils and bacteria, with educational computer games such as Phage Wars, Spore, and Colourshift to demonstrate more subtle and complex biological concepts.