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

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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

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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.