Biological transformation of soil organic phosphorus in a long-term management trial

by

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ABSTRACT

BIOLOGICAL TRANSFORMATION OF SOIL ORGANIC PHOSPHORUS IN A LONG-TERM MANAGEMENT TRIAL

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Biological transformations of phosphorus (P) in the soil are largely dependent on the functioning of the soil microbial community. The Glenlea Long-term Rotation Study site near Winnipeg, Canada was chosen to determine the effect of 20 years of no input organic (ORG), organic with composted manure (ORG+M), conventional (CONV) and restored prairie (PRA) management on alkaline phosphatase activity (ALP), bacterial phoD community composition, abundance and expression, and P availability. During a study at the site, soil chemical analysis revealed that NaHCO₃-P was significantly lower in the ORG system, compared to CONV and PRA. Higher rates of ALP were reported in the ORG and ORG+M plots with a significant negative correlation to NaHCO₃-P in 2011 ($r^2=0.71; P=0.03$) and 2012 ($r^2=0.51; P=0.02$). The significant correlation between phoD gene abundance and ALP activity suggests that it may be a better indicator of activity than phoD diversity.

A greenhouse experiment using soil from the long-term management site examined the effect of form and rate of P amendment. For each soil, treatments of no P, composted cattle manure (low, medium and high), and mineral P as KH₂PO₄ (15, 40, 80 and 160 mg P kg⁻¹ soil) were applied to pots containing 0.9 kg of each soil and planted to Italian ryegrass. Although ALP activity values at day 0 were similar among the ORG,
CONV and PRA soils, by day 30 sampling the ALP was higher in response to P amendments in the ORG (5.38 to 6.23 µmol PNP g⁻¹ hr⁻¹) compared to the CONV (3.26 to 5.63 µmol PNP g⁻¹ hr⁻¹) and PRA (3.22 and 4.71 µmol PNP g⁻¹ hr⁻¹) soils. As with the field trial, the higher ALP rates corresponded with higher phoD gene, but not transcript, abundance. Ryegrass plants harvested after 106 days showed a larger biomass response when amended with P compared to the control (no P) in the ORG and ORG+M than the CONV and PRA soils. These results suggest that management system may play a more significant role in influencing soil microbial and biochemical processes in soils than does the form and rate of P amendment.
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CHAPTER 1: INTRODUCTION

1.1 GENERAL INTRODUCTION

Widespread apparent phosphorus (P) shortages have been reported in organically managed soils across Canada. Organic agriculture systems aim to optimize productivity while avoiding the use of synthetic chemical products and genetically modified plants. In developed countries, farmers with organic certification receive a premium for their produce while farmers in developing countries may be organic by default by not having access to these technologies. In either scenario, it is important to understand the processes involved to maintain crop production while protecting the environment, and also for potentially reducing fertilizer inputs in conventional agricultural production. Since the use of synthetic orthophosphate fertilizers are not approved in organic production, a detailed understanding of P cycling is essential.

As the area in organic production continues to rise, ensuring adequate soil P fertility to maintain crop productivity is a priority. In 2008, 35 million hectares of agricultural land was under organic management internationally, with 8.2 million in arable or permanent cropland (Willer and Kilcher, 2010). The acreage of certified organic farmland in Canada in 2008 was 611,676 hectares, with another 16,880 in transition (Macey, 2010). The number of certified organic producers nationally has risen from 1174 in 1992 to 3816 in 2005, with Alberta, Saskatchewan, and Manitoba accounting for 1700 of the total (Macey, 2010). The majority of this production was in field crops, with livestock, fruits and vegetables, and wild rice as smaller proportions of
Phosphorus is an essential macronutrient for all living organisms as a component of genetic material (Hammond and White, 2008). Although there is abundant P in soil, more than 80% may be in forms unavailable for plant uptake (Brady and Weil, 2007). Phosphorus is a major limiting macronutrient in agricultural production and orthophosphate fertilizer is routinely applied to soil, often at rates in excess of plant requirements. While this insures yields with increases up to 50% (Stewart et al., 2005), there is the potential for P to be transferred off-site and into waterways, thereby contributing to eutrophication (Sharpley et al., 1992). In addition to the possible environmental consequences of P over-application, there is a finite supply of phosphate reserves that are economical to recover, although there remains a high degree of uncertainty as to the approximate quantity.

The predictions of the depletion of phosphate reserves vary greatly, and some studies caution that the existing rock phosphate reserves (primarily Morocco, China and the US) could be depleted in 50-100 years (Smil, 2000, Gunther, 2005), with global production expected to peak around 2030 (Cordell et al., 2008). It is foreseeable that the high quality reserves will become depleted with resulting increases in the cost of food production and possibly yield decreases. Globally, agricultural production and food security depend on having a sufficient supply of affordable orthophosphate fertilizer which will be essential to produce food for a growing population projected to exceed nine billion by 2050 (United Nations, 2013). Currently there are no suitable alternatives available. Organic material such as manure amendments can supply sufficient P for plant growth, but there is an inadequate and often localized supply.
Studies on certified organic farms on the Northern Great Plains of Canada have often reported low concentrations of easily extractible phosphate (Table 1.1), yet the plant biomass typically does not reflect deficiencies. Current on-farm monitoring of plant available P is done using standard soil tests that measure the easily extractable P from a soil sample. However, these soil phosphorus tests may underestimate the amount of P that will be bioavailable over the growing season since they do not consider the microbially-mediated processes involved in P mineralization of less available P pools.

Previous research has largely focused on soil inorganic P, resulting in limited knowledge of organic forms (phosphate esters, nucleic acids and phospholipids), which can be mineralized by microbes to \( \text{H}_2\text{PO}_4 \) (1.1).

![Diagram of phosphate mineralization equilibrium]

In soil the soluble phosphates may be fixed as Fe, Al, or Ca phosphates or immobilized by microbes to organic P making it unavailable to plants (Brady and Weil, 2007). In highly weathered soils where inorganic forms have very low solubility organic P contributes most to plant growth. Organic P may also contribute a large proportion of the nutritional requirements in high-yielding organic systems where P is continually being exported in plant material with few suitable inputs available.
Table 1.1. Studies examining soil test phosphorus levels on organically managed soils in the Northern Great Plains region. Adapted from Woodley et al. 2014.

<table>
<thead>
<tr>
<th>Location</th>
<th>Description</th>
<th>Years organic at initiation</th>
<th>Sampling Period (years)</th>
<th>Soil test P values (kg P ha(^{-1}))</th>
<th>Depth (cm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manitoba(^1)</td>
<td>9 farms, 42 fields</td>
<td>&gt;5</td>
<td>1</td>
<td>15.6 (4-54)(^4)</td>
<td>0 – 20</td>
<td>Entz et al. 2001</td>
</tr>
<tr>
<td>Saskatchewan(^2)</td>
<td>60 fields</td>
<td>&gt;5</td>
<td>1</td>
<td>18.0 (5.7-30.7), 13.1 (4.0-28.6), 12.2 (4.0-28.3)</td>
<td>0-15, 15-30, 30-45</td>
<td>Knight et al. 2010</td>
</tr>
<tr>
<td>Ontario(^1)</td>
<td>15 fields</td>
<td>&gt;10</td>
<td>1</td>
<td>12 (&lt;10-&gt;20)(^3)</td>
<td>0-15</td>
<td>Roberts et al. 2008</td>
</tr>
<tr>
<td>Saskatchewan(^3)</td>
<td>Field trial</td>
<td>6</td>
<td>6</td>
<td>8.7 (7.8-9.4), 7.2 (5.6-8.1), 2.1 (1.3-2.7), 0.9 (0.7-1.3)</td>
<td>0-15, 15-30, 30-60, 60-90</td>
<td>Malhi et al. 2009</td>
</tr>
<tr>
<td>Manitoba(^1)</td>
<td>Field trial</td>
<td>13</td>
<td>1</td>
<td>21.7(^6), 8.0(^7), 11.3(^8)</td>
<td>0-15</td>
<td>Welsh et al. 2009</td>
</tr>
<tr>
<td>Manitoba(^1)</td>
<td>(Glenlea)</td>
<td>18</td>
<td>1</td>
<td>23.4(^6), 3.6(^7), 4.5, 1.7, 1.5, 1.1</td>
<td>0-15, 15-30, 30-60</td>
<td>Bell et al. 2012</td>
</tr>
<tr>
<td>Manitoba(^1)</td>
<td>(Carmen)</td>
<td>9</td>
<td>1</td>
<td>8.4(^6), 8.8(^7), 2.4, 2.7, 1.3, 1.9</td>
<td>0-15, 15-30, 30-60</td>
<td>Bell et al. 2012</td>
</tr>
</tbody>
</table>

\(^1\) NaHCO\(_3\) extractable P (pH 8.5)
\(^2\) Modified Kelowna extraction
\(^3\) Mehlich extractable P
\(^4\) Values in bold are mean values with the range given in parenthesis when available.
\(^5\) mg kg\(^{-1}\).
\(^6\) Grain-only rotation (spring wheat-pea-spring wheat-flax).
\(^7\) Forage-grain rotation (spring wheat-alfalfa-alfalfa-flax).
\(^8\) Forage-grain with composted beef manure applied in 2002 only.
The biogeochemical cycles of carbon (C), nitrogen (N), sulphur (S) and P in soil are mediated by biological activity, and driven by the breakdown of organic matter for use as an energy source and metabolism, and the availability of nutrients (Gregorich et al., 1996). Extracellular phosphatase (EC 3.1.3) is a non-specific enzyme that catalyses the hydrolysis of phosphomonoester and, contributes to P bioavailability in soil. Bacteria play an important role in production of extracellular ALP, whereby phosphate (Pi) that is released and can be utilized by plants or microorganisms. An analysis of sequenced prokaryotic genomes by Zimmerman et al. (2013) estimated that 32% contained at least one of three genes with the potential to produce ALP.

Figure 1.1: Schematic model of the role of soil organisms in the mineralization and immobilization of soil P. Modified from Richardson and Simpson, 2011.

It has been suggested that soils under organic management may encourage higher enzyme activity, resulting in increased P turnover and mineralization (Oberson and
Frossard, 2005). However, as in the case of the Northern Great Plains of Canada, the continuous export of grain with few imports may not be sustainable over the long-term. Prior to initiation of the following research study, a survey of Canadian organic producers identified soil fertility and crop rotations as a critical research priority with soil quality, ecological interactions, and soil life all in the top six priorities selected (OACC, 2009).

The Glenlea Long Term Crop Rotation and Management site, consisting of the oldest organic-conventional comparison plots in Canada, were sampled to evaluate if long-term management system influences potential ALP production, ALP gene harbouring bacteria, and P bioavailability. A previous study at this location that examined soil P pools revealed that soil test P levels were below the agronomic threshold in the grain-forage rotation (Welsh et al., 2009). The organic systems (alfalfa/grain and annual grain), when compared to conventional, had lower levels of easily extractable water and NaHCO₃-extractable P and moderately available NaOH-extractable P, but no differences in the HCl-extractable pool. In comparison, total N throughout the profile (120 cm) did not reveal any significant differences between the alfalfa/crop or annual crop rotations under organic or conventional management or the re-established grassland after 18 years (Bell et al., 2012). The lower long-term yields in the organic/alfalfa crop may be caused by P deficiencies, which could also affect N fixation during the alfalfa phase and subsequent availability to the wheat crop.

1.2 THESIS FORMAT AND OBJECTIVES

The research chapters in this thesis are presented in paper format. The overall objective of this thesis was to assess the effect of long-term management on the
abundance and expression of bacterial \textit{phoD} genes and ALP activity, and determine differences in soil P bioavailability. This was achieved through three studies presented in Chapters 2, 3, and 4, with the general conclusions in Chapter 5.

Chapter 2 was a two-year field study assessing the relationship between \textit{phoD} gene abundance and ALP activity. Soils from a long-term management trial were used to determine the effect on these bacterial communities and their relationship to soil P bioavailability. To our knowledge, this is the first study to measure the abundance of \textit{phoD} bacteria using primers designed by Sakurai et al. (2008). An overview of the mechanisms by which bacteria respond to low soil available phosphate levels and a detailed description of the pho regulon are given. This chapter has been accepted for a special issue on organic P in Geoderma that is currently in press.

Chapter 3 describes a greenhouse study that was designed using the field soils from chapter 2. In chapter 2 we found \textit{phoD} gene abundance to be significantly correlated with potential ALP activity, with differences in management systems related to differences in labile P concentrations. The objective of the greenhouse trial was to assess the effects of long-term management and differences in the type and rates of application of P amendments on potential ALP activity, and \textit{phoD} gene and transcript abundance. Ryegrass plants were grown in 0.9 kg of soil with no P, mineral P, or manure P treatments for 30 days before sampling for nucleic acids and ALP activity. This chapter has been accepted for publication in Soil Biology and Biochemistry.

Chapter 4 provides the analysis of soil and plant factors from the greenhouse study in Chapter 3. The aim of this chapter was to determine the effects of long-term management system on operationally defined soil P pools and to assess if these soils
respond differently to mineral and manure P treatments. Treatments were destructively sampled 106 days after planting ryegrass and the soils analysed for available nutrients and the plants for above and below ground biomass and plant nutrient (N and P) uptake.

Chapter 5 describes the general conclusions, synthesizing the results from the 3 research chapters. The chapter attempts to link changes in the microbial and biochemical processes from chapter 2 and 3 with the plant available P, biomass yields, and plant P uptake results in chapter 4.
CHAPTER 2
LINKING ALKALINE PHOSPHATASE ACTIVITY WITH BACTERIAL PHOD GENE ABUNDANCE IN SOIL FROM A LONG-TERM MANAGEMENT TRIAL

2.1 ABSTRACT

Changes in land management practices may have significant implications for soil microbial communities important in organic P turnover. Soil bacteria can increase plant P availability by excreting phosphatase enzymes that catalyze the hydrolysis of ester-phosphate bonds. Examining the diversity and abundance of ALP gene harbouring bacteria may provide valuable insight into ALP production in soils. This study examined the effect of 20 y of no input organic (ORG), organic with composted manure (ORG+M), conventional (CONV) and restored prairie (PRA) management on soil P bioavailability, ALP activity (ALP), and abundance and diversity of ALP gene (phoD) harbouring bacteria in soils from the northern Great Plains of Canada. Management system influenced bioavailable P (P< 0.001), but not total P, with the lowest concentrations in the ORG systems and the highest in PRA. Higher rates of ALP were observed in the ORG and ORG+M treatments with a significant negative correlation between bioavailable P and ALP in 2011 (r²=0.71; P=0.03) and 2012 (r²=0.51; P=0.02), suggesting that ALP activity increased under P limiting conditions. The phoD gene abundance was also highest in ORG and ORG+M resulting in a significant positive relationship between bacterial phoD abundance and ALP activity (r²= 0.71; P=0.009). Analysis of phoD bacterial community fingerprints showed a higher number of species in
CONV compared to ORG and ORG+M, contrary to what was expected considering greater ALP activity under ORG management. In 2012, banding profiles of ORG+M showed fewer phoD bacterial species following the second manure application, although ALP activity was higher than in 2011. This indicates that a few species may be producing more ALP and that quantitative gene analysis was a better indicator of activity than the number of species present.

**Keywords:** Bioavailable P; enzyme activity; Pho regulon; soil microbial communities; organic agriculture

2.2 INTRODUCTION

Anthropogenic changes in land management influence soil nutrient cycling and availability by altering the physical, chemical and biological properties of soil (Six et al., 1998, Ross et al., 1999, Post et al., 2000, Guo and Gifford, 2002, Lauber et al., 2008 and Osborne et al., 2011). Phosphorus is a key nutrient to all living organisms as a component of essential macromolecules, including nucleic acids and phospholipids, and a requirement for energy, growth and development (Hammond and White, 2008). Globally, P deficiencies limit plant growth in both managed and natural ecosystems. Although P exists in abundance in the soil, it is often present in forms unavailable to plants, which typically utilize inorganic orthophosphate (H$_2$PO$_4^-$ or H$_3$PO$_4$) in soil solution. Phosphate fertilizer is routinely applied to crops above plant growth requirements and the over application of P creates a serious environmental concern as a major contributor to eutrophication when mobilized and transferred into waterways
Chemical and biological P fertilizers transferred by runoff from agricultural lands have been identified as the main cause of the rapid eutrophication of Lake Winnipeg (Schindler et al., 2012), a freshwater lake near our study site with a drainage basin of nearly 1 million km$^2$ (Wassenaar and Rao, 2012). Improved nutrient management and plant utilization of soil P could decrease input requirements, reducing demand on limited accessible global P reserves while decreasing contamination of waterways.

In contrast to possible over application in conventional agriculture, low plant available (i.e. easily extractable) P has been reported on organic farms across Canada (Entz et al., 2001, Martin et al., 2007, Knight et al., 2010, Roberts et al., 2008 and Main et al., 2013). Manure can be a valuable source of P but regular application in the Great Plains region of North America is restricted by large distances and the tendency for producers to specialize in either livestock or grain production (Russelle et al., 2007). Replacement of P is especially challenging in these systems since limited other alternative P input options are available under organic certification (Woodley et al., 2014). We hypothesize that in these systems the turnover of organic P by microorganisms is essential for meeting plant requirements and maintaining long-term soil productivity, as suggested by Wassenaar and Rao (2012).

Organic P accounts for a large proportion of total P in soil and is an important P source for plants and microorganisms but it must be converted into inorganic P before it can be utilized by plants. Soil microorganisms are key drivers in biogeochemical cycling of P through excretion of extracellular enzymes such as phosphatases, a broad group of enzymes that convert organic P into phosphate (Sharpley, 1985 and Tarafdar and Jungk,
Alkaline (EC 3.1.3.1) and acid (EC 3.1.3.2) phosphatases are phosphomonoesterases with a wide substrate specificity capable of hydrolysing ester-phosphate bonds (i.e. mononucleotides and sugar phosphates) (Nannipieri et al., 2011).

Bacteria have been shown to induce ALP production under conditions of low available inorganic P (Apel et al., 2007 and Wanner, 1996) thereby expending energy for enzyme production only when required. During conditions of phosphate deficiency, activity of the phosphate starvation (Pho) regulon is induced and the transportation of phosphate is then executed by an alternate transport system (Vershinina and Znamenskaya, 2002). Genes encoding phosphomonoesterases are included in the suite of genes responsible for P acquisition during phosphate starvation (Vershinina and Znamenskaya, 2002). In bacteria, three homologous genes within the Pho regulon have been identified in the production of ALP: \textit{phoA} (Bradshaw et al., 1981, Hulett et al., 1990, Hulett et al., 1991, Ray et al., 1991, Chang et al., 1986 and Zappa et al., 2001), \textit{phoD} (Gomez and Ingram, 1995) and \textit{phoX} (Wu et al., 2007). Zimmerman et al. (2013) calculated that 31.9% of 3058 sequenced prokaryotic genomes exhibited the genetic potential to produce ALP by containing at least one of the three homologous genes. The protein sequence of ALP was initially characterised in \textit{E. coli} by Bradshaw et al. (1981). Produced by \textit{phoA}, the phosphatase is a homodimer activated by Mg$^{2+}$ and Zn$^{2+}$ and was originally believed to be the main contributor of ALP in marine ecosystems. More recently it was proposed that \textit{phoX} was more widely distributed among marine bacteria, and induced solely upon P starvation (Sebastian and Ammerman, 2009). Differing from \textit{phoA}, monodimers \textit{phoD} and \textit{phoX} are dependent upon Ca$^{2+}$ as a cofactor (Yamane and Maruo, 1978 and Wu et al., 2007). In soil bacteria \textit{phoD} was the most frequent ALP
gene present in metagenomic datasets for 16S rRNA, although \textit{phoA} and \textit{phoX} were also identified (Tan et al., 2013).

Hydrolysis of organic P by enzymes is an important process to the survival, growth and reproduction of bacteria yet little is known about the diversity and abundance of genes encoding phosphatase enzymes in soil and how they are affected by management practices. Community profiling studies have indicated shifts in bacterial \textit{phoD} communities in response to organic matter (Sakurai et al., 2008) or chemical P fertilization rates (Sakurai et al., 2008 and Tan et al., 2013). Shifts in \textit{phoD} bacterial communities coincided with changes in ALP activity (Sakurai et al., 2008) but no studies have quantified \textit{phoD} gene abundance in soil or examined the link between gene abundance and enzyme activity.

A long-term farming system experiment was established in southern Manitoba, Canada, with an alfalfa-alfalfa-wheat-flax rotation under organic or conventional management, and restored native perennial grassland, and it has been maintained for the past 20 years. Studies at this site have found a depletion of easily extractable P under organic management (Welsh et al., 2009 and Bell et al., 2012), and unique bacterial communities associated with the organic system (Li et al., 2012). However, it is unclear whether differences in the bacterial community composition are driving the turnover of organic P in these systems. The objective of this study was to examine soil P bioavailability, ALP activity (ALP), and abundance and diversity of \textit{phoD} bacteria in this system. Using this approach, we were able to evaluate the capability of the \textit{phoD} gene abundance to be used as an indicator of enzyme function.
2.3 MATERIALS AND METHODS

2.3.1 Site Description

A long-term experiment to compare organic and conventional farming systems was established in 1992 at the University of Manitoba Glenlea Research Station located in the Red River Valley of southern Manitoba, Canada (49°38′25″N, 97° 8′28″W, 238 m elevation). The soil is a Humic Vertisol of the Scantenbury and Hoddinott series with 9% sand, 26% silt and 66% clay, an average pH_{H2O} of 7.4 and 7.7% organic matter content (Bell et al., 2012 and Welsh et al., 2009).

The regional climate is temperate moist continental, with long-term (1992-2012) mean annual maximum temperatures of 8.6°C and minimum -2.8 °C and mean annual precipitation of 537.2 mm (Winnipeg, Environment Canada). The typical growing season from late May through September, had average precipitation of 395.6 mm and mean maximum and minimum temperatures of 20.3°C and 7.7°C, respectively. During the 2011 and 2012 growing season (May to September), total rainfall was 215 and 227.5 mm with an average daily maximum temperature of 23.4°C and 23.9°C and minimum 10.1°C and 9.7°C, respectively. Further details of the site and experimental design are given by Welsh et al. (2009) and Bell et al. (2012).

The experiment is a completely randomized design with three replicates. The organic and conventional systems are fully phased i.e. all rotation phases present each year and a restored grassland plot was included in each replicate (25 m x 25 m). Although the rotations have changed over the years, since 2004 the plots (4 m x 25 m) were in a 4-yr rotation of flax-alfalfa-alfalfa-wheat (Linum usitatissimum, Medicago sativa, Triticum aestivum L.). No fertilizers or pesticides were applied to the organic
plots, which is typical in the northern Great Plains. In 2007, the organic plots were split and composted cattle manure was applied in the fall of 2007 and 2011 (10 t ha\(^{-1}\); N 2.52%, P 0.05%, K 2.45%, S 0.25%). Considering the limited availability of composted cattle manure for large-scale farming in this region, the low rates of manure applied to the plots are typical in this area. For the conventionally managed plots, N was applied on wheat plots following alfalfa based on soil test recommendations at an average of 75 kg N ha\(^{-1}\) and P was applied at 20-25 kg P ha\(^{-1}\) when the seeding of crops (Bell et al., 2012). Hard red spring wheat (cv. Waskada) was seeded at a rate of 112 kg ha\(^{-1}\). Crop residue remained on the soil surface until spring tillage and the alfalfa plots were cut two times per year and the biomass removed.

For comparison, restored prairie plots were also included in the trial. These plots were seeded in 1992 to native grasses *Agropyron dasystachum*, *Andropogon gerardii* Vitman, *Elymus lanceolatus* (Scribn. & J.G. Smith) Gould, *Elymus trachycaulus* (Link) Gould ex Shinners, *Panicum virgatum* (L.), *Pascopyrum smithii* (Rydb.) A. Löve, *Sorghastrum nutans* (L.) (Bell et al., 2012). Prairie plots remain undisturbed aside from burning every 4-5 years, with June 2011 the most recent burn event.

For the current study, we sampled the forage-grain rotation under organic no input (ORG), organic with manure (ORG+M) and conventional (CONV) management and the restored prairie grassland (PRA) during the 2011 and 2012 field seasons. The plots sampled in 2011 and 2012 were both in the wheat phase of the rotation, and were not the same plot both years.
2.3.2 Soil sampling and analysis

Soil samples were collected in July 2011 and 2012 to correspond with the flag-leaf growth stage from the wheat phase of the forage-grain rotation, and the prairie grassland plots. Three soil samples were taken along a transect to a 15 cm depth in a diagonal transect across the plot using a Dutch Auger (2.5 cm radius) and bulked into one composite sample per plot. Field moist composite samples were passed through a 4 mm sieve and a subsample was stored at 4°C for enzyme assays and DNA extraction within 72 hours. The remaining sample was air-dried and ground (< 2 mm) prior to chemical analysis. All results were adjusted to oven-dry weight equivalents.

2.3.3 Soil chemical properties

Bioavailable NaHCO₃-P was determined on soil in a 1:20 ratio with 0.5M NaHCO₃ (pH 8.5) and shaken for 30 min (Olsen et al., 1954) in duplicate. Since charcoal was not added before the extraction, carbonates were removed from the extract by acidification with 3M H₂SO₄ and reactive P determined by measuring blue P-molybdate complex reaction using a UV/vis spectrophotometer 6405 (Jenway, Staffordshire, UK) at 880 nm (Murphy and Riley, 1962). Total P by ignition (TP) was determined in the soil in duplicate by igniting samples at 550°C for 4 h and extracted by shaking for 16 h with 0.5M H₂SO₄ (Saunders and Williams, 1955). Molybdate reactive P in the extracts was determined as above. Total N content of soil was measured using an Elementar Vario Max N/C Analyzer (ELEMENTAR Analysensysteme, Hanau, Germany). To determine soil organic carbon, samples were pre-treated with H₂SO₃ to remove carbonates and analysed on a LECO CNS Analyzer (LECO Corporation, Michigan, USA).
2.3.4 Phosphatase activity assay

Potential soil ALP activity was measured within 72 hr of sampling according to the method by Tabatabai and Bremner (1969). Briefly, 1 g soil was incubated in modified universal buffer solution (pH 11.0) with para-nitrophenyl phosphate substrate (Sigma-Aldrich, USA) at 39°C. After 1-hr, reactions were stopped with 0.5M NaOH, samples filtered through Whatman 42 paper and the formation of \( p \)-nitrophenol determined colorimetrically using a spectrophotometer at 420 nm.

2.3.5 DNA extraction

Total genomic soil DNA was extracted from 0.25 g of soil (dry weight equivalent) according to manufacturer’s protocol using the PowerSoil®DNA Isolation Kit (MoBio, Carlsbad, CA, USA). The DNA was stored at -20°C until analysis.

2.3.6 PCR amplification and denaturing gradient gel electrophoresis (DGGE)

A fragment of the bacterial \( \text{phoD} \) gene was amplified with primers ALPS-F730 (5'-CAGTGGGACGACCACGA GGT-3') and ALPS-R1101 (5'--GAGGCCGATCGGCATGTCG -3') (Sakurai et al. 2008). A GC clamp (5'-CGCCCGCCGCGCCCCCGCCGCGCCGCCGTCCCCGCCGCCGCCGCCGG-3') was attached to the reverse primer to prevent complete denaturation during electrophoresis. The PCR reactions were prepared with 2.5 µL of 10x buffer, 2 mM MgCl\(_2\), 200 µM deoxynucleotide triphosphate, 0.4 µM each forward and reverse primer, 0.25 µL \( Taq \) DNA polymerase (Promega, Madison, WI, USA), 1 µL DNA and brought to a final volume of 25 µL with sterile H\(_2\)O.

The PCR was performed on an Eppendorf Mastercycler EP Gradient S using the
following conditions: initial denaturation at 94°C for 4 min, 35 cycles of elongation at 94°C for 45 s, annealing at 57°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 8 min. The presence and size of the PCR products were verified under UV light on a 1% agarose gel stained with ethidium bromide. PCR products were stored at 4°C.

Amplification products were analysed by denaturing gradient gel electrophoresis (DGGE) to profile the composition of the phoD bacterial communities. Analysis to separate bands by DGGE used a D-code system (BioRad, Hercules, CA, USA) with 8% acrylamide denaturing gradient gel (45-80%) in a Tris-acetate-EDTA buffer at 80V for 15 hr. Gels were stained with SYBR green for 15 min, placed under UV illumination and photographed using GeneSnap (Syngene, Cambridge, UK). Select bands were excised, amplified and confirmed for presence and size on 1% agarose gel before sequencing to validate amplification of the target phoD gene. The phoD community fingerprints were reproducible with a separate amplified sample and denaturing gel. All samples within a year were run on a single gel with a negative control on each gel. Diversity analysis was completed by comparing banding patterns using GeneTools (Syngene, Cambridge, UK).

2.3.7 Quantitative PCR analysis of phoD genes

Real-time PCR was used to quantify the bacterial phoD from a standard curve constructed using plasmid from Pseudomonas aeruginosa PA01. Genomic DNA of PA01 was amplified with primers ALPS-F730 and ALPS-1101, cloned with TOPO® TA Cloning® Kit (with pCR®2.1 Vector) and One Shot® TOP10 Chemically Competent E. coli (Invitrogen, Life Technologies Inc., Burlington, Canada). The plasmid was
sequenced for verification before constructing a standard curve for absolute quantification of *phoD* gene copy. The standard curve was prepared in triplicate using 5 serial 10-fold dilutions, and quantification calculated by determining the starting copy number by considering the concentration of the plasmid and number of base pairs (vector plus primer). Quantitative PCR was conducted on a Roche LightCycler®480 Real-time PCR System in triplicate using 5 µl SYBR green master mix (BIORAD), 0.4 µL each ALPS-F730 and ALPS-R1101 10µmol primers, and 0.4 µL DNA in a 10 µL reaction. Cycling conditions were as follows: 1 cycle at 94°C for 4 min, 40 cycles of 94°C for 45 s, 57°C for 30 s, 72°C for 1 min, and 1 cycle at 72°C for 8 min. Data was collected after annealing at 57°C. Immediately following the run, a melt curve analysis was conducted to check the specificity of the reaction by heat denaturing for 40 cycles at 0.2°C/s from 55 to 95°C. The amplification of the PCR reactions had an efficiency of 1.95, where 2 is the highest quality representing doubling each amplification cycle (Tellmann and Geulan, 2006) and an error value of 0.009 calculated as the mean squared error of the standard curve. No amplification was detected in the negative controls.

**2.3.8 Statistical analysis**

Statistical analyses for response variables were performed using the GLM procedure of JMP 11 (SAS Institute Inc., Cary, NC). The significance of the effects of year, management system and their interactions were tested with two-way analysis of variance. Shapiro-Wilks’ test was used to test for normality and non-normal data was log_{10} transformed before analysis. Significant differences between soils or treatments were determined by Tukey’s test, with same letters within a year representing no
significant difference at $P<0.05$. The DGGE banding patterns were used to create similarity matrices. The ordination of *phoD* bacterial communities to visualize multivariate distances between treatments was plotted using non-metric multidimensional scaling (nMDS) analysis with the PC-ORD v.5.0 software package (MJM software, Gleneden Beach, OR). The Autopilot Slow and Thorough option was selected to measure the Sørensen distance with the first run originating from a random starting point, which recommends a starting configuration for the final ordination to obtain the minimum stress achievable (McCune and Grace, 2002). Satisfactory final stress values of 14.3 and 9.9 were obtained for the 2011 and 2012 data analyses and the ordination displayed in a two dimensional format.

2.4 RESULTS

2.4.1 Soil chemical properties

Management system and year did not have a significant interactive effect on soil properties but the sampling year was significant ($P<0.05$) for all properties except total P and organic C (Table 2.1). This difference was likely a spatial, rather than temporal difference, since different plots were sampled in 2011 and 2012. Management system had a significant effect on P properties and processes but not C and N concentration (g kg$^{-1}$). Olsen P concentrations were lowest in the ORG systems and highest in PRA at the July sampling in both 2011 and 2012 (Table 2.1) with significant differences between both management system ($P<0.001$) and sampling year ($P<0.001$; Table 2.2). The ORG+M plots showed increased Olsen P values of 2.3 mg P kg$^{-1}$ after the one manure application in 2007. Although a fall manure application (2011) did not result in a
significant change, Olsen P increased by 4.4 mg P kg\(^{-1}\) and total P were significantly
\((P<0.05)\) in the ORG+M compared to ORG treatment in 2012. The PRA system had
significantly higher Olsen P values than all cropping systems, except CONV in 2012.

Table 2.1: ANOVA table for soil properties and processes.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Soil properties</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Olsen P (mg kg(^{-1}))</td>
<td>Total P (mg kg(^{-1}))</td>
<td>Organic C (g kg(^{-1}))</td>
<td>Total N (g kg(^{-1}))</td>
<td>ALP phoD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year (Y)</td>
<td>***†</td>
<td>ns</td>
<td>ns</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Management (M)</td>
<td>***</td>
<td>*</td>
<td>ns</td>
<td>ns</td>
<td>***</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Y x M</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>
† *, **, *** significant at 0.05, .01 and 0.001 probability, respectively. ns Not significant

Table 2.2: Soil nutrient concentrations in soil samples (0-15 cm) collected from the
wheat phase of the forage-grain rotation in July 2011 and 2012.

<table>
<thead>
<tr>
<th>Year</th>
<th>Management</th>
<th>Soil chemical properties</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Olsen P (mg kg(^{-1}))</td>
<td>Total P (mg kg(^{-1}))</td>
<td>Organic C (g kg(^{-1}))</td>
<td>Total N (g kg(^{-1}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>ORG†</td>
<td>5.4 ± 0.7c††</td>
<td>633.5 ± 53.8</td>
<td>32.6 ± 4.5</td>
<td>2.3 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ORG+M</td>
<td>7.7 ± 0.3bc</td>
<td>630.1 ± 7.5</td>
<td>34.2 ± 3.0</td>
<td>2.3 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CONV</td>
<td>9.3 ± 1.2b</td>
<td>565.6 ± 24.6</td>
<td>34.3 ± 0.3</td>
<td>2.2 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PRA</td>
<td>22.2 ± 1.8a</td>
<td>655.6 ± 55.7</td>
<td>34.4 ± 4.1</td>
<td>2.1 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>ORG</td>
<td>7.6 ± 1.0c</td>
<td>647.8 ± 31.0b</td>
<td>35.6 ± 3.3</td>
<td>2.6 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ORG+M</td>
<td>12.0 ± 2.6bc</td>
<td>704.6 ± 8.1a</td>
<td>34.5 ± 2.8</td>
<td>3.0 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CONV</td>
<td>19.3 ± 4.4ab</td>
<td>649.6 ± 21.0ab</td>
<td>36.7 ± 0.5</td>
<td>2.7 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PRA</td>
<td>29.6 ± 6.6a</td>
<td>666.8 ± 25.4ab</td>
<td>39.2 ± 1.4</td>
<td>2.7 ± 0.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
† Management systems are organic no input (ORG), organic with manure added (ORG+M), conventional (CONV) and prairie (PRA).
†† Values are treatment means ± standard deviation (n=3) with same letters within a year representing no significant difference at \(P<0.05\) as determined by Tukey’s test.
2.4.2 Phosphatase activity and phoD gene abundance

Phosphatase activity was significantly higher in the ORG and ORG+M treatments compared to CONV and PRA in 2011 (Figure 2.1a). In 2012, ALP of the ORG+M following the manure addition in the previous fall was significantly higher than all other treatments. The PRA soils showed the lowest levels of ALP in both years. The phoD gene copy numbers were high in all samples, ranging from $3.0 \times 10^6$ (PRA) to $8.2 \times 10^6$ (ORG+M) and $6 \times 10^6$ (CONV) to $1.3 \times 10^7$ (ORG+M) per g of dry soil in 2011 and 2012, respectively (Figure 2.1b). The phoD gene abundance showed similar trends to ALP activity although in 2011 there was little difference between the ORG and CONV soil and PRA was significantly lower (Figure 2.1b).

2.4.3 Relationship between soil variables and phoD abundance

Soil samples with low Olsen P had corresponding increases in ALP activity but the relationships between the mean values were not significant (Figure 2.2). However, the individual data points did show a significant negative correlation between Olsen P and ALP activity in both 2011 ($P=0.006$) and 2012 ($P=0.008$). A significant positive relationship was observed between bacterial phoD abundance and ALP activity (Figure 2.3).
Figure 2.1: Effect of management system on a) potential alkaline phosphatase activity (ALP) and b) bacterial phoD gene abundance in soil samples collected from the wheat phase of the forage-grain rotation in July 2011 and 2012. Abundance is expressed per g dry soil. Values are treatment means with bars representing standard deviation (n=3) with same letters within a year representing no significant difference at $P<0.05$ as determined by Tukey’s test. An absence of letters indicates no significant difference.

2.4.4 Community composition and abundance of bacterial phoD

The bacterial phoD gene DGGE profiles revealed a large number of bands for each management system with more phoD species observed in 2011 than 2012 (Figure 2.4). Management system affected the number of phoD species present as indicated by bands in the DGGE profiles, with the highest numbers observed in CONV plots, averaging 32.0 in 2011 and 34.3 in 2012. The average number of bands in the ORG+M (25.3, 23.0) community profiles were similar to the PRA system (25.7, 23.5) and the lowest numbers observed in the ORG plots (21.3 and 20.0) in 2011 and 2012,
respectively. A PCR product from one sample was sequenced to confirm that the amplified gene was the target $phoD$ ALP producing bacterial gene ($Pseudomonas$ sp. UW4, GenBank CP003880.1, 94% identity). The different banding patterns both within and among the management systems suggest differences in bacterial $phoD$ community composition from that sampling location.

![Figure 2.2](image1.png)

Figure 2.2: Relationship between Olsen P and potential alkaline phosphatase activity in 2011 and 2012. Values are treatment means (n=3).

![Figure 2.3](image2.png)

Figure 2.3: Relationship between soil bacterial $phoD$ gene abundance and potential alkaline phosphatase activity (ALP) in 2011 and 2012. Values are treatment means (n=3).
Figure 2.4: DGGE analysis of *phoD* bacterial population in a) 2011 and b) 2012. Each lane represents a sample from organic (O), manure-amended organic (M), conventional (C) and prairie (P) management systems with each line representing a different species of bacteria. The number below each lane represents the number of bands identified in the sample.

Communities of *phoD* bacteria were affected by management systems. Ordination by nMDS based on DGGE profiles revealed similar variations in both years (Figure 2.5). The ORG plots had the greatest deviation between replicates and were clearly separated from the other treatments. The ORG, CONV and PRA profiles all occupied separate quadrats while the ORG+M was oriented closest to the PRA (Figure 2.5a). In 2012, the ordination by nMDS reveals separation of *phoD* communities by management (Figure 2.5b). There was a clear shift of the ORG+M towards the CONV resulting from the prior fall manure application, in comparison to the plots in 2011 with the manure treatment applied four years prior.
2.5 DISCUSSION

Soil chemical properties in our study indicated that management system had a significant effect on Olsen P in both years, while none was observed for C and N. Many studies have demonstrated the conversion from native grasslands to annual cropping systems depletes bioavailable P (Hedley et al., 1982, Tiessen et al., 1984, Sharpley and Smith, 1985 and Motavalli and Miles, 2002), as also indicated in our study with lower concentrations in the agricultural systems. The soil nutrient values we report are comparable to previous studies at this location showing lower Olsen P values in the ORG no input system (Bell et al., 2012 and Welsh et al., 2009). Using a modified Hedley fractionation, Welsh et al. (2009) demonstrated depletion of easily extractable P fractions in the ORG system, compared to CONV cropping or PRA, but little difference in the more recalcitrant P fractions. Considering the high requirements of P by alfalfa...
production, it is somewhat surprising that there was little difference in Olsen P values from the ORG plots sampled in 2004 (8.0 mg kg\(^{-1}\); Welsh et al., 2009) and our results from 2012 (7.6 mg kg\(^{-1}\)), when no inputs were applied. Welsh et al. (2009) estimated a cumulative P budget (input-output) for the first 13 years as a -118 kg P ha\(^{-1}\) deficit in the ORG systems, averaging approximately 9 kg P ha\(^{-1}\) removed per year. The alfalfa biomass is cut and removed for hay twice per growing season with little organic matter returned to the soil for those two years of the rotation. Available P values in the PRA plots, as also reported by Welsh et al. (2009), were very high. Since there were no inputs or exports from the restored PRA system, high levels of available and potentially available P were likely present at the beginning of the trial.

There was a significant effect of sampling year with lower levels of Olsen P and soil N for all systems during the first sampling year, 2011. The 2011 growing season began with a cool wet spring followed an extensive dry period preceding sampling, totalling only 10mm of precipitation in July. During the growing season from May to August there were only 147 mm of precipitation in 2011 compared to 224 mm in 2012, with little inter-annual difference in mean monthly temperatures. Crop productivity was affected with lower than average wheat yields, with the ORG and CONV systems yielding 52% and 20% lower in 2011 than 2012 (Martin Entz, personal communication). This presumably resulted in less P being removed in biomass and leaving more available nutrients for the subsequent year. In addition, the drying and rewetting of soil has been found to create large increases in water-extractable P (Turner and Haygarth, 2001, Styles and Coxon, 2006 and Bünemann et al., 2013). The majority of this may be released from the microbial biomass but Bünemann et al. (2013) also reported non-microbial
contribution to labile P (but not C) in sterilized soils. The climatic variations between 2011 and 2012 may also have resulted in differences in enzyme activity and the $phoD$ bacterial communities between years.

Olsen P levels were negatively correlated with ALP activities at Glenlea in both years, implying that phosphatase production was induced at low phosphate levels (Zhang et al., 2012). During the drought conditions in 2011, ALP in the ORG and ORG+M plots were significantly higher than the CONV and PRA soil. In comparison, elevated rates of ALP activity were observed in all soils in 2012, ranging from a 24% increase in ORG to 114 % increase in CONV soil. These increases in potential ALP activity corresponded with higher concentrations of bioavailable P and a significant increase in ALP gene abundance. Sardans et al. (2008) also found that drought decreased phosphatase activity in a manipulation forest study over 6 years. Seasonal variation in microbial populations and activities has been well documented, along with the effects of temperature and moisture (Bardgett et al., 1999, Smit et al., 2001, Papatheodorou et al., 2004 and Hamel et al., 2006), although some of the differences in our study may be attributed to spatial variability.

We observed increases in ALP activity in the ORG+M treatment in the growing season following a fall manure treatment in 2011, with the $phoD$ gene abundance also responding positively. Parham et al. (2003) reported higher phosphatase activities in soils with composted cattle manure applied every fourth year for a century compared to chemical P fertilizer, as well as promoting microbial activities including microbial biomass. Birkhofer et al. (2008) also found improved soil quality, microbial biomass, and natural pest control in plots of long-term organic farming with barnyard manure
applied although wheat yields were 23% lower than those receiving mineral fertilizers and herbicides. Increases in temperature and moisture will increase microbial activity but is ultimately dependent on substrate availability. In addition to providing substrate for enzyme hydrolysis, organic matter in the manure may increase binding sites allowing accumulation of enzymes in the soil (Burns, 1982).

Since P cycling is influenced by a combination of chemical and biological processes, linking phosphatase activity directly to P pools and changes in P bioavailability is challenging. In our study, even after 21 years of exporting biomass from the ORG system the Olsen P values were still 7.6 mg kg\(^{-1}\) in 2012, compared to 8.0 mg kg\(^{-1}\) in 2004 (Welsh et al., 2009). It is likely that the higher ALP activity in these plots, compared to CONV and PRA, are contributing to P cycling in this system, indicating that organic P plays a major role in P availability in these systems. Given the challenges in linking potential ALP activity to organic P cycling, and plant nutrition (Speir and Cowling, 1991), it is especially difficult to quantify the contribution of ALP gene abundance and diversity to the cycling of P.

The relationship between ALP activity and nutrient concentrations in the soil is still not well understood. Previous studies have compared the effect of management system on phosphatase activity but correlations with P, C and N have been inconsistent across experiments. Positive correlations between phosphatase activity and SOC were reported by a number of authors (Speir, 1977, Frankenberger and Dick, 1983, Dick et al., 1988 and Saha et al., 2008) but we did not observe a relationship with SOC in 2011\((P=0.27)\) or 2012 \((P=0.30)\), supporting the results of Zhang et al. (2012). A correlation with total N was observed in 2012 only, while Dick et al. (1988) found ALP
was highly correlated with total N.

The restored PRA system, which consisted of native grasses, differed considerably from the agricultural systems. The plots were burned every 4-5 years but were not subjected to tilling, cutting, or addition of fertilizers or pesticides throughout the prior 20-year duration of the experiment. Considering that higher phosphatase activity has often been observed in uncultivated compared to cultivated soils (Zhang et al., 2012), it can be speculated that the low ALP activity in 2011 may have been negatively affected by the spring burn. Ajwa et al. (1999) reported a decrease in ALP when a tall grass prairie was exposed to yearly burns at four sampling dates compared to no burning. Interestingly, the addition of N fertilizer to the burned treatments resulted in a further reduction in ALP activity in the spring and early summer (Ajwa et al., 1999). The effects of burning on the soil microbial populations are likely confined to the top 2.5 cm and Dick et al. (1988) found only a weak correlation, however the tillage used in that study may have diluted the effects.

The microbial community responded to low P conditions under ORG management by increasing ALP, for which phoD gene abundance is at least partially responsible. The diversity of bacterial phoD genes presumably affects the function. The phoD community profiling by DGGE demonstrated different banding patterns in the management systems evaluated. Soil amendments, particularly repeated organic amendments, have been shown to increase diversity of the bacterial community (Sun et al., 2004 and Parham et al., 2003) often demonstrating a greater effect on the total microbial community than management system (Esperschütz et al., 2007 and Lynch, 2014). We had hypothesized that the number of phoD species would be lower in soils
under CONV management but observed the opposite effect. Although the values were not significant, total organic C concentrations were higher in the CONV and PRA systems, corresponding with lower ALP activity and \textit{phoD} gene abundance, and more bands present in the community fingerprints representing \textit{phoD} species. One explanation is the possibility that soil C is more accessible to microbes under CONV, since the higher productivity in the CONV system returns more C to the soil, compared to ORG management (Birkhofer et al., 2008). Tan et al. (2013) reported higher richness and Shannon-Wiener diversity indices for 16S rRNA and \textit{phoD} communities in pastures receiving high rates of P fertilization after 42 years, compared to a no-P soil. We observed a lower number of \textit{phoD} bands in the 2012 plots following the fall manure application, although an increase in microbial activity and diversity has been previously demonstrated with manure addition (Parham et al., 2003, Esperschütz et al., 2007, Sakurai et al., 2008 and Chaudhry et al., 2012).

Shifts in \textit{phoD} bacterial communities have been significantly correlated with ALP and fertilization management (Sakurai et al., 2008 and Chhabra et al., 2013). Our results from the community fingerprinting of \textit{phoD} bacterial communities over the two years show a separation of ORG, which also had the highest variability, based on nMDS ordination. Interestingly, the ORG+M was closer to CONV in the plots samples in 2012 following the fall manure application. Differences in bacterial diversity important in other aspects of P-solubilisation have also been reported. Mander et al. (2012) demonstrated that bioavailable soil P levels significantly affected the frequency that this P-solubilisation was represented in the soil bacterial community by identifying cultured bacteria based on sequence analysis of 16S rRNA genes. The taxonomy and abundance
of bacteria under low or high P status differed between the three long-term pasture
management sites, with eleven families common to all.

Although studies examining the effects of farm management systems on
functional gene abundance and diversity important in P cycling are rare, improved access

to sequencing technology has resulted in numerous studies examining 16S rRNA in soil.
A recent study using pyrosequencing to investigate the influence of ORG and CONV
systems on bacterial communities at our field site reported that, in general, 
Proteobacteria were more common in the ORG farming system compared to CONV (Li
et al., 2012). Similarly, after 16 years of contrasting management, Proteobacteria,
Bacteroidetes, and Gemmatimonadetes were significantly more abundant in organically
amended soils, while Actinobacteria dominated under conventional management and
Acidobacteria under fallow grassland (Chaudhry et al., 2012). Although these studies
have demonstrated that long-term management resulted in a shift in microbial
communities, they do not provide information on specific species present or the
functioning of these bacteria.

When comparing differentially fertilized pasture soils using pyrosequencing of
16S rDNA, Tan et al. (2013) reported that the phoD community was restricted to a few
phyla, particularly those related to Alphaproteobacteria. This may be more related to
primer biases towards Alphaproteobacteria than the actual distribution of phoD in the
soil since the primers were designed using isolates of Caulobacter crescentus CB15,
Corynebacterium glutamicum, Mesorhizobium loti, Nostoc sp., Pseudomonas aeruginosa
PAO1, and Sinorhizobium meliloti 1021 (Sakurai et al., 2008). However, the significant
positive correlation we observed between phoD gene abundance and ALP activity over
the two years signifies that these species may be important in the production of extracellular alkaline phosphatases in these soils. Even though the use of DGGE in our study revealed differences in \textit{phoD} community composition, this may be disclosing more dominant populations and further analysis would be required to comment on the diversity, evenness and richness of the community.

Sampling protocol is an important consideration in interpreting differences in bacterial diversity and function since temporal shifts in microbial communities can be significant (Németh et al., 2014). However, when evaluating the effect of long-term management systems it is not always necessary to sample at multiple times over the growing season (Bissett et al., 2013). By sampling at the flag-leaf stage of wheat growth over two years we saw similar trends in both ALP activity and \textit{phoD} gene abundance. In a nutrient study of spring wheat in three locations of the northern Great Plains, maximum accumulation of P occurred by the beginning of heading (Miller et al., 1994 and Malhi et al., 2006) and our sampling time was chosen to correspond with peak P uptake in wheat plants.

2.6 CONCLUSIONS

In conclusion, this study demonstrates that long-term management may impact P cycling by differences in the abundance and diversity of \textit{phoD} bacterial communities. Even low rates of compost manure had a significant effect on potential ALP activity and \textit{phoD} gene abundance. One of the main limitations to understanding the response of bacteria to P starvation is the diverse set of genes responsible for P utilization as well as the phylogenetic diversity that provides challenges for designing universal primer sets.
Quantification of the *phoD* gene resulted in a significant positive correlation with potential ALP activity in the soil, regardless of management system. It is important to note that although this correlation was observed, a few *phoD* bacteria containing highly inducible ALP genes may be responsible for the increased ALP activity, rather than gene abundance alone. Thus, both the *phoD* community composition and gene abundance must be considered. In a subsequent study we use a high throughput sequencing approach to characterize the composition of the *phoD* community and quantify changes in *phoD* gene transcripts in response to P amendment, in order to understand the community involved in ALP activity.

Organic management systems are often commended for improved soil quality and increased microbial diversity and function, which may at least partially be attributed to longer and more diverse crop rotations. However, in this study the crop rotations were identical and differed only in the rate and type of inputs applied. The lack of inputs available under organic certification for large-scale production in the northern Great Plains challenges the claim of sustainability of these systems, considering the low bioavailable P levels. The increased *phoD* gene abundance and potential ALP activity contribute to replenishing the easily extractable pool but does not address the long-term issue of soil P replenishment, nor the effect on crop productivity. Although the PRA system consisted of grasses native to the area and the only management was prescribed burns every 4-5 years, it was most similar to the CONV systems for the microbial parameters measured indicating that the *phoD* community responded to the level of bioavailable P regardless of whether it was applied as orthophosphate.
3.1 ABSTRACT

Bacterial transformation of phosphorus (P) compounds in soil is largely dependent on soil microbial community function, and is therefore sensitive to anthropogenic disturbances such as fertilization or cropping systems. However, the effect of soil management on the transcription of bacterial genes that encode phosphatases, such as \textit{phoD}, is largely unknown. This greenhouse study examined the effect of long-term management and P amendment on potential alkaline phosphatase (ALP) activity and \textit{phoD} gene (DNA) and transcript (RNA) abundance. Soil samples (0-15 cm) were collected from the Glenlea Long-term Rotation near Winnipeg, Manitoba, to compare organic, conventional and prairie management systems. In the greenhouse, pots of soil from each management system were amended with P as either soluble mineral fertilizer or cattle manure and then planted with Italian ryegrass (\textit{Lolium multiforum}). Soils from each pot were sampled for analysis immediately and after 30 and 106 days. Significant differences among the soil/P treatments were detected for inorganic P, but not the organic P in NaHCO\textsubscript{3}-extracts. At day 0, ALP activity was similar among the soil/P treatments, but was higher after 30 days for all P amendments in soil from organically managed plots. In contrast, ALP activity in soils under conventional and prairie management responded to increasing rates of manure only, with significant effects from medium and high manure application rates at 30 and 106 days. Differences in ALP
activity at 30 days corresponded to the abundance of bacterial phoD genes, which were also significantly higher in soils under organic management. However, this correlation was not significant for transcript abundance. Next-generation sequencing allowed the identification of 199 unique phoD operational taxonomic units (OTUs) from the metagenome (soil DNA) and 35 unique OTUs from the metatranscriptome (soil RNA), indicating that a subset of phoD genes was being transcribed in all soils.

3.2 INTRODUCTION

The biochemical conversion of organic phosphorus (P) compounds to orthophosphate by enzymes is an important step in the global P cycle. Although abundant P exists in the soil in various forms as organic and inorganic P, it is utilized by microorganisms and plants as orthophosphate ($H_2PO_4^{2-}$ or $H_2PO_4^-$) in soil solution. Since soil orthophosphate is limiting for plant productivity in many terrestrial ecosystems, the production of extracellular enzymes facilitating mineralization of organic P compounds (phosphatases) may play an important role in plant nutrition (Speir and Cowling, 1991; Tarafdar and Claassen, 1998).

Various phosphatases exist in soil and contribute to the mineralization of P compounds. Phosphatases are a broad group of enzymes capable of releasing orthophosphate from organic P forms. The phosphomonoesterases (EC 3.1.3) include acid and alkaline phosphatases, phytases and nucleotidases (Nannipieri et al., 2011), and mineralize orthophosphate monoesters such as sugar phosphates, phytate and nucleotides. Extracellular alkaline phosphatase (EC 3.1.3.1 - ALP) is a non-specific enzyme that catalyses the hydrolysis of ester-phosphate bonds of many orthophosphate monoesters.
(Nannipieri et al., 2011), excluding phytate, thereby contributing to P bioavailability in soil.

Bacteria play an important role in the biochemical cycling of P by excreting phosphatases (Tabatabai, 1994) to obtain orthophosphate essential for functioning and growth. Some bacteria contain phosphate starvation-inducible (psi) genes as part of the phosphate starvation-(pho) regulon, which are responsible for the synthesis of phosphomonoesterases and phosphodiesterase (Vershinina and Znamenskaya, 2002). The production of ALP by bacteria has been demonstrated to be induced by P starvation (Apel et al., 2007; Vershinina and Znamenskaya, 2002; Wanner, 1996). As part of the Pho regulon, the phoD gene is one of three homologous genes (phoA, phoD, phoX) that have been identified in coding for ALP production in bacteria (Gomez and Ingram, 1995). An analysis of sequenced prokaryotic genomes by Zimmerman et al. (2013) estimated that 32% contained at least one of phoA, phoD or phoX. A previous study on bacterial communities identified the phoD gene to be a key ALP gene in soils, although phoA and phoX were also present (Tan et al., 2013). Shifts in phoD bacterial communities have been reported in response to manure and mineral P fertilization (Sakurai et al., 2008; Tan et al., 2013). In addition, long-term management can influence both the phoD community and gene abundance (Fraser et al., 2014) although no information on the active phoD community has been reported in soils.

In many regions, the productivity of high yielding farming systems may be limited by P availability. Over time, the continuous export of P in grain and animal products depletes more labile P forms (Welsh et al., 2009), thus creating a dependency on P inputs to maintain yields. Conventional farming systems routinely apply
orthophosphate fertilizer, which may impact the abundance, diversity and functioning of the microbial community (Fanin et al., 2014; Fraser et al., 2014). High rates of mineral P may inhibit transcription by the Pho regulon (Oshima et al., 1996), thus suppressing ALP activity by bacteria (Spiers and McGill, 1979; Saha et al., 2008; Zhang et al., 2012). In contrast, organic farming depends largely on animal manures when available as a source of nutrients, especially P. The addition of animal manure to soil may result in increased enzyme activity by inclusion of humic-enzyme complexes with manure (Burns et al., 1982) and/or increased activity of the entire microbial community (Watts et al., 2010; Lynch, 2014). Although we did not characterize the P compounds in the manure used in this study, Gagnon et al. (2012) reported that 59% of the total P in composted cattle manure was accounted for in the resin P and NaHCO$_3$-P extracts of a Hedley fractionation. Turner (2004) characterized P forms in cattle manure using $^{31}$P NMR and reported that 64% of the NaOH-EDTA extractable P was present as orthophosphate, and of that fraction, approximately 14.6% was in the form of orthophosphate monoesters. Therefore, it is likely that only a small fraction of this may be present as phosphate esters that can be hydrolysed by ALP.

To evaluate the effect of management system on the response of bacterial $phoD$ gene and transcript abundance to the rate and type (manure P, mineral P) of treatment, soils were collected from a long-term farming system experiment for a greenhouse study. Located in the great Northern Plains region of Canada, the experimental site was established in 1992 and included plots of alfalfa-crop rotations and restored native prairie. In a previous field study at this site, we observed that management system had a significant effect on ALP activity, and also produced differences in $phoD$ gene
abundance and community composition in soils sampled at the flag-leaf stage of wheat growth (Fraser et al., 2014). Plots managed as certified organic with a one-time manure application (12 mg P kg$^{-1}$ soil) showed differences in measured microbial properties, with significantly higher ALP activity and $phoD$ gene abundance compared to the restored prairie plots. The organic field sample with a manure application demonstrated a shift in the $phoD$ bacterial community, but not gene abundance, to be more similar to the conventional managed system than the organic no input system (Fraser et al., 2014). It is unclear whether the $phoD$ bacterial community responded to the P in the manure or if the organic matter addition increased overall activity of the total microbial community.

The objective of the current study was to evaluate the impact of various rates of manure P or mineral P on bacterial alkaline phosphatase ($phoD$) gene and transcript abundance and potential ALP activity. Amendments were applied to soils from a long-term management trial under organic, conventional, and prairie management to determine if there are inherent differences in the microbial response to P amendment rate and type. We also evaluated the use of $phoD$ transcript abundance, compared to DNA, as an indicator of ALP activity in soil, and used next-generation Illumina sequencing to characterize the genetic similarity of the $phoD$ genes and transcripts associated with the organic, conventional and prairie field soils.

3.3 MATERIALS AND METHODS

3.3.1 Experimental design and soil collection

The soils used in this study were collected from the grain-forage rotation of the Glenlea Long-Term Crop Rotation and Management Station (49°38'25”N, 97° 8’28”W, 39
238 m elevation) near Winnipeg, Manitoba, Canada. The site is Canada’s longest running organic and conventional comparison trial going into its 20th growing season at the time of sampling.

Briefly, the soil at the site is a heavy clay Humic Vertisol comprised of 66% clay, 26% silt and 9% sand in the topsoil (Welsh et al., 2009). The organic and conventional cropping systems consisted of a four-year rotation of flax (*Linum usitatissimum*), alfalfa (*Medicago sativa*) twice, and wheat (*Triticum aestivum* L. cv. Waskada). The organic plots received no inputs for the duration of the trial while the conventional received N based on fall soil testing and P applied at a yearly at a rate of approximately 20-25 kg P ha⁻¹. Flax and wheat residues were left on the plots and the alfalfa biomass was cut and removed twice per growing season. The restored prairie plots include a mix of native perennial grasses (see Bell et al. (2012) for the list of plant species) and received no management since inception, with the exception of a prescribed burn every four to six years with the most recent relative to sample collection for this study occurring in 2005. For a detailed description of the experiment see Welsh et al. (2009) and Bell et al. (2012).

Prior to seeding wheat in 2011, approximately 12.0 kg of soil (0-15 cm depth) was collected from across each plot (n=3), air-dried and mechanically ground to break large clumps (< 4 mm). A greenhouse experiment was initiated comparing soil from the organic, conventional and prairie treatments. The three field replicates of each soil (11.5 kg dry soil) were combined and pre-incubated for fourteen days at 40% gravimetric moisture (24°C day, 18°C night) to restore microbial activity. Containers were loosely covered with black plastic, the soil mixed daily and germinated weeds removed. Following pre-incubation, 500 g of soil were collected from each pooled soil for initial
analysis (ALP, *phoD* gene abundance). The NaHCO₃-extractable P was analysed on air-dried soils before the 14-day incubation.

For soils from each of the management systems (organic, conventional, prairie), eight P treatments were applied to pots containing 0.9 kg of soil with four replicates arranged in a randomized complete block design. To calculate nutrient requirements for the manure treatments, three subsamples of composted cattle manure were analysed at the Nova Scotia Agriculture Lab with the results in g kg⁻¹ as follows: total P of 0.99, total N 3.82, NH₄⁺-N 0.22, K 1.34, Mg 2.02, Ca 9.60, Na 0.12 and a pH of 7.1. Macronutrients were solubilised in H₂O and added to all treatments for a final level of: 150 mg N kg⁻¹, 200 mg K kg⁻¹, 35 mg Ca kg⁻¹ and 15 mg Mg kg⁻¹. Microelements were added at a rate of (mg kg⁻¹): 2 Cu, 1 Mn, 1 B, and 0.1 Mo (Oberson et al., 2010).

Phosphorus was added to 0.9 kg of soil in pots as: control (no P), manure (low, medium, high) and KH₂PO₄ (15, 40, 80, 160 mg P kg⁻¹ soil). The manure treatments were applied based on total P of the manure at 18, 120, 240 mg P kg⁻¹ soil for the low, medium and high treatments, respectively. Based on the range of apparent P use efficiencies in the literature (Eghball and Power, 1999; Oberson et al., 2010), we assumed that 30% of the total P in the composted manure would be taken up by the plant (6, 40 and 80 mg P kg⁻¹ soil).

Fifteen Italian ryegrass (*Lolium multiflorum*) seeds were planted and thinned to 10 evenly spaced plants after emergence. Soil moisture was maintained at 60% field capacity and the greenhouse conditions set to 25°C day and 18° night with a 16 hour photoperiod.
At 30 days, approximately 25 g (fresh weight) soil was sampled at three locations in the pot to make one composite sample. Approximately 2.5 g of the soil was immediately placed in a 15 ml centrifuge tube containing 5 ml Lifeguard™ Soil Preservation Solution (MoBio, Carlsbad, CA, USA) and stored at -20°C until nucleic acid extraction. Fresh soil was stored at 4°C for potential ALP activity assays within 48 h and the remainder dried, ground and analysed for NaHCO₃-extractable P. After 106 days the pots were destructively sampled and the soil analysed for ALP.

3.3.2 Labile P

Labile P was determined by extracting soil with 0.5M NaHCO₃ (pH 8.5) in a 1:20 soil to solution ratio in duplicate for 30 min (Olsen et al., 1954) and then filtering extracts through Whatman no. 42 filter paper. The samples were acidified with 3M H₂SO₄ to remove carbonates and molybdate reactive P (NaHCO₃-extractable orthophosphate) determined with a UV/vis spectrophotometer 6405 (Jenway, Staffordshire, UK) at 880 nm (Murphy and Riley, 1962). Total P was determined in an aliquot of the extract by autoclave digestion with ammonium persulfate and 0.9M H₂SO₄ (EPA, 1971). Molybdate unreactive P, which we assumed represented predominantly NaHCO₃-extractable organic P compounds, was calculated as the difference between total P and inorganic P of the extract.

3.3.3 Phosphatase activity assay

Soil samples at 0, 30 and 106 days were analysed for potential ALP activity within 48 hr of sampling following the method of Tabatabai and Bremnar (1969). Assays
were conducted by incubating 1 g of soil for 1 hr at 37°C at pH 11.0 using para-nitrophenol phosphate (Sigma-Aldrich, USA) as a substrate. Samples were filtered with Whatman 42 filter paper, diluted within the range of the standard curve, and the color intensity of the p-nitrophenol measured using a spectrophotometer at 420 nm.

3.3.4 Nucleic acid extractions and reverse transcription

Soil samples in Lifeguard™ Soil Preservation Solution (MoBio, Carlsbad, CA, USA) were centrifuged for 5 min at 2500 g and the solution poured off. The RNA and DNA were co-extracted according to manufacturer’s instructions using RNA PowerSoil® Total RNA Isolation Kit, followed by the RNA PowerSoil® DNA Elution Accessory Kit (MOBIO, Carlsbad, CA) to acquire metagenomic and metatranscriptomic DNA and RNA. Extracted DNA was stored at -20°C until analysis. The RNA extracts were immediately subjected to DNase treatment to remove any residual DNA by adding 1 µl RNase-Free DNase and 5 µl RNase-Free Buffer RDD (Qiagen, Venlo, Netherlands) to 43 µl of RNA. After 10 min the DNase was inactivated by adding 1µl 50 mM EDTA and heating to 70°C for 10 min. RNA was converted to cDNA according to manufacturer’s instructions using the High Capacity cDNA Reverse Transcription Kit (Invitrogen™, Carlsbad, CA, USA) with random hexamer primers and stored at -20°C. The absence of DNA contamination was confirmed by the lack of a PCR product when visualized on an agarose gel and from a sample in the RT-qPCR with no reverse transcription enzyme.

3.3.5 Quantification of gene and transcript abundance

For absolute quantification of phoD, a plasmid standard was created by cloning
amplified genomic DNA of *Pseudomonas aeruginosa* PA01 (ALPS-F730 and ALPS-1101) with a TOPO® TA Cloning® Kit (with pCR®2.1 Vector) and One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen™, Carlsbad, CA, USA) and sequenced for verification. The standard curve was prepared using five serial ten-fold dilutions, and the number of gene copies calculated by measuring the concentration of the plasmid and number of base pairs (vector plus primer).

Bacterial *phoD* genes and transcripts were amplified with primers ALPS-F730 (5’-CAGTGGGACGACCACGAGGT-3’) and ALPS-R1101 (5’-GAGGCCGATCGGCG ATGTCG -3’) (Sakurai et al., 2008) on a LightCycler®480 Real-time PCR System (Roche, Basel, Switzerland) as described in Fraser et al. (2014). In brief, plates were set up using an epMotion 5075 VAC Automated Pipetting System (Eppendorf, Hamburg, Germany). The PCR reactions were prepared with 5 µl of 2x i-Taq Universal SYBR® Green Supermix (BIORAD, Hercules, CA, USA), 0.4 µl (10 µmol) of each ALPS-F730 and ALPS-R1101 primers, and 0.4 µl template in a 10 µl reaction. Cycling conditions were as follows: 94° for 4 min, 40 cycles of 94°C for 45 sec, 57°C for 30 sec, 72°C for 1 min, and 72°C for 8 min. Data was collected after annealing at 57°C. Immediately following the run, a melt curve analysis was conducted to check the specificity of the reaction by heat denaturing for 40 cycles at 0.2°C/sec from 55 to 95°C.

The DNA samples were diluted 1:20 before analysis and each sample was run in two separate wells on a 384 well plate with the standard curve and negative controls included in triplicate. For quantification of transcript abundances, low cDNA template levels resulted in a primer dimer that could not be eliminated by optimisation, interfering
with direct quantification. A set of cDNA samples were run in triplicate to confirm the presence of transcripts by gel electrophoresis and visual analysis of the melt curves. To increase the template to a quantifiable concentration, a second set of cDNA was spiked with a known amount of DNA that was subtracted from the final concentration to calculate \( \text{phoD} \) transcript abundance. The amplification of the PCR reactions had an efficiency of 1.96, where 2 is the highest quality representing a doubling at each amplification cycle (Tellmann and Geulan, 2006) and an error value of 0.04 calculated as the mean squared error of the standard curve. No amplification was detected in the negative controls.

3.3.6 Next generation sequencing (NGS)

To confirm that the quantified gene and transcript abundance was \( \text{phoD} \), sequences were generated for the control (no P) soil samples from the organic, conventional and prairie plots. Amplicon libraries were prepared in three serial PCR stages in a total of 60 PCR cycles, 35 cycles to generate amplicons in the first stage, 10 cycles to add adapter tags in the second stage, and 15 cycles to add index tags in the third stage. Community composition is not expected to be altered by the last 25 cycles. In Stage 1, PCR was conducted in a 40 µl reaction split into two wells. Each reaction contained 1X Platinum® Taq buffer, 2 mM MgCl\(_2\), 0.08 µL of Platinum® Taq DNA Polymerase (Invitrogen, Burlington ON, Canada), and 2 mM dNTPs, 0.2 µM (10 pmol µL\(^{-1}\)) of each ALPS-F730 and ALPS-R1101 primers, 0.08 µl T4g32 reagent (New England Biolabs, Whitby, ON, Canada), and 0.8 µl of soil DNA or cDNA. The PCR conditions for Stage 1 were as follows: initial denaturation 94°C for 1 min, 35 cycles
incorporating 94°C for 10 sec, 57°C for 30 sec, 72°C for 40 sec, 72°C for 5 min. The duplicate PCR reactions were pooled, then purified using spin columns (UltraClean GelSpin DNA Extraction Kit, MoBio Laboratories Inc., Carlsbad CA, USA) and eluted in 30 µl of 1/10 strength elution buffer. Reactions were stored up to three days at 4°C until use in Stage 2.

In Stage 2, PCR was conducted to add proprietary Illumina adapter sequences (Illumina Inc., San Diego, CA). Each reaction (25 µl) was the same as Stage 1 PCR except 0.3 µl of Platinum® Taq (Invitrogen, Burlington, ON, Canada), 0.2 µM of each primer with Illumina adapter attached to 5’ ends, and 4 µl of purified Stage 1 amplicon as template, and only 10 cycles at 57°C annealing temperature were used. The PCR reactions were purified using spin columns as described above. In Stage 3, Illumina index tags were added to the ends of the amplicons for each sample. A different combination of tags was used for each sample. Primers (10 µM) consisting of Illumina’s proprietary index tags and adapters were purchased from the University of Guelph Advanced Analysis Centre- Genomics (Guelph, ON, Canada). Single 25 µl PCR reactions were used containing the same reagents as in Stage 2 save that 0.2 µl of Platinum® Taq (Invitrogen, Burlington, ON, Canada) and 1 µl of purified Stage 2 amplicon were added as the DNA template. The PCR cycling conditions were as for Stage 2 except 15 total cycles were used.

The PCR reactions were purified using spin columns and eluted in 50 µl of 1/10 strength elution buffer and frozen at -20°C until submission to the University of Guelph Advanced Analysis Centre - Genomics (Guelph, ON, Canada) for sequencing. Multiplexed sample sequencing was conducted using an Illumina MiSeq Reagent Kit v3.
(600-cycle) (Illumina Inc., San Diego, CA) producing paired end reads 300 bp in length. Unprocessed FASTQ files were obtained for subsequent analysis.

3.3.6.1 Bioinformatics pipeline

Overlapping paired end reads were assembled using PEAR software (Zhang et al., 2013). Primers and poor quality sequences were removed using cutadapt software (Martin, 2011). After quality filtering, a range of 11537 to 54468 reads in between 312-379 bp in length was recovered (Supplemental Table S1). The UPARSE pipeline was used to cluster sequences into centroid operational taxonomic units (OTUs) (Edgar, 2013). A 75% sequence similarity threshold was used for OTU clustering (Tan et al., 2013). This resulted in 199 and 35 OTUs for DNA and RNA, respectively. Reads were mapped back to OTUs using USEARCH software (Edgar, 2010). Reads per OTU were standardized by the number of sample reads to allow comparison among samples. The OTU centroid sequences were submitted to GenBank with accession numbers KP188592 to KP188790.

3.3.6.2 NGS Data analysis

The OTU centroid sequences were identified using BLASTx (Camacho et al., 2008) against NCBI's nr (protein) database. The BLASTx algorithm translates nucleotide sequences and conducts pairwise comparisons against protein sequences to determine best possible matches. Only sequences verified to be phoD were included in subsequent analysis.
The MOTHUR software (Schloss et al., 2009) was used to calculate proportions of shared OTUs between samples, to summarize the distances between samples in terms of the Morisita-Horn similarity coefficient, a beta similarity measure, and to generate a UPGMA tree distinguishing samples based on the Morisita-Horn similarity coefficient. The similarity tree was visualized using the iTOL portal (Letunic and Bork, 2006).

3.3.7 Statistical analysis

Soil P data, enzyme activities, gene and transcript abundance data were analyzed using a two-factor (Soil x P amendment) ANOVA in a general linear model (GLM) in JMP 11 (SAS Institute Inc., Cary, NC). All values were converted to oven-dry weight equivalents. Variables were tested for normality using Shapiro-Wilkes test and log transformed when required. Significant differences among soils or treatments were determined using student’s t-test ($P<0.05$).

3.4 RESULTS

3.4.1 Labile soil P

At initiation of the greenhouse trial, concentrations of soil NaHCO$_3$-extractable inorganic P were 5.80, 10.39 and 19.07 µg g$^{-1}$ (Table 3.1) in the organic, conventional and prairie soils, respectively. For the control pots with no P added, NaHCO$_3$-extractable inorganic P increased to 6.30 µg g$^{-1}$ (organic), 15.5 µg g$^{-1}$ (conventional) and 34.6 µg g$^{-1}$ (prairie) when analysed 30 days after planting ryegrass (Fig. 3.1). The NaHCO$_3$-extractable inorganic P at 30 days was significantly different among soils and P
treatments ($P<0.001$). For the NaHCO$_3$-extractable organic P the main effects of soil ($P<0.001$) and P treatment ($P<0.05$) were significant, but there was no difference in the response of the soils to each P treatment (Table 3.2). Concentrations of NaHCO$_3$-extractable inorganic and organic P increased as expected compared to the control (no P) treatment for all soils (Fig. 3.1).

<table>
<thead>
<tr>
<th>Inorganic P (µg g$^{-1}$)</th>
<th>ALP (µmol pNPP g$^{-1}$ soil hr$^{-1}$)</th>
<th>phoD gene abundance (copy # g$^{-1}$ soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORG 5.80 ± 1.2</td>
<td>2.40 ± 0.3</td>
<td>2.07E+6</td>
</tr>
<tr>
<td>CONV 10.39 ± 3.4</td>
<td>2.75 ± 0.3</td>
<td>2.09E+6</td>
</tr>
<tr>
<td>PRA 19.07 ± 3.5</td>
<td>2.81 ± 0.4</td>
<td>2.59E+6</td>
</tr>
</tbody>
</table>

**Table 3.1**: Concentration of soil NaHCO$_3$-extractable inorganic P from air-dried soils and potential alkaline phosphatase (ALP) activity and phoD gene abundance in soils sampled at day 0 following a pre-incubation period (14 days at 40% water-holding capacity). Values are treatment means ± standard deviation (n=3), except phoD (n=2).

**3.4.2 Alkaline phosphatase activity**

Comparison of ALP activity at day 0, following a 14-day pre-incubation at 40% gravimetric moisture, showed little difference among soils from the organic, conventional and prairie plots (2.40, 2.75, 2.81 µmol pNPP g$^{-1}$ soil hr$^{-1}$; Table 3.1). After 30 days, the ALP activity in soil sampled from the control pots (no P) increased to 5.39, 3.34, and 3.82 µmol pNPP g$^{-1}$ soil hr$^{-1}$ for soils from the organic, conventional and prairie plots, respectively (Fig. 3.2). After 30 days of ryegrass growth in the greenhouse, ALP activity showed significant effects of both soil and P treatment ($P<0.001$) (Fig. 3.2). These effects were also observed at the 106 day harvest sampling ($P<0.05$) (Table 3.2).
Figure 3.1: Concentration of soil NaHCO$_3$-extractable inorganic P and organic P in soil samples at 30 days after seeding in soil from a) organic (ORG), b) conventional (CONV) and c) prairie (PRA) management systems. Lines represent the baseline NaHCO$_3$-extractable inorganic P value at day 0. Values are treatment means ± standard error (n=4) of the total with letters indicating significantly different means ($P<0.05$). Bar patterns represent P treatments of no P (black), manure P (stripes) and mineral P (checkers).
Soil ALP activity was higher and showed less variation in response to P treatment (no P, manure P or mineral P) in soil from the organic compared to conventional and prairie plots. Although ALP activity was similar for all soils at day 0, by 30 days after planting ryegrass the organic soil showed a rapid short-term change when high rates of manure were applied (Fig. 3.2). In the organic soil, potential ALP activity was significantly higher than the conventional and the prairie soils for all P treatments \( (P<0.05) \) except for medium and high manure treatment where no differences were seen.

### 3.4.3 Quantification of phoD gene abundance and transcription activity

By quantifying the \( \text{phoD} \) gene abundance we observed a significant effect of soil management \( (P<0.001) \), which was substantially higher in organic, compared to conventional and prairie (Fig. 3.3). There was also a significant effect of P treatment \( (P<0.01) \) with high mineral P application corresponding with lower gene abundance, compared to high rates of manure. However, the soils from organic, conventional, and prairie systems did not respond differently to the applied P treatments (Table 3.2). These differences were significant for the no P, high manure, and 15, 40 and 80 mg P kg\(^{-1}\) of mineral P. The \( \text{phoD} \) gene abundance in the conventional and prairie soils did not show a consistent response to the rates or types of applied P treatments.

The \( \text{phoD} \) transcript copy numbers were detected in all soils sampled and demonstrated that soils responded significantly differently to P fertilization (Fig. 3.3; \( P<0.01 \)). However, when the main effects were separated and the means compared, there were no clear trends with respect to either soil, P rate, or difference between no P, manure P and mineral P.
Table 3.2: Analysis of variance (ANOVA) table for soil properties and processes at 30 and 106 days.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>30 days</th>
<th>106 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaHCO₃</td>
<td>NaHCO₃</td>
</tr>
<tr>
<td></td>
<td>Inorganic P</td>
<td>Organic P</td>
</tr>
<tr>
<td>Block***††</td>
<td>ns†††</td>
<td>***</td>
</tr>
<tr>
<td>Soil (S)</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>P Treatment (T)</td>
<td>***</td>
<td>*</td>
</tr>
<tr>
<td>S x T</td>
<td>***</td>
<td>ns</td>
</tr>
</tbody>
</table>

† Potential alkaline phosphatase activity
†† Block refers to the arrangement of replications of treatments imposed in the greenhouse
†††*, **, *** significant at 0.05, .01 and 0.001 probability
Figure 3.2: Potential alkaline phosphatase activity measured in soils amended with manure P and mineral P (KH$_2$PO$_4$) in soils under organic (ORG), conventional (CONV) and restored prairie management (PRA) at 0, 30 and 106 days after planting ryegrass in the greenhouse. Data points represent treatment means (n=4) and the same letters within a sampling time and soil type represent no significant difference at $P<0.05$ as determined by student’s t-test. Where no letters are present there were no significant differences between treatments within a soil and amendment type.
Figure 3.3: Differences in bacterial *phoD* gene and transcript abundance in response to P fertilization treatment in long-term organic (ORG), conventional (CONV), and prairie (PRA) managed soils at day 30 of ryegrass grown in a greenhouse trial. Values are P treatment means ± standard error (n=4) with letters indicating significantly different means ($P<0.05$) within a soil. An asterisk indicates soil type with the significantly highest value within a P treatment ($P<0.05$). Bar patterns represent P treatments of no P (black), manure P (stripes) and mineral P (checkers).
3.4.4 Identification of phoD gene abundance and transcription activity

Next generation sequencing of DNA from the control (no P) treatments revealed 102 phoD OTUs in common among all the systems with a total of 163, 153, and 137 in soils from the organic, conventional and prairie plots, respectively (Fig. 3.4a). The metagenome from the organic soil had the highest number (26) of distinct phoD OTUs while the prairie soil had the lowest number (5; Fig. 3.4a). The phoD RNA sequences (from metatranscriptome) showed only eight active phoD OTUs in common (ALPS-1, 5, 6, 7, 52, 53, 66 and 74), of which three were identified using a cutoff of 70% similarity in blastx as Methylobacterium, Methyloferula, and Skermanella (Fig. 3.4b). Soils from the conventional system had the highest number of active phoD OTUs (23), followed by organic (19) and prairie (17). There were nine active phoD OTUs found only in soil from the conventional system (ALPS-20, 51, 58, 65, 90, 134, 137, 174, 198) classified as phoD from Streptomyces, Mesorhizobium, Bradyrhizobium, Kribella and five unclassified. A UPGMA tree showing groupings of the conventional and prairie management systems based on the Mosisita-Horn similarity coefficient demonstrates a greater similarity between the conventional and prairie plots for both the RNA and DNA phoD samples (Fig. 3.4c).

3.4.5 Relationship of phoD gene abundance to ALP activity and labile P

Soil samples with high potential ALP activity generally had correspondingly high rates of gene abundance. A significant positive relationship was found between ALP activity and phoD gene abundance (Fig. 5; \( P<0.001, r^2=0.76 \)), but not transcript abundance (\( P=0.17, r^2=0.08 \); data not shown). A similar lack of relationship was found
Figure 3.4: Venn diagram of bacterial \textit{phoD} OTUs in a) DNA and b) RNA (cDNA) showing sharing between soils from long-term organic (ORG), conventional (CONV) and prairie (PRA) management systems. Active sequences from the cDNA community were identified to the Genus level using BLASTx c) UPGMA tree distinguishing samples based on the Morisita-Horn similarity coefficient of DNA (d) and RNA (r).
Figure 3.5: Relationship between soil bacterial \textit{phoD} gene abundance and potential alkaline phosphatase (ALP) activity in soils sampled 30 days after planting ryegrass in the greenhouse. Values are treatment means (n=4).

between \textit{phoD} gene and transcript abundance ($P=0.72$, $r^2=0.03$; data not shown).

Soil samples with low concentrations of NaHCO$_3$-extractable organic P likewise had higher rates of \textit{phoD} gene abundance (Fig. 5), resulting in a significant negative relationship ($P<0.001$, $r^2=0.40$). No relationship was observed between \textit{phoD} gene abundance and soil NaHCO$_3$-extractable inorganic P ($P=0.88$, $r^2=0.001$). Since \textit{phoD} gene abundance and ALP activity were significantly correlated, similar relationships existed with a significant correlation between ALP activity and NaHCO$_3$-extractable organic P ($P<0.01$, $r^2=0.31$), but not NaHCO$_3$-extractable inorganic P ($P=0.45$, $r^2=0.03$; data not shown).
3.5 DISCUSSION

High rates of ALP activity were observed in ryegrass pots with soil from long-term plots under organic management. Interestingly, the ALP activity in soils with P applied showed that the no P and mineral P treatments in soils with organic management did not vary from the manure P treatments; however, differences between mineral and manure P were seen in soils with conventional and prairie management. High rates of ALP activity were observed after 30 days in organic soils regardless of P treatment type or rate. Differences among soils from the organic plots compared to that of the conventional and prairie plots, even when no P was added, were much greater than those observed in a previous field study with these soils (Fraser et al., 2014). The addition of nutrients at seeding may have affected microbial growth. As well, the more favorable...
temperature (24°C day, 18°C night) and moisture (60% field capacity) conditions maintained in the greenhouse compared to the field and/or plant roots may have stimulated the rhizosphere communities within the pot. Other studies have reported increases in ALP activity following manure application (Parham et al., 2003; Saha et al., 2008; Zhang et al., 2014). After 25 years of applying mineral fertilizers or pig manure, Zhang et al. (2014) found that pig manure significantly increased phosphatase activity but no effect was observed between the control and a fertilizer treatment (150 kg N ha\(^{-1}\), 75 kg P\(_2\)O\(_5\) ha\(^{-1}\), 75 kg K\(_2\)O ha\(^{-1}\)). Although enzymes may be added with manure, we speculate that this was not a major contributor in our study since there were no significant differences in ALP activity even among the high manure rates and no P added in organic pots.

Negative correlations have been reported between labile P and ALP activity, suggesting that enzyme production is induced at conditions of low bioavailable P (Zhang et al., 2012; Fraser et al., 2014). However, in this greenhouse study, we observed similar increases in the ALP activity levels within the organic, conventional and prairie soils regardless of the applied P rate. The potential ALP activities at day 0 were similar for all of the soils, but 30 days after the P treatments were applied there were differences in the ALP activity and \(phoD\) gene abundance among soils with different management histories. Although the application of manure P to the conventional and prairie soils resulted in significantly higher ALP activity, there was little change associated with mineral P addition compared to the control for all soils.

While some studies have reported the inhibition of phosphatase activities with the application of mineral P (Saha et al., 2008), others contradict these results by reporting an
increase in ALP activity with application of mineral P (Kanchikerimath and Singh, 2001). In the present study, no significant impact of the addition of mineral P was observed. This lack of response may be explained by measurement of activity in the assay beyond that induced under P starvation, such as activity from phosphatases stabilized on soil particles or constitutively released by microbial cells (Nannipieri et al., 2011). Phosphatases may persist in the soil with a reported half-life of 14 days (Pettit et al., 1977; Tadano et al., 1993). If ALP activity is induced only at low phosphate levels, we should have seen a strong correlation between the NaHCO$_3$-extractable inorganic P concentrations in soil and ALP activity. It is also important to note that other phosphatases not measured in this study (i.e. acid phosphatases, phytases, phosphodiesterases) may also be responsible for hydrolyzing P compounds in the NaHCO$_3$-extractable fraction.

Although it is impossible to distinguish phosphatases excreted by active microbial cells from those stabilized on soil surfaces using the potential ALP assay, there were few differences among ALP activity in organic, conventional and prairie managed soils at day 0. Therefore, differences in potential ALP enzyme activity at day 30 are likely due to differences in microbial communities, since the $phoD$ gene abundances were significantly correlated with potential ALP activities in soils from different management systems. Concomitant changes in $phoD$ gene abundance and ALP activity confirm the results in our preceding field study (Fraser et al., 2014). However, there was little evidence for changes in $phoD$ gene abundance in response to P addition treatments in this greenhouse study. Also, it is important to note that quantification of the $phoD$ gene does not take into account ALP produced by other bacterial genes (i.e. $phoA$, $phoX$), which could also
contribute to the potential ALP activity, or phoD genes that were not amplified with the ALPS primer set. By detecting phoD OTUs in the soil metatranscriptome and quantifying their abundance we confirm that phoD was being transcribed and thereby assume that the bacterial phoD is contributing to alkaline phosphatase activity, even though gene transcript values were not correlated to either gene abundance or ALP activity.

Despite significantly higher gene abundance in soils under organic versus conventional and prairie management, a corresponding increase in phoD gene transcripts was not observed. Somewhat surprisingly, P addition, even at high rates, did not significantly impact transcript abundance in the organic and conventionally-managed soils compared to the control. It is possible that differences in phoD bacterial community composition among management systems may play a role. In the preceding field study, a higher number of DGGE bands were detected in soils under conventional and prairie management, compared to organic management, while the corresponding ALP activity was significantly lower in these plots compared to the organically-managed plots (Fraser et al., 2014). Since phoD bacteria are taxonomically diverse, different groups (or OTUs) may respond differently to P treatments, with a few OTUs being highly induced and potentially driving ALP production. In this greenhouse study, comparisons of similarity among phoD gene sequences using the Morista-Horn index indicate greater similarity between the conventional and prairie systems compared to the organic system (Fig 4c). The Morista-Horn index is recommended due to its relative independence from sample size and diversity (Magurran, 2004). A similar trend was seen in the phoD transcripts, although a much lower number of OTUs were identified in the metatranscriptome (35
OTUs).

It is challenging to relate gene abundance and activity directly to enzyme activity since the ALP assay measures potential ALP activity under optimal conditions and does not differentiate extracellular enzymes. Despite more phoD RNA OTUs in soil from conventional (23) versus organic (19) management, and similar transcript abundance, there was higher potential ALP activity at 30 days in the organically managed soil. This indicates a lesser number of OTUs in the organically managed soil, resulting in higher ALP activity at 30 days. Although phoD is not a phylogenetic marker, it is possible to identify the closest similarities to known phoD sequences based on NCBI BLAST. The OTUs unique to the organically-managed soil (ALPS-27, 56, 72, 132) matched most closely to Sedimentitalea, Cyanothece, Aquabacterium, and Acidovorax phoD sequences. It is likely that as databases for phoD sequences expand that a stronger identification will be possible.

Potential ALP production was strongly correlated with phoD gene abundance, but not transcript copy number. We had hypothesized that phoD transcription would be inhibited by high rates of P, especially in a highly soluble form. The lack of response of transcript abundance both among soils and to rate and type of P treatment was unexpected considering the substantial differences in potential ALP activity among all factors, with a quick response of ALP production in soils from organic management at the 30 day sampling. The transcript abundance for the organically-managed soil with manure P treatments was lowest, while ALP activity was significantly higher than activity detected in soils under conventional and prairie management. This may partially be explained by the sampling time, since the extraction of RNA to measure transcript
abundance as a measure of gene expression measures only a brief moment in time. Although Ca\(^{2+}\) is a cofactor for ALP production by the \textit{phoD} gene (Yamane and Maruo, 1978; Wu et al., 2007), it is unlikely that this was a limiting factor since it was added in nutrient solution to all pots.

To our knowledge, this is the first study reporting \textit{phoD} expression of bacterial communities in soil with the ALPS-F730 and ALPS-R1101 primers. If expression of the \textit{phoD} gene had occurred only when the Pho regulon was induced by phosphate starvation (Apel et al., 2007; Wanner, 1996), a negative correlation between mineral P rate and \textit{phoD} gene expression would have resulted. However, we saw this trend only in the organically-managed soil suggesting that the response may be influenced by both labile soil P concentrations and the bacterial species present. If response of \textit{phoD} is dependent on P availability, an increase in both gene and transcript abundance would be expected in the pots with no P added, especially considering the addition of other nutrients and the optimum growing conditions in the greenhouse. In this greenhouse study, only \textit{phoD} gene abundance, but not expression, showed a significant negative correlation with labile P. The possibility that other factors are affecting the expression of the Pho regulon has been proposed, such as oxidative stress under nutrient rich conditions as reported by Darbon et al. (2012) in \textit{Streptomyces lividans}. For future studies, measuring the gene and transcript levels with a more universal \textit{phoD} primer set, as well as \textit{phoA} and \textit{phoX} genes, may provide a more complete understanding of ALP production and the bacterial response to manure and mineral P fertilization.

In addition, the data presented here was from a short-term study with ALP activity and \textit{phoD} gene and transcript abundance measured at 30 days after P application and it is
possible that differences would only be detected over a longer time period. Although other studies have reported short-term changes in microbial communities (Lazcano et al., 2013; Xiong et al., 2014), results in the present study indicate that long-term soil management had a much greater effect on ALP activity and gene abundance than P treatment after 30 days.

3.6 CONCLUSIONS

Gaining a better understanding of functional genes such as phoD may help predict the effects of anthropogenic changes on the biological processes important in P turnover. In production-based systems, understanding nutrient cycling and availability is essential to minimize inputs while increasing yields. Potential ALP was similar in the organically-managed soil regardless of P treatment applied, while the soils from conventional and prairie management increased only at higher rates of manure application. The conventional soil reacted similarly to the restored prairie soil despite being in the same cropping rotation as the organic management, while the restored prairie remained uncultivated since the inception of the field trial. In accordance with a previous field study, the ALP activity was significantly correlated with phoD gene abundance thereby providing an indication of bacterial ALP production in these soils.
SOIL PHOSPHORUS BIOAVAILABILITY AS INFLUENCED BY LONG-TERM MANAGEMENT AND APPLIED PHOSPHORUS SOURCE

4.1 ABSTRACT

Soil P availability may be significantly impacted by land management practices, thereby impacting plant P uptake in response to P amendments. The aim of the study was to determine if long-term management influences the soil P pools and plant bioavailability in response to amendment with manure P or mineral P. Soil samples (0-15 cm) were collected from the grain-forage rotation (flax-alfalfa-alfalfa-wheat) of the Glenlea Long-term Rotation near Winnipeg, Manitoba to compare organic (ORG), organic with composted manure (ORG+M), conventional (CONV) and restored prairie (PRA) management systems. For each soil, the response of Italian ryegrass (Lolium multiflorum) to treatments of a control (no P), composted cattle manure (6, 40 and 80 mg P kg⁻¹ soil) and mineral P as KH₂PO₄ (15, 40, 80 and 160 mg P kg⁻¹ soil) was assessed over a 106-day greenhouse trial. All other nutrients were applied in solution. An initial sequential P fractionation indicated that after 19 years of management, the ORG soil had lower concentrations of both labile and moderately labile P fractions compared to the CONV and PRA soils. A one-time manure application (ORG+M) in 2007 at 10 tonnes dry matter ha⁻¹ produced little difference from the ORG soil. There was a greater response of ryegrass biomass in the ORG and ORG+M pots than the CONV and PRA to both the manure and soluble P additions compared to the control. The ORG and ORG+M soils with low manure treatments had the highest apparent shoot P recovery and
the PRA soil was lowest with -9.0% for the low manure treatment. According to the analysis of apparent shoot P recovery by ryegrass, there was no differential response to P treatment in the soils from different long-term management system.

4.2 INTRODUCTION

Long-term management can influence soil P forms and bioavailability. Since P is an essential macronutrient limiting plant productivity in many ecosystems, agricultural production is heavily dependent on synthetic P fertilizers for crop yields. Organic production systems are often perceived as more sustainable, since they avoid the use of chemical fertilizers and pesticides. However, current yields obtained by organic farming may not be sufficient to feed the world. Mäder et al. (2002) found a 20% decrease in crop yields in organic compared to conventional systems over a 21-yr period. During this period there was a corresponding 34 to 53% decrease in fertilizer and energy consumption. As the global population continues to increase, so does our dependency on P fertilizer.

Phosphorus management in organic systems is especially challenging. Unlike nitrogen, which may be added to the soil by incorporating nitrogen-fixing legumes into the crop rotation, there are few options available for P under organic certification. Rock phosphate and bone meal are mostly insoluble in the Northern Great Plains since the soils typically have a higher pH and are often calcareous (Arcand et al., 2010). Although manure is an adequate source of P and essential to maintaining yields in many organic systems worldwide, this region is limited by large farm sizes and long distances to a manure source (Lynch et al., 2014). These limitations to P replenishment in organic
cropping systems have lead to reports of low available P both on-farm and long-term research trials (Enzt et al., 2001, Roberts et al., 2007, Malhi et al., 2009, Miller et al., 2008, Welsh et al., 2009, Knight et al., 2010). However, corresponding plant P deficiencies have not been reported despite the constant export of P at harvest, suggesting that the labile P pools are being replenished by less labile forms and play an important role in maintaining adequate plant available P. Soil tests are routinely used to estimate plant available P, but this does not take into account the less available forms that may become available over the growing season.

In contrast to reports of low plant available P concentrations in soils, the over application of P in mineral or manure P has created serious environmental problems worldwide. It is common for producers to apply a basal rate of P at seeding, regardless of soil P concentrations. If P levels are considered, only the labile easily extractible pool is measured by provincial soil test laboratories. Excess P will be sorbed to soil particles, immobilized, converted to less available forms, or be removed in runoff. These losses have been identified as a major source of eutrophication of water bodies (Sharpley et al., 1992, Schindler et al., 2012), causing potentially toxic algal blooms. Manure has traditionally been applied based on nitrogen recommendations (Sharpley et al., 1998, Miller et al., 2011), resulting in an over application of P. In addition, the costs associated with transporting manure often result in high rates applied in close proximity to the source. Both the previous management of the site and the P source being applied should be considered to maximize plant utilization of P, while minimizing potential negative environmental impacts.

Since anthropogenic practices influence soil P availability, and ultimately plant P
uptake and yields, we collected soils from a long-term field experiment near Winnipeg, Canada for a greenhouse experiment. The aim of the study was to determine differences in P bioavailability and apparent P recovery in ryegrass between long-term ORG, ORG+M, CONV and PRA management when amended with varying rates of manure P or mineral P. The objectives of the experiment were to: (1) evaluate initial differences in extractible soil P pools; (2) examine the relationship between P bioavailability, plant P uptake and yield in response to varying rates of manure and chemical P amendments; and, (3) determine the effect of long-term management on the apparent P recovery of compost manure and chemical P amendments.

4.3 MATERIALS AND METHODS

4.3.1 Management treatment description and soil sample collection

The Glenlea farming system experiment is part of the University of Manitoba Glenlea Research Station located in southern Manitoba, Canada in the Red River valley (49°38’25”N, 97° 8’28”W). The experiment is a randomized complete block design with split plots (fully phased) and three reps. The plots used in this study were a forage-grain rotation under organic no input (ORG), organic-manure amended (ORG+M), and conventional (CONV) management (Table 4.1) with a 4-yr rotation of flax-alfalfa-alfalfa-wheat (*Linum usitatissimum*, *Medicago sativa*, *Triticum aestivum* L.). In addition, each block included restored prairie plot (PRA; 45 x 60 m) seeded to *Agropyron dasytachum*, *Andropogon gerardii* Vitman, *Elymus lanceolatus* (Scribn. And Smith) Gould, *Elymus trachycaulus* (Link) Gould ex Shinners, *Panicum virgatum* (L.), *Pascopyrum smithii* (Rydb.) A. Löve, *Sorghastrum nutans* (L.) (Bell et al., 2012) to be used as a reference.
The soil is a heavy clay Humic Vertisol of the Scantenbury and Hoddinott series with 9% sand, 26% silt and 66% clay, an average \( \text{pH}_{\text{H}_2\text{O}} \) of 7.4 and 7.7% organic matter content (Bell et al. 2012; Welsh et al. 2009). Further details of the site and experimental design are given by Welsh et al. (2009) and Bell et al. (2012).

In May 2011, soil samples (0-15 cm; \( n=20 \)) were collected prior to the wheat phase of the Glenlea Long Term Crop Rotation and Management research plots at Glenlea, Manitoba for initial soil chemical analysis and soil P fractionation. Additional bulk soil (0-15 cm depth) was collected (~12.0 kg per plot), dried and mechanically ground (< 4 mm) for the greenhouse trial.

4.3.1.1 Manure analysis

Composted cattle manure was supplied by the Agriculture and Agri-Food Canada Beef Research Unit at Brandon, Manitoba (Welsh et al., 2009). Air-dried samples were analysed for nutrient content (Table 4.2).

4.3.1.2 Soil phosphorus fractionation

Soil samples were air dried and finely ground prior to chemical analysis (< 1 mm). Soil samples (0-15 cm) were sequentially extracted in duplicate to fractionate total P into pools differing in bioavailability using a modified procedure by Hedley et al. (1982), eliminating the microbial P extraction. These fractions have been operationally defined with decreasing availability for plant uptake as: (1) Resin P-P\(_i\), exchangeable
Table 4.1: Management systems and restored native prairie at the Glenlea Long Term Crop rotation and Management site.

<table>
<thead>
<tr>
<th>Rotation†</th>
<th>Systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grain-forage</td>
<td><strong>Flax – alfalfa – alfalfa – wheat</strong></td>
</tr>
<tr>
<td>Organic – no input</td>
<td>ORG</td>
</tr>
<tr>
<td>Organic + manure</td>
<td>ORG+M</td>
</tr>
<tr>
<td>Conventional</td>
<td>CONV</td>
</tr>
</tbody>
</table>

† Soils (0-15cm) were collected in spring 2011, preceding the wheat phase of the grain-forage rotation under three management systems.

Table 4.2: Composted beef manure selected properties.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (%)</td>
<td>58.0 (7.5)</td>
</tr>
<tr>
<td>pH</td>
<td>7.1 (0.06)</td>
</tr>
</tbody>
</table>

Nutrient Content (g kg\(^{-1}\))††

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total P</td>
<td>0.99 (0.06)</td>
</tr>
<tr>
<td>Total N</td>
<td>3.82 (0.05)</td>
</tr>
<tr>
<td>NH(_4^+)-N</td>
<td>0.22 (0.01)</td>
</tr>
<tr>
<td>K</td>
<td>1.34 (0.1)</td>
</tr>
<tr>
<td>Mg</td>
<td>2.02 (0.1)</td>
</tr>
<tr>
<td>Ca</td>
<td>9.60 (0.7)</td>
</tr>
<tr>
<td>Na</td>
<td>0.12 (0.02)</td>
</tr>
</tbody>
</table>

† Completed by Nova Scotia Department of Agriculture analytical services (mean and standard deviation, n=3).
†† Reported values before adjusting for dry matter percent.
with solution; (2) 0.5 M NaHCO₃ (pH 8.5) Pᵣ and Pₒ, sorbed on soil minerals and some microbial P; (3) 0.1 M NaOH-Pᵣ, Pₒ, associated with Fe and Al oxyhydroxides; (4) 0.1 M NaOH after ultrasonification-Pᵣ, Pₒ, removing P at internal surfaces of aggregates (sNaOH Pᵣ and Pₒ); (5) 1 M HCl-Pᵣ apatite mineral and some occluded P and (6) residual P by dry ashing. Although the reactive P is mostly orthophosphate, it may contain some organic compounds and the unreactive P may include inorganic polyphosphates (Shand et al., 2000) for clarity, molybdate reactive P was considered to be inorganic P (Pᵣ) and unreactive P as organic P (Pₒ).

Bicarbonate extractable P was determined on soil in a 1:20 ratio with 0.5M NaHCO₃ (pH 8.5) and shaken for 30 min (Olsen et al., 1954). Since activated charcoal was not added to the during the extraction, the extracts were acidified with 3M H₂SO₄ to remove carbonates and inorganic P measured colorimetrically with a UV/vis spectrophotometer 6405 (Jenway, Staffordshire, UK) by acid-molybdate reaction at 880 nm (Murphy and Riley, 1962). Total soil P was determined by igniting samples at 500°C for 4h and extracting with 0.5M H₂SO₄ (Saunders and Williams, 1955). Total C and N content of soils were measured using an Elementar Vario Max N/C Analyzer (ELEMENTAR Analysensysteme, Hanau, Germany).

4.3.2 Greenhouse experiment

Soil samples from the three replicates for each management system (ORG, ORG+M, CONV, PRA) were pooled together and mixed to make one composite sample per system. Soils were pre-incubated at 40% gravimetric moisture in the greenhouse and after 14 days, pots were filled with 0.9 kg dry weight equivalent (D.W.E.).
experimental design was a completely randomized design with four soils (ORG, ORG+M, CONV, PRA), eight P treatments (Table 4.3), and four replicates.

Table 4.3: Phosphorus amendments and application rates for greenhouse study assessing the response of Italian ryegrass in soils from organic, organic with compost, conventional and prairie management.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Addition</th>
<th>Available P (mg P kg⁻¹ soil)</th>
<th>Total P (mg P kg⁻¹ soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No P</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Low Manure</td>
<td>Composted cattle manure</td>
<td>6†</td>
<td>18</td>
</tr>
<tr>
<td>Med Manure</td>
<td>Composted cattle manure</td>
<td>40†</td>
<td>120</td>
</tr>
<tr>
<td>High Manure</td>
<td>Composted cattle manure</td>
<td>80†</td>
<td>240</td>
</tr>
<tr>
<td>Low soluble P</td>
<td>KH₂PO₄</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Medium soluble P</td>
<td>KH₂PO₄</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>High soluble P</td>
<td>KH₂PO₄</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Very High soluble P</td>
<td>KH₂PO₄</td>
<td>160</td>
<td></td>
</tr>
</tbody>
</table>

†Assuming 30% of total P in the compost is available.

Taking into consideration the nutrient concentrations in the manure amendments (Table 4.2), macronutrients were solubilised in H₂O and added to all treatments for a final level of: 150 mg N kg⁻¹, 200 mg K kg⁻¹, 35 mg Ca kg⁻¹ and 15 mg Mg kg⁻¹. Microelements were added at a rate of (mg kg⁻¹): 2 Cu, 1 Mn, 1 B, and 0.1 Mo (Oberson et al., 2010).

In each pot, fifteen Italian ryegrass (*Lolium multiflorum*) seeds were added and thinned to 10 plants after emergence. Soil moisture was maintained at 60% field capacity and the greenhouse conditions set to 25°C day and 18° night with a 16 h photoperiod. Plants received soluble nutrients (except P) after each cut (days 30, 62, 75 and 89) to ensure that other nutrients were not limiting plant growth.
4.3.2.1 Soil and plant sampling and analysis

Ryegrass plants were cut approximately 2 cm above the soil at 30, 62, 75 and 89 days with the final harvest at 106 days after planting (DAP). Plant samples from all sampling dates were combined into one composite sample per pot before being dried, weighed, ground (< 2 mm) and analysed for P and N content. At the final harvest, pots were destructively sampled and soil was separated from the roots that were washed before analysis. Plant shoots and roots were oven dried, weighed and ground (<1 mm) for tissue analysis. Plant tissue P was determined by ashing at 500°C for 4 h and extracted by heating in 10% HNO₃/30% HCl (Jones and Case, 1990). Total P in plant tissue extracts was measured by inductively coupled plasma (ICP) spectroscopy by the Nova Scotia Department of Agriculture analytical services lab. Total C and N content of soil and N content of plant tissue was measured using an Elementar Vario Max N/C Analyzer (ELEMENTAR Analysensysteme, Hanau, Germany). Extractable NaHCO₃ inorganic and organic P and total P in the soils were determined as described above. Cumulative shoot P uptake was determined for each pot and used to determine apparent P use efficiency calculated as:

\[
\text{APUE} = \frac{[(\text{shoot P uptake}_{\text{treatment}} - \text{shoot P uptake}_{\text{control}})]}{\text{total P applied}}
\]

(Eghball and Power, 1999)

4.2.4 Statistical analysis

Statistical analyses were completed using the GLM procedure of JMP 11 (SAS Institute Inc., Cary, NC). The significance of the effects of block, soil, amendment treatment, and soil x treatment interaction were included in the model. Variables were
tested for normality using S-W and log transformed when required. Significant differences between soils or treatments were determined using Tukey’s test (P<0.05).

4.3 RESULTS

4.3.1 Soil P fractions

The sequential P extraction revealed lower concentrations of both soil labile and moderately labile P fractions in the ORG soil compared to the PRA and CONV systems (Table 4.4). The greatest differences were seen in the Pi for the Resin Pi, NaHCO₃-Pi and the NaOH-Pi. For these extractions the CONV soil was 53%, 50% and 45% higher than the ORG, while the PRA had P concentrations 74%, 82% and 77% more than ORG. In contrast, the residual P fraction was highest in the ORG (272.2 mg kg⁻¹) and ORG+M soils (266.5 mg kg⁻¹) while the PRA soil was lowest (186.5 mg kg⁻¹). In the soils sampled from the PRA plots, the NaOH-Po was nearly double that of the agriculturally managed soils, basically the difference from the residual P pool. The sum of P concentrations in the fractions was 539.6, 550.6, 559.8, and 612.3 mg P kg⁻¹ for the ORG, ORG+M, CONV, and PRA soils, respectively. This represented P recoveries between 78% and 89% when compared to total P by ignition.
Table 4.4: Sequentially extracted P and total P, C and N concentration in soil samples (0-15 cm) taken in May 2011. Values are treatment means and standard deviation (n=3).

<table>
<thead>
<tr>
<th>Soil Property</th>
<th>ORG</th>
<th>ORG+M</th>
<th>CONV</th>
<th>PRA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inorganic P (Pi)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resin Pi</td>
<td>10.2 ± 1.8</td>
<td>16.4 ± 4.6</td>
<td>21.5 ± 5.9</td>
<td>38.5 ± 5.0</td>
</tr>
<tr>
<td>NaHCO₃-Pi</td>
<td>4.6 ± 0.9</td>
<td>7.0 ± 2.4</td>
<td>12.7 ± 2.6</td>
<td>25.2 ± 5.3</td>
</tr>
<tr>
<td>NaOH-Pi</td>
<td>14.3 ± 1.7</td>
<td>17.6 ± 2.8</td>
<td>33.3 ± 9.4</td>
<td>60.8 ± 13.0</td>
</tr>
<tr>
<td>sNaOH-Pi</td>
<td>4.8 ± 1.2</td>
<td>6.2 ± 0.7</td>
<td>10.1 ± 3.3</td>
<td>17.6 ± 3.4</td>
</tr>
<tr>
<td>HCl-Pi</td>
<td>114.4 ± 4.9</td>
<td>114.4 ± 13.1</td>
<td>98.6 ± 6.0</td>
<td>105.5 ± 2.8</td>
</tr>
<tr>
<td><strong>Organic P (Po)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaHCO₃-Po</td>
<td>16.1 ± 4.6</td>
<td>17.8 ± 1.9</td>
<td>24.0 ± 3.7</td>
<td>25.5 ± 2.4</td>
</tr>
<tr>
<td>NaOH-Po</td>
<td>79.5 ± 13.2</td>
<td>82.1 ± 6.8</td>
<td>79.1 ± 13.4</td>
<td>133.4 ± 7.9</td>
</tr>
<tr>
<td>sNaOH-Po</td>
<td>23.1 ± 0.4</td>
<td>22.6 ± 1.3</td>
<td>25.9 ± 6.1</td>
<td>19.4 ± 1.7</td>
</tr>
<tr>
<td>Residual P</td>
<td>272.7 ± 18.3</td>
<td>266.5 ± 31.5</td>
<td>254.6 ± 38.2</td>
<td>186.5 ± 30.8</td>
</tr>
<tr>
<td><strong>∑ P Fractions (mg kg⁻¹)</strong></td>
<td>539.6 ± 39.5</td>
<td>550.6 ± 54.7</td>
<td>559.8 ± 53.4</td>
<td>612.3 ± 29.3</td>
</tr>
<tr>
<td><strong>Total P (mg kg⁻¹)†</strong></td>
<td>693.8 ± 13.6</td>
<td>702.9 ± 54.4</td>
<td>668.9 ± 37.1</td>
<td>688.0 ± 11.5</td>
</tr>
<tr>
<td>Fraction P Recovery (%)‡</td>
<td>78</td>
<td>78</td>
<td>84</td>
<td>89</td>
</tr>
<tr>
<td>NaHCO₃-Pi (mg kg⁻¹)</td>
<td>5.8 ± 1.2</td>
<td>7.2 ± 1.5</td>
<td>10.4 ± 3.4</td>
<td>19.1 ± 3.5</td>
</tr>
<tr>
<td><strong>Total C (g kg⁻¹)</strong></td>
<td>30.8 ± 1.9</td>
<td>28.5 ± 1.2</td>
<td>29.2 ± 0.8</td>
<td>28.6 ± 3.1</td>
</tr>
<tr>
<td><strong>Total N (g kg⁻¹)</strong></td>
<td>3.0 ± 0.4</td>
<td>2.8 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>2.8 ± 0.2</td>
</tr>
</tbody>
</table>

† Total soil P by ignition (Saunders and Williams 1955)
‡ % of total P recovered in P fractions
4.3.2 Soil chemical properties in the greenhouse

Soil chemical properties assessed at 106 DAP differed considerably between the applied P treatment and among the soils from the different management systems, but an interaction was seen only for soil N (Table 4.5). Soil NaHCO$_3$-P concentrations were significantly different for the main effects of soil and management (Table 4.5). As more P was added as manure or fertilizer P, the concentration of NaHCO$_3$ extractible P in the soil at final harvest increased regardless of management system. The PRA soil was significantly higher for all the P amendment treatments with a control (0P) NaHCO$_3$-Pt value of 32.0 mg P kg$^{-1}$ compared to the lowest value of 9.9 mg P kg$^{-1}$ for ORG soil (Figure 4.1). The values increased accordingly with the rate of P applied, with the very high mineral P treatment values equaling 68.4 and 41.3 mg P kg$^{-1}$ for soils from the PRA and ORG management systems.

The amount of P supplied by manure (as % of total P applied) over the 106 d of ryegrass growth was determined by considering the initial concentration of Pi in the soil, the P removed in plant biomass, the amount of Pi remaining in the soil at harvest, and the amount of total manure P added. The percent of P available in the soils for low, medium and high manure P treatments were: 107, 54 and 48 for ORG; 169, 66 and 56 for ORG+M; 176, 78, and 64 for CONV; and 182, 77, and 61 for PRA.

4.3.3 Plant biomass and nutrient uptake

The results for the shoot and root dry matter biomass are presented in Table 4.5 and 4.6. The management by treatment interaction was significant for all plant responses. In the control (no P) treatment the shoot biomass, shoot P uptake and N uptake were
significantly lower in the ORG than all other management systems. Since differences in N uptake, correspond to differences in P uptake it is assumed that N was not a limiting factor for plant growth. Shoot biomass yields were highest for the CONV soil for all of the treatments but did not consistently increase in response to the manure treatment and were significant in the control and low, medium and high KH$_2$PO$_4$ treatment only, with the ORG system being significantly lower. Shoot biomass in the CONV and PRA soils with the mineral P treatments increased only 2.2 g and 1.7 g pot$^{-1}$ between the highest rate and the control, compared to an increase of 6.6 g in the ORG pots. The corresponding shoot P uptake (Table 4.5) shows similar trends to the shoot biomass, indicating that the differences in the P uptake are a function of the plant growth (Figure 4.2). The plant N uptake displays the same significant differences as the plant P uptake, with the control, 15, 40 and 80 mg kg$^{-1}$ KH$_2$PO$_4$ treatments being higher in the CONV soil compared to the ORG. The root biomass and root P uptake showed significant differences for management, treatment, and an interaction of the effects (Table 4.5).

4.3.4 Relationship between P added and plant nutrition

There was higher shoot P uptake in the manure P treatments than the KH$_2$PO$_4$ for all of the soils (Figure 4.3 and Table 4.6). For the manure P treatments in the ORG, ORG+M and CONV soils, the P uptake increased at similar rates considering the total amount of P applied. Despite the P uptake in the PRA soil control (0P) being equal to the CONV, it did not increase at the same rate. The mineral P treatment results showed the same trend where the increase in the CONV pots was linear.
Table 4.5: ANOVA table for soil and plant properties at ryegrass harvest† from soils under long-term organic, organic-manure amended, conventional and restored prairie management with treatments: control, low manure, medium manure, low mineral P, medium mineral P, high mineral P and very high mineral P.

<table>
<thead>
<tr>
<th>ANOVA (p-value)††</th>
<th>Soil</th>
<th>Plant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaHCO₃-P (mg kg⁻¹)</td>
<td>Total C (g kg⁻¹)</td>
</tr>
<tr>
<td>Rep</td>
<td>***</td>
<td>**</td>
</tr>
<tr>
<td>Management (M)</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Treatment (T)</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>M x T</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

† Harvest was 106 days after the ryegrass was planted.
†† *, **, *** significant at 0.05, .01 and 0.001 probability, respectively. ns Not significant.
Figure 4.1: Concentration of soil NaHCO₃-inorganic P (Pi) and organic P (Po) in soils sampled from (a) ORG, (b) ORG+M, (c) CONV and (d) PRA at harvest (106 days). Values are treatment means ± standard deviation of the total P in the extract (n=4). An asterisk above a P treatment indicates a significant difference at P<0.05 between soil as determined by Tukey’s test.
Table 4.6: Above and below-ground ryegrass dry matter production, P uptake and N and C content after 106 days determined for soils taken from the different management systems. Values are treatment means and standard deviation (n=4) with same letters within a treatment representing no significant difference at p<0.05 as determined by Tukey’s test.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low Manure</th>
<th>Medium Manure</th>
<th>High Manure</th>
<th>15 KH₂PO₄</th>
<th>40 KH₂PO₄</th>
<th>80 KH₂PO₄</th>
<th>160 KH₂PO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot Biomass (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORG</td>
<td>4.1 ± 0.7b</td>
<td>7.0 ± 2.6</td>
<td>9.0 ± 1.9</td>
<td>8.7 ± 2.7</td>
<td>5.8 ± 1.1b</td>
<td>7.7 ± 1.6b</td>
<td>8.8 ± 1.8b</td>
<td>10.7 ± 2.4</td>
</tr>
<tr>
<td>ORG+M</td>
<td>8.2 ± 1.8a</td>
<td>9.1 ± 4.8</td>
<td>9.5 ± 3.2</td>
<td>10.8 ± 4.6</td>
<td>8.8 ± 2.8ab</td>
<td>10.4 ± 1.7ab</td>
<td>11.3 ± 2.0ab</td>
<td>11.6 ± 1.8</td>
</tr>
<tr>
<td>CONV</td>
<td>11.0 ± 0.8a</td>
<td>10.8 ± 1.6</td>
<td>11.2 ± 0.5</td>
<td>11.7 ± 1.8</td>
<td>11.8 ± 2.1a</td>
<td>12.7 ± 1.0a</td>
<td>13.1 ± 1.0a</td>
<td>13.2 ± 1.1</td>
</tr>
<tr>
<td>PRA</td>
<td>9.3 ± 1.6a</td>
<td>7.8 ± 1.5</td>
<td>10.0 ± 1.4</td>
<td>9.7 ± 1.9</td>
<td>9.3 ± 2.3ab</td>
<td>10.2 ± 1.7ab</td>
<td>10.6 ± 1.4ab</td>
<td>11.0 ± 0.7</td>
</tr>
<tr>
<td>Root Biomass (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORG</td>
<td>12.5 ± 0.3b</td>
<td>17.5 ± 5.5</td>
<td>24.7 ± 6.0</td>
<td>31.3 ± 1.9</td>
<td>14.5 ± 3.3ab</td>
<td>16.6 ± 0.9</td>
<td>17.2 ± 1.2</td>
<td>22.9 ± 4.9</td>
</tr>
<tr>
<td>ORG+M</td>
<td>18.5 ± 1.6a</td>
<td>17.6 ± 4.6</td>
<td>23.0 ± 5.9</td>
<td>30.7 ± 6.5</td>
<td>26.7 ± 16.3a</td>
<td>17.0 ± 3.4</td>
<td>22.9 ± 9.6</td>
<td>24.4 ± 6.1</td>
</tr>
<tr>
<td>CONV</td>
<td>15.1 ± 6.0ab</td>
<td>17.3 ± 3.8</td>
<td>32.4 ± 5.5</td>
<td>38.3 ± 4.3</td>
<td>13.3 ± 0.9b</td>
<td>15.9 ± 2.5</td>
<td>22.0 ± 6.3</td>
<td>23.4 ± 1.1</td>
</tr>
<tr>
<td>PRA</td>
<td>16.9 ± 3.8ab</td>
<td>20.3 ± 3.2</td>
<td>27.6 ± 6.2</td>
<td>37.2 ± 9.5</td>
<td>16.7 ± 3.4ab</td>
<td>16.1 ± 3.5</td>
<td>17.4 ± 1.4</td>
<td>27.0 ± 4.8</td>
</tr>
<tr>
<td>Shoot P uptake (mg pot⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORG</td>
<td>6.7 ± 0.5b</td>
<td>15.3 ± 4.7</td>
<td>28.5 ± 8.9</td>
<td>40.7 ± 7.6</td>
<td>11.8 ± 2.4b</td>
<td>17.0 ± 3.8b</td>
<td>21.2 ± 6.3b</td>
<td>33.0 ± 12.6b</td>
</tr>
<tr>
<td>ORG+M</td>
<td>19.9 ± 4.1a</td>
<td>21.7 ± 11.5</td>
<td>37.0 ± 14.0</td>
<td>48.3 ± 19.4</td>
<td>20.7 ± 5.8a</td>
<td>27.1 ± 1.9a</td>
<td>37.4 ± 1.7a</td>
<td>43.6 ± 7.8ab</td>
</tr>
<tr>
<td>CONV</td>
<td>22.0 ± 1.2a</td>
<td>25.3 ± 2.4</td>
<td>48.6 ± 6.0</td>
<td>61.7 ± 9.8</td>
<td>25.4 ± 4.9a</td>
<td>29.4 ± 2.7a</td>
<td>40.5 ± 2.7a</td>
<td>55.7 ± 3.3a</td>
</tr>
<tr>
<td>PRA</td>
<td>22.4 ± 2.0a</td>
<td>20.2 ± 2.8</td>
<td>43.0 ± 5.1</td>
<td>52.3 ± 11.9</td>
<td>22.5 ± 5.4a</td>
<td>26.3 ± 5.8a</td>
<td>31.7 ± 4.2a</td>
<td>44.2 ± 1.9ab</td>
</tr>
<tr>
<td>Root P uptake (mg pot⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORG</td>
<td>31.6 ± 18.4</td>
<td>22.4 ± 6.4</td>
<td>32.8 ± 10.8</td>
<td>39.7 ± 10.2</td>
<td>24.1 ± 12.0</td>
<td>20.5 ± 4.8</td>
<td>19.1 ± 3.3</td>
<td>28.5 ± 15.0</td>
</tr>
<tr>
<td>ORG+M</td>
<td>27.5 ± 8.5</td>
<td>24.9 ± 12.1</td>
<td>29.2 ± 11.8</td>
<td>34.0 ± 21.1</td>
<td>23.4 ± 3.3</td>
<td>20.2 ± 7.6</td>
<td>29.8 ± 16.4</td>
<td>30.0 ± 10.6</td>
</tr>
<tr>
<td>CONV</td>
<td>17.5 ± 10.5</td>
<td>22.2 ± 5.7</td>
<td>36.3 ± 1.8</td>
<td>50.7 ± 9.4</td>
<td>13.7 ± 2.6</td>
<td>12.7 ± 2.2</td>
<td>20.4 ± 4.6</td>
<td>26.9 ± 4.6</td>
</tr>
<tr>
<td>PRA</td>
<td>20.5 ± 5.5</td>
<td>31.1 ± 10.6</td>
<td>35.6 ± 24.5</td>
<td>51.3 ± 14.8</td>
<td>21.3 ± 9.7</td>
<td>18.8 ± 5.0</td>
<td>17.0 ± 4.3</td>
<td>29.7 ± 5.3</td>
</tr>
<tr>
<td>Plant N uptake (mg pot⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORG</td>
<td>228.0 ± 14.5b</td>
<td>416.5 ± 85.3</td>
<td>443.5 ± 61.0</td>
<td>485.5 ± 62.9</td>
<td>316.1 ± 57.9b</td>
<td>410.5 ± 79.9b</td>
<td>464.4 ± 90.6b</td>
<td>531.2 ± 62.1</td>
</tr>
<tr>
<td>ORG+M</td>
<td>487.9 ± 48.9a</td>
<td>511.7 ± 158.8</td>
<td>485.8 ± 46.1</td>
<td>516.1 ± 166.9</td>
<td>516.4 ± 87.0ab</td>
<td>525.6 ± 80.4ab</td>
<td>576.5 ± 36.4ab</td>
<td>584.7 ± 24.9</td>
</tr>
<tr>
<td>CONV</td>
<td>561.9 ± 7.7a</td>
<td>544.6 ± 27.9</td>
<td>547.4 ± 28.1</td>
<td>583.6 ± 35.6</td>
<td>561.1 ± 42.4a</td>
<td>581.0 ± 12.7a</td>
<td>592.3 ± 7.7a</td>
<td>604.4 ± 12.5</td>
</tr>
<tr>
<td>PRA</td>
<td>491.1 ± 43.5a</td>
<td>424.0 ± 65.3</td>
<td>483.9 ± 18.8</td>
<td>494.3 ± 48.4</td>
<td>471.2 ± 78.2ab</td>
<td>504.0 ± 57.1ab</td>
<td>527.3 ± 19.0ab</td>
<td>536.4 ± 3.9</td>
</tr>
</tbody>
</table>
Figure 4.2: Italian ryegrass cumulative biomass (a, c, e, g) and plant P uptake (b, d, f, h) after 106 days in soils under long-term organic (ORG), manure-amended organic (ORG+M), conventional (CONV), and prairie (PRA) management. Values are treatment means and bar represent standard deviation (n=4).
Figure 4.3: Relationship between Italian ryegrass shoot P uptake and total P added to pots (a, b) and biomass (c, d) after 106 days in soils under long-term organic (ORG), manure-amended organic (ORG+M), conventional (CONV), and prairie (PRA) management. Each point represents the treatment mean (n=4).
Figure 4.4: Apparent P use efficiency (APUE) in soils amended with manure P (low L, medium M, high H) or mineral P (15, 40, 80, 160 g kg\(^{-1}\)) calculated as APUE = \([\text{shoot P uptake}_{\text{treatment}} - \text{shoot P uptake}_{\text{control}}]/\text{total P applied}\). Values represent the treatment mean (n=4) ± standard deviation.

Despite the control (0P) pots ORG+M, CONV and PRA soils being very similar (19.9, 22.0, 22.4 mg P pot\(^{-1}\)), the plant uptake at 160 mg P kg\(^{-1}\) was higher (55.7 CONV, 43.6 ORG+M, 44.2 PRA). The shoot APUE of the manure and mineral P treatments for the ORG, ORG+M, CONV and PRA soils are displayed in Figure 4.4. Although the management and treatment showed significant differences (P<0.001), the interaction between the factors was not. The ORG and ORG+M soils with low manure, and ORG, ORG+M and CONV soils with low mineral P had the highest APUE % ranging from 41-51%. The PRA soil values were the lowest with -9% in the low manure P and the highest
4.4 DISCUSSION

The sequential P fractionation of the Glenlea Long-term Rotation trial soils demonstrated that the P concentrations were being depleted in the ORG management system in the labile and moderately labile fractions only, when compared to the CONV and PRA system. A previous study at Glenlea examining soil P pools revealed soil test P levels below the agronomic threshold in the grain-forage rotation of the ORG system (Welsh et al., 2009). The ORG soil, when compared to CONV, had lower levels of resin P and NaHCO$_3$-extractable P and moderately available NaOH extractable P, but no difference in the more recalcitrant fractions or total P after 18 years. However, it was not investigated if these soils from different management systems respond differently to various rates of mineral and manure amendments. In an experiment looking at the effect of intensive plant production on eight different soil types, Guo et al. (2000) also found that the labile P fractions were most affected, along with NaOH-Pi. They also concluded that the residual HCl extractable P became available in a relatively short period of time in the less weathered soils. Interestingly, a similar study analysing differences between conventional and organic cropping in P pools after 13 years showed differences in NaHCO$_3$-Pi but not Po (representing organic P; Oberson et al., 1993).

Although several limitations have been reported with sequential P fractionation procedures (Condron and Newman, 2011), it does provide us with information on the potential bioavailability of P in soils under different management systems. In this study, a modified Hedley fractionation was used. Since we were not looking for the particular P
species, which could be provided by $^{31}$P NMR, these operationally defined pools provided a useful indication of bioavailability. In agreement with other studies (Entz et al., 2001, Welsh et al., 2009, Knight et al., 2010), we found that reported deficiencies in soil test P are not representative of all of the P that may become available by microbial and/or chemical processes. Although abundant total P exists in the soil, replacement of P in long-term organic systems is essential for maintaining yields, considering both labile and moderately labile P pools may be depleted, especially where alfalfa biomass is continuously removed as in this rotation sequence.

The total soil C and N values of the soils before the greenhouse study were comparable to Bell et al. (2012) for these plots after 18 years in the ORG (29.7 ± 0.6; 2.59 ± 0.05), CONV (32.9 ± 0.05; 2.74 ± 0.07), and PRA soils (34.4 ± 2.1; 2.58 ± 0.09). After 106 days, the effect of management and P treatment resulted in significant differences in soil C and N, and an interaction for total N suggests that the soils responded differently to the P treatments. When adjusting the amounts of nutrients applied, we used the total amount of N in the manure sampled since the amount of plant available is difficult to estimate (Sørensen et al., 1994). Also, additional carbon is added to the manure P treated pots, changing the C:N:P ratios, which may affect nutrient cycling and availability.

The soil NaHCO$_3$-P concentrations were significantly different for both the management and treatment but no interaction was observed. The NaHCO$_3$-Pi values at harvest, after 106 days in the 0P treatment are very similar to the starting values with only the ORG+M soil increasing from 7.2 to 9.7 mg P kg$^{-1}$, even with P being removed by the plant. Presumably this may be attributed to the mineralization of the manure that
was applied in the field. Considering that 160 mg P kg\(^{-1}\) was added to the pots for the very high P treatment and the values of the total \text{NaHCO}_3-P increased only by 31.4, 35.4, 28.7 and 35.6 mg P kg\(^{-1}\) soil for the ORG, ORG+M, CONV, and PRA soils, respectively, some of the available P is being immobilised or converted to less available forms. Eghball and Power (1999) found significantly lower soil P concentrations when manure was applied according to plant P requirements and supplemented with N fertilizer, compared to N based manure applications.

It has been documented that fertilization (manure or mineral) can change the relative concentrations of inorganic and organic P pools (Hedley et al., 1982, Keller et al., 2012, Malik et al., 2012, Turner et al., 2004). While fertilizer P, applied as orthophosphate typically increases labile inorganic P, manure P also adds to the labile organic P pool. Turner et al. (2004) characterized P form in cattle manure using \(^{31}\)P NMR and reported the 79% of the extractable P was present as phosphate, accounting for 98% in the \text{NaHCO}_3 extract, which found that the P in the manure was largely available for plant uptake. In our experiment, the rates of manure P were applied to the pots with the assumption that 30% of composted manure P would taken up by the plant, but the P available in the manure was much higher considering the amount removed in shoot biomass and the \text{NaHCO}_3-Pi remaining in the soil after 106 d. In the low manure treatments, more P was available than the manure P applied indicating that other sources of soil P were also being mineralized. In the medium and high manure treatments, 48% - 78% of the P was either removed by plant biomass or detected in the harvest \text{NaHCO}_3-Pi extracts. These P supplies rates from the manure are likely higher than what would occur in the field, considering the optimum temperature and moisture conditions in the
There was a greater response of ryegrass biomass in the ORG and ORG+M soils than the CONV and PRA soils to both the manure and soluble P addition compared to the control. This was expected considering the lower levels of labile P in these systems. Shoot biomass yields for the CONV soil were significantly higher than the ORG in the control and low, medium and high KH$_2$PO$_4$ treatment. Between 80 and 160 kg P g$^{-1}$ soil, the shoot biomass did not increase more than 0.4 g in any soil except the ORG (8.8 to 10.7 g), indicating application at the higher rates does not result in a corresponding yield increase. The shoot biomass, but not root, increased significantly in the manure treatment for soils from all management systems but there was little increase in the shoot or root biomass between the 0, 15, 40 and 80 mineral P treatment. The manure addition increases organic matter in the soil and has been demonstrated to increase the pH of soils (Whalen et al., 2000).

The concept of sufficiency level of soil nutrients assumes that P addition will increase yields to a maximum level after which point the response to addition rates diminishes (Bray, 1944). In our study, the larger differences in plant P uptake than biomass, especially in the medium and high manure treatment possibly indicate the luxury uptake of P in these plants. The linear regressions of total P and shoot P uptake of resulted in $r^2$ values between 0.94 to 0.99 in the manure P treatments and 0.98 to 0.995 in the mineral P treatments, demonstrating that even an increase a linear increase in P uptake even at the high application rates. Cordovil et al. (2007) found in a pot study that the ryegrass biomass and plant P uptake both increased with increasing rates of N added as manure. Other studies have found higher plant N and P uptake in manure treated soils.
compared to mineral fertilizer (Eghball and Power, 1999). In contrast, Oberson et al. (2010) reported that ryegrass grown in the greenhouse amended with mineral P had more biomass and higher P uptake than the same rate of manure P. In designing these studies, it is challenging to predict the amount of P that will be immediately available, which would allow application at a rate comparable to a soluble mineral P fertilizer.

The range of apparent P use efficiencies in our study (-9% to 51%) was similar to other reported values for manure and mineral P treatments. The highest P use efficiencies for aboveground biomass (51% and 47%) were found in the low rate treatments of the ORG and ORG+M pots, which are likely a reflection of the lower labile P values and the efficient uptake by ryegrass plants. Eghball and Power (1999) found an APUE range of 12% in manure (applied for N recommendations) to 40% P in the mineral P treatment, as averaged over four years. They predicted that the high APUE in the mineral P treatment was likely a result of high initial labile P, which limited the number of sorption sites and therefore less adsorption. This contradicts what we found in soil from the PRA management where the soils had the highest NaHCO$_3$-P, but the lowest values of APUE. The low rate of manure P resulted in a negative value suggesting that not enough P was added, since it was not significantly different from the control treatment, or possible immobilization by microorganisms or complexation reactions with organic matter.

4.5 CONCLUSIONS

Long-term management significantly affected the plant response to the different types and rates of P amendments. The initial characterization of the soils involving a
sequential P fractionation indicated that the levels of easily extractable P were depleted in the ORG soil, compared to the CONV and PRA soils. When each of the soils were amended with mineral or manure P and planted to ryegrass for 106 days in the greenhouse, the results indicated that the manure treatments resulted in higher plant P uptake. The P uptake in the mineral P treatments resulted in a more linear trend, with plants in the PRA soil having very similar P uptake to the ORG+M soil, despite the much higher initial NaHCO₃-P values. Although available P was lower in the ORG soil at the initiation of the greenhouse trial, there was no significant response of management by P treatment for NaHCO₃-P. Overall, the interaction of long-term management with the P treatment did not have a significant effect on the apparent P recovery of ryegrass in the greenhouse trial, suggesting no differential response of soils to form of P supplied as affected by framing system.
CHAPTER 5
GENERAL CONCLUSIONS

This thesis examined the contribution of \textit{phoD} gene abundance and expression to potential ALP activity, and soil P dynamics in a long-term management trial. Both the field study and the greenhouse experiment demonstrated that ALP activity and \textit{phoD} gene abundance were higher in the ORG managed soils. Since the composted manure application in the field study in Chapter 2 to the ORG+M soil resulted in a significant increase in ALP activity compared to the ORG soil with no manure, it was expected that the manure P treatments in the greenhouse would also result in higher ALP activity. Interestingly, the ALP activity in the soils from the ORG soil reacted similarly regardless of the rate of manure and mineral P treatment after 30 days of ryegrass growth. The ALP in the CONV and PRA soils increased with higher rates of manure P at 30 days and continued to increase until the harvest sampling. The ALP activity in the mineral P treatments of these soils increased very little at 30 days and then increased at the same rate as the control pots at 106 days.

The few studies that have been published on ALP gene harbouring bacteria in soil have attempted to link the ALP activity to shifts in the bacterial \textit{phoD} microbial community and changes in P availability (Sakurai et al., 2008, Tan et al., 2013). There have been no other studies published that considered the \textit{phoD} gene abundance using quantitative PCR. In Chapter 2 of this thesis, there were obvious differences between management systems in the \textit{phoD} microbial community profiles based on DGGE analysis. Although this technique does not show fine-scale differences or provide
information on species, the profiles did indicate fewer species present in the ORG plots despite having significantly higher ALP activity. The *phoD* gene abundance did, however, show a significant correlation with ALP activity across soils for all the management systems indicating that this may be a better indicator of activity than shifts in microbial community composition.

Not only can ALP activity influence P availability in the soil, low levels of phosphate may induce ALP activity. There have been contradictory reports on the effect of phosphate levels on the expression of genes within the pho regulon and phosphatase activity. Corresponding with the negative correlation between ALP and available P in Chapters 2 and 3, Juma and Tabatabai (1978), Nannipieri et al. (1978), Olander and Vitousek (2000) Zhang et al. (2012), Tan et al. (2013) all saw similar trends, while other studies have shown no effect of phosphate concentrations on phosphatase activity (Schneider et al., 2001).

Since a negative correlation was observed between the NaHCO$_3$-extractable organic P and both ALP activity and *phoD* gene abundance in the field, it was expected that the applied manure, and especially the mineral P amendment would suppress the *phoD* gene expression in the greenhouse trial. However, the transcript abundance was not correlated to other measured variables and it was not significantly higher in the ORG soil, as was demonstrated by the ALP activity and *phoD* gene abundance. Waldrip et al. (2011) suggested that phosphatase activity is only coupled with phosphate when concentrations are at very high or very deficient levels, which could be the case in the greenhouse experiment. We were able to confirm expression of the *phoD* gene in all soils, thus indicating that the gene abundance is a better indicator of the potential ALP
activity. The challenges faced in optimization of the quantitative PCR for the RNA (cDNA) samples substantiate the need for the ALPS primers to be redesigned. This should be considered a priority before subsequence studies are conducted on the *phoD* gene, especially if gene expression is to be included in the analysis.

In interpreting the results from the ALP assays in Chapters 2 and 3, it is important to consider the limitations to the analysis. The most important consideration is that the phosphatase activity is a measure of the potential, not actual activity (Nannipieri, 2011). The conditions under which the assays are conducted at optimal moisture and temperature are very different from the field conditions. Especially in the Northern Great Plains of Canada where there are large temperature fluctuations, and moisture may be a limiting factor to both microbial and plant growth. In addition, these assays were conducted at a pH of 11.0 to eliminate any possibility of acid phosphatase enzymes being measured which is many magnitudes higher than the neutral pH found in the soil from the Glenlea field experiment. Although these are important when considering the absolute quantities of phosphatase activity, for the results reported in Chapters 2 and 3 all of the samples were treated consistently and therefore the differences in the relatively quantities showed significant differences in activity in the soils from the different management systems with the ORG soil consistently being higher.

The other important consideration is that the assays do not give us any information on whether the enzyme is currently being excreted. The enzymes could be stabilized on microbial cells, dead cells, spores, stabilized on reactive particles such as clay, iron oxides or hydroxides or be free enzymes, although these are probably fairly insignificant (Burns, 1982, Nannipieri, 2011). However, in this study the results from the
phoD gene abundance in both the field and the greenhouse study were significantly correlated with the ALP activity and consistently higher in the ORG soil, compared to the CONV and PRA soils. However, the phosphatase activity measured in the assays may include stabilized enzymes, or enzymes being excreted by plant roots or fungi, since there is no way to differentiate between the sources based on the current method. Even if bacteria excreted the phosphatases, they may be produced by the phoA, phoD or phoX, of which we measured only the phoD for gene abundance and expression. The phoD gene was chosen since it was demonstrated to be the most frequent ALP gene present in metagenomic databases of soil bacteria (Tan et al., 2013).

It is difficult to directly link enzyme activity and microbial functioning to the changes in plant nutrient uptake. In Chapters 2 and 3, we did see higher ALP and phoD gene abundance in ORG, which corresponded with higher apparent P use efficiency in the low manure P and mineral P treatments in Chapter 4. Although ALP activity and phoD gene abundance were significantly higher in soil under ORG management, there was no significant increase in the available P in the ORG soil compared to the CONV and PRA soils. There were significant differences between both treatment and soil for all of the measured soil and plant factors in Chapter 4, including the shoot and root P uptake in the greenhouse trial. Tarafdar and Jungk (1987) did report a significant correlation between the depletion of organic P and phosphatase activity in the rhizosphere of clover (r=0.97, P<0.01) and wheat (r=0.97, P<0.001) when measured within 0.8 mm and 1.5 mm in clover and wheat, respectively. Within this zone of close proximity to the root, it was observed that the phosphatases (likely produced by the root) were responsible for the reduction in the organic P, but that this decreased as distance from the root zone
increased.

The strong correlation between potential ALK activity and \textit{phoD} gene abundance, indicates that there was a bacterial contribution of alkaline phosphatase in the soils from both the field and greenhouse study. Biological conversion of labile organic P into orthophosphate in soil solution is an important process in the phosphorus cycle, including bacterial alkaline phosphatase production (Figure 5.1). Although other genes may also be contributing to this process, the presence of \textit{phoD} transcripts was confirmed in all soils from this greenhouse study. Future research including other phosphatase producing genes, as well as associations across trophic levels, will add to this knowledge of the biological cycling of phosphorus.

![Figure 5.1: Schematic diagram of phosphorus fractions in the P cycle as measured by the Hedley sequential fractionation method. The bacterial \textit{phoD} gene contributes to the conversion of labile organic P into orthophosphate in soil solution. \textit{Pi} represents molybdate reactive P and \textit{Po} represents molybdate unreactive P. Modified from Chauhan et al. 1981.](image-url)
Identifying a correlation between plant-excreted phosphatases and nutrient uptake may be more relevant than with microbial excreted enzymes, since the P becomes available directly in the root zone. In Chapters 2 and 3, we sampled bulk soil, not just rhizosphere, meaning that increases in P availability may have been more accessible to other microbes than to the plants themselves. Eventually, the turnover of this microbial P would also be available for plant update. The methods that were used in this study to determine P availability were not sensitive enough to determine changes in P speciation due to enzyme hydrolysis.

The studies in the thesis provided insight into the relationship between NaHCO₃-P, ALP activity and \textit{phoD} gene abundance. The differences between variables measured from the ORG, CONV and PRA management systems, substantiate the need to maintain long-term management trials for monitoring the effects of anthropogenic activities.
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