Geoderma 257–258 (2015) 115–122

Contents lists available at ScienceDirect

Geoderma

journal homepage: www.elsevier.com/locate/geoderma

Linking alkaline phosphatase activity with bacterial *phoD* gene abundance in soil from a long-term management trial

Tandra Fraser [a], Derek H. Lynch [b], Martin H. Entz [c], Kari E. Dunfield [a,*]

[a] School of Environmental Sciences, University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada
[b] Department of Plant and Animal Sciences, Dalhousie University, Agriculture Campus, Truro, Nova Scotia B2N 5E3, Canada
[c] Department of Plant Science, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada

ARTICLE INFO

Article history:
Received 7 July 2014
Received in revised form 16 October 2014
Accepted 21 October 2014
Available online 30 October 2014

Keywords:
Bioavailable P
Enzyme activity
Pho regulon
Soil microbial communities
Organic agriculture

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 which catalyze the hydrolysis of ester-phosphate bonds. Examining the diversity and abundance of alkaline phosphatase gene harboring bacteria may provide valuable insight into alkaline phosphatase production in soils. This study examined the effect of 20 years of no input organic (ORG), organic with composted manure (ORG + M), conventional (CONV) and restored prairie (PRA) management on soil P bioavailability, alkaline phosphatase activity (ALP), and abundance and diversity of ALP gene (*phoD*) harboring 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 ($r^2 = 0.71$; $P = 0.03$) and 2012 ($r^2 = 0.51$; $P = 0.02$), 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 ($r^2 = 0.71$; $P = 0.009$). 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 is 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.

1. 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 and Kwon, 2000; Guo and Gifford, 2002; Lauber et al., 2008; Osborne et al., 2011). Phosphorus (P) 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, phosphorus 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 ($H_2PO_4^{2-}$ or $H_2PO_4^-$) in soil solution. Phosphate fertilizer is routinely applied to crops above plant growth requirements. This over-application of P creates a serious environmental concern as a major contributor to eutrophication when mobilized and transferred into waterways (Sharpley et al., 1992; Withers and Haygarth, 2007). 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 phosphorus reserves while decreasing contamination of waterways.

In contrast to possible over-application in conventional agriculture, low plant available phosphorus (easily extractable) has been reported on organic farms across Canada (Entz et al., 2001; Martin et al., 2007; Knight et al., 2010; Roberts et al., 2008; 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

* Corresponding author.
E-mail address: dunfield@uoguelph.ca (K.E. Dunfield).

http://dx.doi.org/10.1016/j.geoderma.2014.10.016
0016-7061/

available under organic certification (Woodley et al., 2014). We hypothesize that in these systems the turnover of organic P by microorganisms is essential for requirements and maintaining long-term productivity (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. 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; Tarafdar and Jungk, 1987). Alkaline (EC 3.1.3.1) and acid (EC 3.1.3.2) phosphatases are phosphomonoesterases with a wide substrate specificity capable of hydrolyzing 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; 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 alkaline phosphatase: *phoA* (Bradshaw et al., 1981; Hulett et al., 1990, 1991; Ray et al., 1991; Chang et al., 1986; Zappa et al., 2001), *phoD* (Gomez and Ingram, 1995) and *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 characterized in *Escherichia coli* by Bradshaw et al. (1981). Produced by *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 *phoX* was more widely distributed among marine bacteria, being induced solely upon P starvation (Sebastian and Ammerman, 2009). Differing from *phoA*, monodimers *phoD* and *phoX* are dependent upon Ca^{2+} as a cofactor (Yamane and Maruo, 1978; Wu et al., 2007). In soil bacteria *phoD* was the most frequent ALP gene present in metagenomic datasets for 16S rRNA, although *phoA* and *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 *phoD* communities in response to organic matter (Sakurai et al., 2008) or chemical P fertilization rates (Sakurai et al., 2008; Tan et al., 2013). Shifts in *phoD* bacterial communities coincided with changes in ALP activity (Sakurai et al., 2008) but no studies have quantified *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, where an alfalfa–crop rotation under organic or conventional management, and restored native perennial grassland, has been maintained for the past 20 years. Studies at our site have indicated a depletion of easily extractable P under organic management (Welsh et al., 2009; 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 structure are driving the turnover of organic P in these systems. The objective of this study was to examine soil P bioavailability, alkaline phosphatase activity (ALP), and abundance and diversity of *phoD* bacteria in this system. Using this approach, we were able to evaluate the capability of the *phoD* gene abundance to be used as an indicator of enzyme function.

2. Methods

2.1. 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 Scanterbury and Hoddinott series with 9% sand, 26% silt and 66% clay, an average pH_{H_2O} of 7.4 and 7.7% organic matter content (Bell et al., 2012; 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 seasons (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 of 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. Although the rotations have changed over the years, since 2004 the plots 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 region with large distances and limited amendment options under organic certification. In 2007, the organic plots were split and conventional 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^{-1} N ha^{-1} and P applied at 20–25 kg P ha^{-1} annually at seeding (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 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, and *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.

2.2. Soil sampling and analysis

Soil samples were collected from the wheat phase of the forage–grain rotation in July 2011 and 2012 to correspond with the flag-leaf growth stage, or 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 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 h. The remaining sample was air dried and finely ground prior to chemical analysis. All results were adjusted to oven-dry weight equivalents.

2.3. Soil chemical properties

Bioavailable (Olsen) P was determined on soil in a 1:20 ratio with 0.5 M $NaHCO_3$ (pH 8.5) and shaken for 30 min (Olsen et al., 1954) in duplicate. Extracts were acidified with 3 M H_2SO_4 to remove carbonates 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). Although this reactive P fraction may include small amounts of organic P, we predict these amounts to be negligible and will refer to reactive P as inorganic P for simplicity. Total P (TP) concentration in the soil was determined in duplicate by igniting samples at 550 °C for 4 h and extracted by shaking for 16 h with 0.5 M H_2SO_4 (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 H_2SO_3 to remove carbonates and analyzed on a LECO CNS Analyzer (LECO Corporation, Michigan, USA).

2.4. Phosphatase activity assay

Potential soil ALP activity was measured within 72 h 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 h, reactions were stopped with 0.5 M NaOH, filtered with Whatman 42 paper and the formation of *p*-nitrophenol determined colorimetrically using a spectrophotometer at 420 nm.

2.5. DNA extraction

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

2.6. PCR amplification and denaturing gradient gel electrophoresis (DGGE)

A fragment of the bacterial *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′-CGCCCGCCGCGCCCCGCGC CCGTCCCGCCGCCCCCGCCCG-3′) was attached to the reverse primer to prevent complete denaturation during electrophoresis. The PCR reactions were prepared with 2.5 μL of 10× 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_2O.

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 analyzed by denaturing gradient gel electrophoresis (DGGE) to profile the structure 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 80 V for 15 h. 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).

2.7. 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 10 μmol 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 Geulen, 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.

2.8. Statistical analysis

Statistical analysis for response variables was 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. 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). These coordinates were used as a starting point for a second run. 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.

3. Results

3.1. 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 (Table 1). 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 1) with significant differences between both management system ($P < 0.001$) and sampling year ($P < 0.001$; Table 2). The ORG + M plots showed increased Olsen P values of 2.3 mg P kg^{-1}

Table 1
ANOVA table for soil properties and processes.

ANOVA Source of variation	Soil properties					
	Olsen P (mg kg^{-1})	Total P (mg kg^{-1})	Organic C (g kg^{-1})	Total N (g kg^{-1})	ALP	*phoD*
Year (Y)	***[a]	ns	ns	***	***	***
Management (M)	***	*	ns	ns	***	***
Y × M	ns	ns	ns	ns	ns	ns

ns: not significant.
[a] *, **, ***: significant at 0.05, .01 and 0.001 probability, respectively.

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 increased 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.

3.2. 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 (Fig. 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 3.0×10^6 (PRA) to 8.2×10^6 (ORG + M) and 6×10^6 (CONV) to 1.3×10^7 (ORG + M) per g of dry soil in 2011 and 2012, respectively (Fig. 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 (Fig. 1b). In 2012, there was large variability between replicates for the ORG and ORG + M treatments, in particular.

3.3. 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 (Fig. 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 ($P = 0.009$; Fig. 3). No significant correlation between *phoD* abundance and Olsen P or total P was observed (data not shown).

3.4. 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 (Fig. 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 was 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

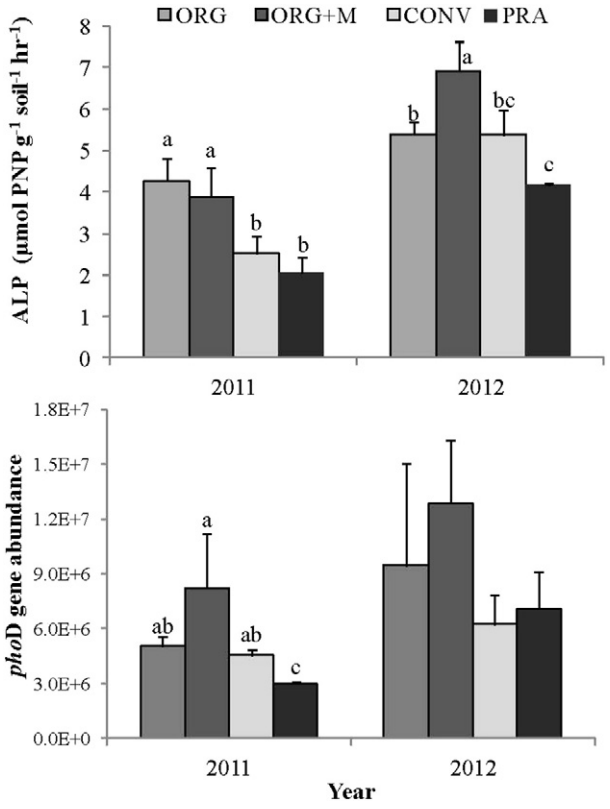

Fig. 1. Effect of management system on a) potential alkaline phosphatase activity (ALP) and b) bacterial *pho*D gene abundance in soil samples collected from the wheat phase of the forage–grain rotation in July 2011 and 2012. Abundance is expressed per g dry soil. Values are treatment means with bars 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.

Table 2
Soil nutrient concentration in soil samples collected from the wheat phase of the forage–grain rotation in July 2011 and 2012.

Year	Management	Soil properties			
		Olsen P (mg kg^{-1})	Total P (mg kg^{-1})	Organic C (g kg^{-1})	Total N (g kg^{-1})
2011	ORG	5.4 ± 0.7c[a]	633.5 ± 53.8	32.6 ± 4.5	2.3 ± 0.3
	ORG + M	7.7 ± 0.3bc	630.1 ± 7.5	34.2 ± 3.0	2.3 ± 0.4
	CONV	9.3 ± 1.2b	565.6 ± 24.6	34.3 ± 0.3	2.2 ± 0.1
	PRA	22.2 ± 1.8a	655.6 ± 55.7	34.4 ± 4.1	2.1 ± 0.4
2012	ORG	7.6 ± 1.0c	647.8 ± 31.0b	35.6 ± 3.3	2.6 ± 0.1
	ORG + M	12.0 ± 2.6bc	704.6 ± 8.1a	34.5 ± 2.8	3.0 ± 0.1
	CONV	19.3 ± 4.4ab	649.6 ± 21.0ab	36.7 ± 0.5	2.7 ± 0.1
	PRA	29.6 ± 6.6a	666.8 ± 25.4ab	39.2 ± 1.4	2.7 ± 0.3

[a] 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.

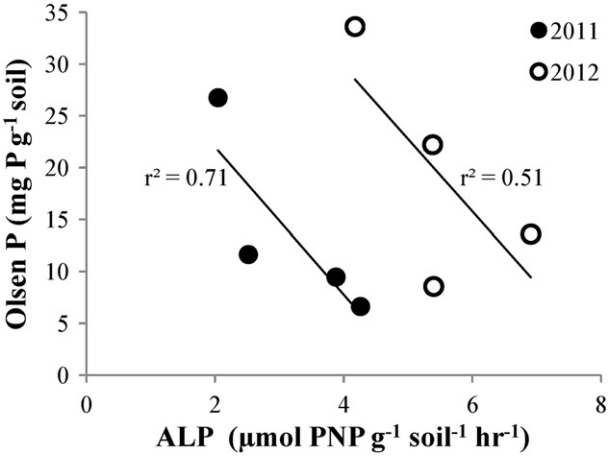

Fig. 2. Relationship between Olsen P and potential alkaline phosphatase activity in 2011 and 2012. Values are treatment means (n = 3).

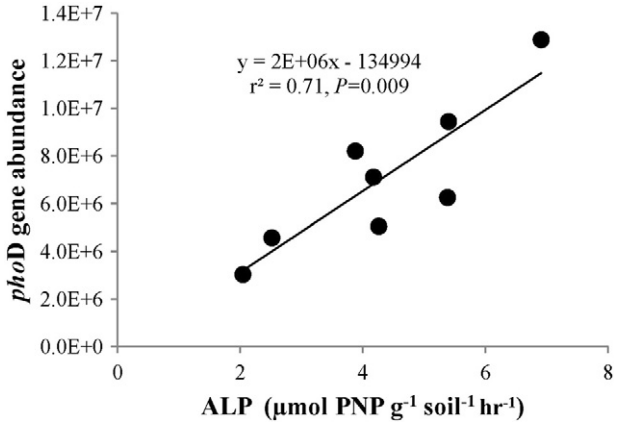

Fig. 3. Relationship between soil bacterial *phoD* gene abundance and potential alkaline phosphatase activity (ALP) in 2011 and 2012. Values are treatment means (n = 3).

and among the management systems suggest differences in bacterial *phoD* community structure from that sampling location.

Communities of *phoD* bacteria were affected by management systems. Ordination by nMDS based on DGGE profiles revealed similar variations in both years (Fig. 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 (Fig. 5a). In 2012, the ordination by nMDS reveals separation of *phoD* communities by management (Fig. 5b). There was a clear shift in the ORG + M towards the CONV resulting from the prior fall manure application.

4. Discussion

Soil chemical properties in our study indicated that management system had a significant effect on Olsen P in both years and total P only after a second manure application, while none was observed for C and N. Many studies have demonstrated the conversion from native grasslands to annual cropping systems deplete bioavailable P (Hedley et al., 1982; Tiessen et al., 1984; Sharpley and Smith, 1985; Motavalli and Miles, 2002), as also indicated in our study with lower concentrations in the agricultural systems. The 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; 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 change in Olsen P values between 2004 in the ORG plots (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 by an extensive dry period preceding sampling, totalling only 10 mm of precipitation in July. During the growing season from May to August there was only 147 mm of precipitation in 2011 compared to 224 mm in 2012, with little interannual 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 have been found to create large increases in water-extractable P (Turner and Haygarth, 2001; Styles and Coxon, 2006; 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 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 was significantly higher than the CONV and PRA soils. 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; Hamel et al., 2006).

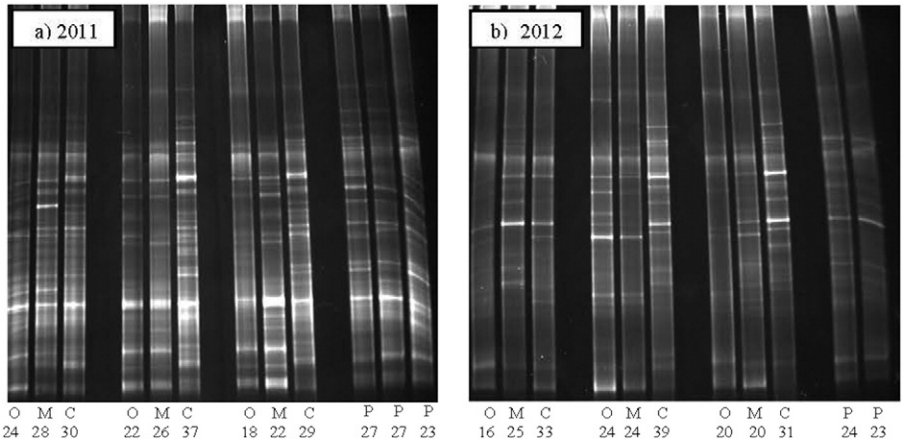

Fig. 4. DGGE analysis of *phoD* bacterial population in a) 2011 and b) 2012. Each lane represents a sample from organic (O), manure-amended organic (M), conventional (C) and prairie (P) management systems with each line representing a different species of bacteria. The number below each lane represents the number of bands identified in the sample.

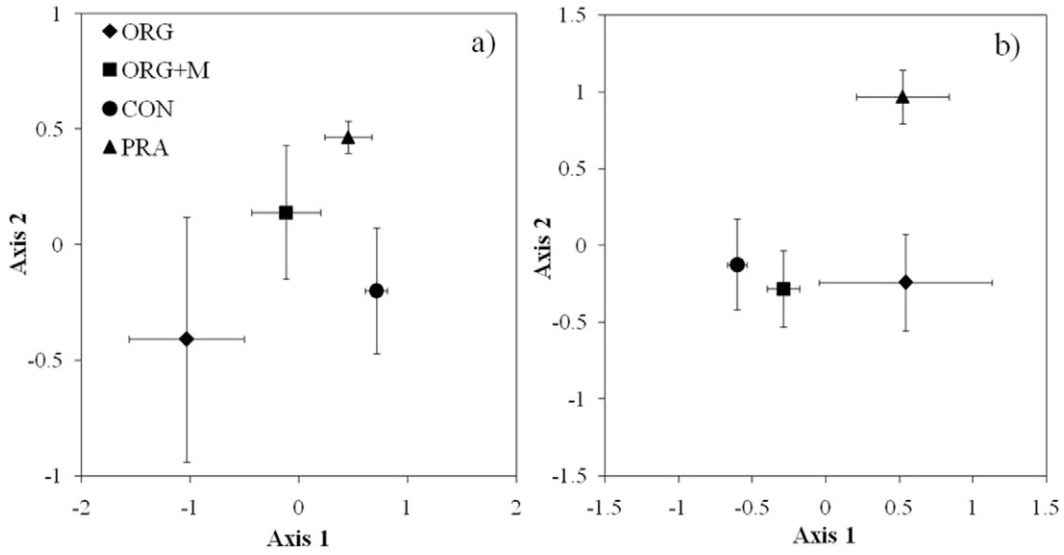

Fig. 5. NMS ordination of DGGE profiles for *phoD* amplicons from soil samples collected in a) 2011 and b) 2012 from plots under organic (♦), manure-amended organic (■), conventional (●) and prairie (▲) management. The data points represent treatment means (n = 3) with error bars indicating the standard errors of the means.

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 4th 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, is contributing to phosphorus 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; 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 that 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; Parham et al., 2003) often demonstrating a greater effect on the total microbial community than management system (Esperschütz et al., 2007; 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 *phoD* gene abundance, and more bands present in the community fingerprints representing *phoD* species. One explanation is the possibility that soil C is more accessible to microbes under CONV compared to ORG management (Birkhofer et al., 2008). Tan et al. (2013) reported higher richness and Shannon–Wiener diversity indices for 16S rRNA and *phoD* communities in pastures receiving high rates of P fertilization after 42 years, compared to a no-P soil. We observed a decrease in the number of *phoD* bands after a second 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; Chaudhry et al., 2012).

Shifts in *phoD* bacterial communities have been significantly correlated with ALP and fertilization management (Sakurai et al., 2008; Chhabra et al., 2013). Our results from the community fingerprinting of *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 shifted closer to CONV after the second manure application. Differences in bacterial diversity important in other aspects of P-solubilization have also been reported. Mander et al. (2012) demonstrated that bioavailable soil P levels significantly affected the frequency that this P-solubilization 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., *P. 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 *phoD* community structure, 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 *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; Malhi et al., 2006) and our sampling time was chosen to correspond with peak P uptake in wheat plants.

In conclusion, this study demonstrates that long-term management may impact P cycling by differences in the abundance and diversity of *phoD* bacterial communities. Even low rates of compost manure had a significant effect on potential ALP activity and *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 which 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. 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.

Acknowledgments

We are indebted to Dr. Bernhard Benkel for technical advice and access to the Atlantic Research Centre for Agricultural Genomics. We would like to thank Dr. Paul Voroney for analyzing soils for TOC, as well as Kamini Khosla and Katherine Rutherford for technical help in the laboratory. Funding was provided by the Canadian Agri-Science Clusters Initiative, Growing Forward of Agriculture and Agri-Food Canada and the Canada Research Chairs Program.

References

Ajwa, H.A., Dell, C.J., Rice, C.W., 1999. Changes in enzyme activities and microbial biomass of tallgrass prairie soil as related to burning and nitrogen fertilization. Soil Biol. Biochem. 31, 769–777.

Apel, A.K., Sola-Landa, A., Rodríquez-García, A., Martin, J.F., 2007. Phosphate control of *phoA*, *phoC* and *phoD* gene expression in *Streptomyces coelicolor* reveals significant differences in binding of *PhoP* to their promoter regions. Microbiology 153, 3527–3537.

Bardgett, R.D., Lovell, R.D., Hobbs, P.J., Jarvis, S.C., 1999. Seasonal changes in soil microbial communities along a fertility gradient of temperate grasslands. Soil Biol. Biochem. 31, 1021–1030.

Bell, L.W., Sparling, B., Tenuta, M., Entz, M.H., 2012. Soil profile carbon and nutrient stocks under long-term conventional and organic crop and alfalfa–crop rotations and re-established grassland. Agric. Ecosyst. Environ. 158, 156–163.

Birkhofer, K., Martijn Bezemer, T., Bloem, J., Bonkowski, M., Christensen, S., Dubois, D., Ekelund, F., Fließbach, A., Gunst, L., Hedlund, K., Mäder, P., Mikola, J., Robin, C., Setälä, H., Tatin-Froux, F., Van der Putten, W.H., Scheu, S., 2008. Long-term organic farming fosters below and aboveground biota: implications for soil quality, biological control and productivity. Soil Biol. Biochem. 40, 2297–2308.

Bissett, A., Richardson, A.E., Baker, G., Kirkegaard, J., Thrall, P.H., 2013. Bacterial community response to tillage and nutrient additions in a long-term wheat cropping experiment. Soil Biol. Biochem. 58, 281–292.

Bradshaw, R.A., Cancedda, F., Ericsson, L.H., Neumann, P.A., Piccoli, S.P., Schlesinger, M.J., Shriefer, K., Walsh, K.A., 1981. Amino acid sequence of *Escherichia coli* alkaline phosphatase. Proc. Natl. Acad. Sci. U. S. A. 78, 3473–3477.

Bünemann, E.K., Keller, F., Hoop, D., Jud, K., Boivin, P., Frossard, E., 2013. Increased availability of phosphorus after drying and rewetting of a grassland soil: processes and plant use. Plant Soil 370, 511–526.

Burns, R.G., 1982. Enzyme activity in soil — location and a possible role in microbial ecology. Soil Biol. Biochem. 14, 423–427.

Chang, C.N., Kuang, W.J., Chen, E.Y., 1986. Nucleotide sequence of the alkaline phosphatase gene of *Escherichia coli*. Gene 44, 121–125.

Chaudhry, V., Rehman, A., Mishra, A., Chauhan, P.S., Nautiyal, C.S., 2012. Changes in bacterial community structure of agricultural land due to long-term organic and chemical amendments. Microb. Ecol. 64, 450–460.

Chhabra, S., Brazil, D., Morrissey, J., Burke, J., O'Gara, F., Dowling, D.N., 2013. Fertilization management affects the alkaline phosphatase bacterial community in barley rhizosphere soil. Biol. Fertil. Soils 49, 31–39.

Dick, R.P., Rasmussen, P.E., Kerle, E.A., 1988. Influence of long-term residue management on soil enzyme activities in relation to soil chemical properties of a wheat–fallow system. Soil Biol. Biochem. 6, 159–164.

Entz, M.H., Guilford, R., Gulden, R., 2001. Crop yield and soil nutrient status on 14 organic farms in the eastern portion of the northern Great Plains. Can. J. Plant Sci. 81, 351–354.

Esperschütz, J., Gattinger, A., Mäder, P., Schloter, M., Fließbach, A., 2007. Response of soil microbial biomass and community structures to conventional and organic farming systems under identical crop rotations. FEMS Microbiol. Ecol. 61, 26–37.
Frankenberger, W.T., Dick, W.A., 1983. Relationships between enzyme activities and microbial growth and activity indices in soil. Soil Sci. Soc. Am. J. 47, 945–951.
Gomez, P.F., Ingram, L.O., 1995. Cloning, sequencing and characterization of the alkaline phosphatase gene (phoD) from *Zymomonas mobilis*. FEMS Microbiol. Lett. 125, 237–245.
Guo, L.B., Gifford, R.M., 2002. Soil carbon stocks and land use change: a meta analysis. Glob. Chang. Biol. 8, 345–360.
Hamel, C., Hanson, K., Selles, F., Cruz, A.F., Lemke, R., McConkey, B., Zentner, R., 2006. Seasonal and long-term resource-related variations in soil microbial communities in wheat-based rotations of the Canadian prairie. Soil Biol. Biochem. 38, 2104–2116.
Hammond, J.P., White, P.J., 2008. Sucrose transport in the phloem: integrating root responses to phosphorus starvation. J. Exp. Bot. 59, 93–109.
Hedley, M.J., Stewart, W.B., Chauhan, B.S., 1982. Changes in inorganic and organic soil phosphorus fractions induced by cultivation practices and by laboratory incubations. Soil Sci. Soc. Am. J. 46, 970–976.
Hulett, F.M., Bookstein, C., Jensen, K., 1990. Evidence for two structural genes for alkaline phosphatase in *Bacillus subtilis*. J. Bacteriol. 172, 735–740.
Hulett, F.M., Kim, E.E., Bookstein, C., Kapp, N.V., Edwards, C.W., Wyckoff, H.W., 1991. *Bacillus subtilis* alkaline phosphatases III and IV. Cloning, sequencing, and comparisons of deduced amino acid sequence with *Escherichia coli* alkaline phosphatase three dimensional structure. J. Biol. Chem. 266, 1077–1084.
Knight, J.D., Buhler, R., Leeson, J.L., Shirtliffe, S., 2010. Classification and fertility status of organically managed fields across Saskatchewan, Canada. Can. J. Soil Sci. 90, 667–678.
Lauber, C.L., Strickland, M.S., Bradford, M.A., Fierer, N., 2008. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biol. Biochem. 40, 2407–2415.
Li, R., Khafipour, E., Krause, D.O., Entz, M.H., de Kievit, T.R., Fernando, W.G.D., 2012. Pyrosequencing reveals the influence of organic and conventional farming systems on bacterial communities. PLoS One 7 (12), e51897. http://dx.doi.org/10.1371/journal.pone.0051897.
Lynch, D.H., 2014. Sustaining soil organic carbon, soil quality and soil health in organic field crop management systems. In: Martin, R.C., MacRae, R. (Eds.), Managing Energy, Nutr. and Pests in Org. Field Crops. CRC Press, pp. 107–132.
Main, M., Lynch, D.H., Voroney, R.P., Juurlink, S., 2013. Soil phosphorus effects on forage harvested and nitrogen fixation on Canadian organic dairy farms. Agron. J. 105, 1–9.
Malhi, S.S., Johnston, A.M., Schoenau, J.J., Wang, Z.H., Vera, C.L., 2006. Seasonal biomass accumulation and nutrient uptake of wheat, barley and oat on a Black Chernozem soil in Saskatchewan. Can. J. Plant Sci. 86, 1005–1014.
Mander, C., Wakelin, S., Young, S., Condron, L., O'Callaghan, M., 2012. Incidence and diversity of phosphate-solubilising bacteria are linked to phosphorus status in grassland soils. Soil Biol. Biochem. 42, 1425–1436.
Martin, R.C., Lynch, D.H., Frick, B., van Straaten, P., 2007. Phosphorus status on Canadian organic farms. J. Sci. Food Agric. 87, 2737–2740.
McCune, B., Grace, J.B., 2002. Analysis of Ecological Communities. MjM Software Design, Gleneden Beach, OR, US.
Miller, R.O., Jacobsen, J.S., Skogley, E.O., 1994. Aerial accumulation and partitioning of nutrients by hard red spring wheat. Commun. Soil Sci. Plant Anal. 25, 1891–1911.
Motavalli, P.P., Miles, R.J., 2002. Soil phosphorus fractions after 111 years of animal manure and fertilizer applications. Biol. Fertil. Soils 36, 35–42.
Murphy, J., Riley, I.P., 1962. A modified single solution method for the determination of phosphate in natural waters. Anal. Chim. Acta. 27, 31–36.
Nannipieri, P., Giagnoni, L., Landi, L., Renella, G., 2011. Role of phosphatase enzymes in soil. In: Bünemann, E.K., et al. (Eds.), Phosphorus in ActionSoil Biology 26. Springer-Verlag, Berlin Heidelberg, pp. 215–243.
Németh, D.D., Wagner-Riddle, C., Dunfield, K.E., 2014. Abundance and gene expression in nitrifier and denitrifier communities associated with a field scale spring thaw N_2O flux event. Soil Biol. Biochem. 73, 1–9.
Olsen, S.R., Cole, C.V., Watanbe, F.S., Dean, L.A., 1954. Estimation of Available Phosphorus in Soils by Extraction With Sodium Bicarbonate. Circ No 939. USDA, Washington D.C.
Osborne, C.A., Zwart, A.B., Broadhurst, L.M., Young, A.G., Richardson, A.E., 2011. The influence of sampling strategies and spatial variation on the detected soil bacterial communities under three different land-use types. FEMS Microbiol. Ecol. 78, 70–79.
Papatheodorou, E.M., Argyropoulou, M.D., Stamou, G.P., 2004. The effects of large- and small-scale differences in soil temperature and moisture on bacterial functional diversity and the community of bacterivorous nematodes. Appl. Soil Ecol. 25, 37–49.
Parham, J.A., Deng, S.P., Da, H.N., Sun, H.Y., Raun, W.R., 2003. Long-term cattle manure application in soil. II. Effect of soil microbial populations and community structure. Biol. Fertil. Soils 38, 209–215.
Post, W.M., Kwon, K.C., 2000. Soil carbon sequestration and land-use change: processes and potential. Glob. Chang. Biol. 6, 317–327.
Ray, J.M., Bhaya, D., Block, M.A., Grossman, A.R., 1991. Isolation, transcription, and inactivation of the gene for an atypical alkaline phosphatase of *Synechococcus* sp. strain PCC 7942. J. Bacteriol. 173, 4297–4309.
Roberts, C.J., Lynch, D.H., Voroney, R.P., Martin, R.C., Juurlink, S.D., 2008. Nutrient budgets of Ontario organic dairy farms. Can. J. Soil Sci. 88, 107–114.
Ross, D.J., Tate, K.R., Scott, N.A., Feltham, C.W., 1999. Land-use change: effects of soil carbon, nitrogen and phosphorus pools and fluxes in three adjacent ecosystems. Soil Biol. Biochem. 31, 808–813.
Russelle, M.P., Entz, M.H., Franzluebbers, A.J., 2007. Reconsidering integrated crop–livestock systems in North America. Agron. J. 99, 325–334.
Saha, S., Prakash, V., Kundu, S., Kumar, N., Lal Mina, B., 2008. Soil enzymatic activity as affected by long term application of farm yard manure and mineral fertilizer under rainfed soybean-wheat system in N-W Himalaya. Eur. J. Soil Biol. 44, 309–315.
Sakurai, M., Wasaki, J., Tomizawa, Y., Shinano, T., Osaki, M., 2008. Analysis of bacterial communities on alkaline phosphatase genes in soil supplied with organic matter. Soil Sci. Plant Nutr. 54, 62–71.
Sardans, J., Peñuelas, J., Ogaya, R., 2008. Experimental drought reduced acid and alkaline phosphatase activity and increased organic extractable P in soil in a *Quercus ilex* Mediterranean forest. Eur. J. Soil Biol. 44, 509–520.
Saunders, W.M.H., Williams, E.G., 1955. Observations on the determination of total organic phosphorus in soils. J. Soil Sci. 6, 254–267.
Schindler, D.W., Hecky, R.E., McCullough, G.K., 2012. The rapid eutrophication of Lake Winnipeg: greening under global change. J. Great Lakes Res. 38, 6–13.
Sebastian, M., Ammerman, J.W., 2009. The alkaline phosphatase PhoX is more widely distributed in marine bacteria than the classical PhoA. ISME J. 3, 563–572.
Sharpley, A., 1985. Phosphorus cycling in unfertilized and fertilized agricultural soils. Soil Sci. Soc. Am. J. 49, 905–911.
Sharpley, A.N., Smith, S.J., 1985. Fractionation of inorganic and organic phosphorus in virgin and cultivated soils. Soil Sci. Soc. Am. J. 49, 127–130.
Sharpley, A.N., Smith, S.J., Jones, O.R., Berg, W.A., Coleman, G.A., 1992. The transport of bioavailable phosphorus in agricultural runoff. J. Environ. Qual. 21, 30–35.
Six, J., Elliot, E.T., Paustian, K., Doran, J.W., 1998. Aggregation and soil organic matter accumulation in cultivated and native grassland soils. Soil Sci. Soc. Am. J. 62, 1367–1377.
Smit, E., Leeflang, P., Gommans, S., van den Broek, J., van Mil, S., Wernars, K., 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Appl. Environ. Microbiol. 67, 2284–2291.
Speir, T.W., 1977. Studies on a climosequence of soils in tussock grasslands. II. Urease, phosphatase and sulfatase activities in topsoils and their relationships with other properties including plant available sulfur. N. Z. J. Sci. 20, 159–166.
Speir, T.W., Cowling, J.C., 1991. Phosphatase activities of pasture plants and soils: relationship with plant productivity and soil P fertility indices. Biol. Fertil. Soils 12, 189–194.
Styles, D., Coxon, C., 2006. Laboratory drying of organic-matter rich soils: phosphorus solubility effects, influence of soil characteristics, and consequences for environmental interpretation. Geoderma 136, 120–135.
Sun, H.Y., Deng, S.P., Raun, W.R., 2004. Bacterial community structure and diversity in a century-old manure-treated agroecosystem. Appl. Environ. Microbiol. 71, 5868–5874.
Tabatabai, M.A., Bremner, J.M., 1969. Use of *p*-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol. Biochem. 1, 301–307.
Tan, H., Matthieu, B., Mooij, M.J., Rice, O., Morrissey, J.P., Dobson, A., Griffiths, B., O'Gara, F., 2013. Long-term phosphorus fertilisation increased the diversity of the total bacterial community and the *pho*D phosphorus mineraliser group in pasture soils. Biol. Fertil. Soils 49, 661–672.
Tarafdar, J.C., Jungk, A., 1987. Phosphatase activity in the rhizosphere and its relation to the depletion of soil organic phosphorus. Biol. Fertil. Soils 3, 199–204.
Tellmann, G., Geulen, O., 2006. LightCycler® 480 Real-Time PCR System: innovative solutions for relative quantification. Gene Expr. 4, 16–18.
Tiessen, H., Stewart, J.W.B., Cole, C.V., 1984. Pathways of phosphorus transformation in soils of differing pedogenesis. Soil Sci. Soc. Am. J. 48, 853–858.
Turner, B.L., Haygarth, P.M., 2001. Biogeochemistry: phosphorus solubilisation in rewetted soils. Nature 411, 258.
Vershinina, O.A., Znamenskaya, L.V., 2002. The Pho regulons of bacteria. Microbiology 71, 497–511.
Wanner, B.L., 1996. Signal transduction in the control of phosphate-regulated genes of *Escherichia coli*. Kidney Int. 49, 964–967.
Wassenaar, L.I., Rao, Y.R., 2012. Lake Winnipeg: the forgotten great lake. J. Great Lakes Res. 38, 1–5.
Welsh, C., Tenuta, M., Flaten, D.N., Thiessen-Martens, J.R., Entz, M.H., 2009. High yielding organic crop management decreases plant-available but not recalcitrant soil phosphorus. Agron. J. 101, 1027–1035.
Withers, P.J.A., Haygarth, P.M., 2007. Agriculture, phosphorus and eutrophication: a European perspective. Soil Use Manag. 23, 1–4.
Woodley, A., Audette, Y., Fraser, T., Arcand, M., Voroney, P., Knight, D., Lynch, D.H., 2014. Nitrogen and phosphorus fertility management in organic field crop production. In: Martin, R.C., MacRae, R. (Eds.), Managing Energy, Nutrients and Pests in Organic Field Crops. CRC Press, pp. 59–106.
Wu, J.R., Shien, J.H., Shieh, H.K., Hu, C.C., Gong, S.R., Chen, L.Y., Chang, P.C., 2007. Cloning of the gene and characterization of the enzymatic properties of the monomeric alkaline phosphatase (PhoX) from *Pasteurella multocida* strain X-73. FEMS Microbiol. Lett. 267, 113–120.
Yamane, K., Maruo, B., 1978. Purification and characterization of extracellular soluble and membrane-bound insoluble alkaline phosphatases possessing phosphodiesterase activities in *Bacillus subtilis*. J. Bacteriol. 134, 100–107.
Zappa, B., Rolland, J., Flament, D., Gueguen, Y., Boudrant, J., Dietrich, J., 2001. Characterization of a highly thermostable alkaline phosphatase from the *Euryarchaeon Pyrococcus abyssi*. Appl. Environ. Microbiol. 67, 4504–4511.
Zhang, A., Chen, Z., Zhang, G., Chen, L., Wu, Z., 2012. Soil phosphorus composition determined by ^{31}P NMR spectroscopy and relative phosphatase activities influenced by land use. Eur. J. Soil Biol. 52, 73–77.
Zimmerman, A.E., Martiny, A., Allison, S.D., 2013. Microdiversity of extracellular enzyme genes among sequenced prokaryotic genomes. ISME J. 7, 1187–1199.