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Effects of poultry manure amendment on phosphorus uptake by ryegrass, soil phosphorus fractions and phosphatase activity

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Abstract Poultry manure (PM) contains a large proportion of phosphorus (P) in mineral-associated forms that may not be readily available for plant uptake. In addition, PM application influences both chemical and biotic processes, and can affect the lability of native soil P. To investigate the effects of PM on soil P availability, we grew ryegrass (*Lolium perenne*) in greenhouse pots amended with poultry manure. Biomass was harvested at 4, 8, and 16 weeks following PM application, with soil separated into rhizosphere and bulk fractions. Soil was sequentially extracted by H_2O, 0.5 M $NaHCO_3$, 0.1 M NaOH, and 1 M HCl, and inorganic P (P_i) and enzymatically hydrolyzable organic P (P_{oe}) were quantitated. Root P concentrations were 37% higher and total P uptake 59% higher with PM application than Control. At week 16, there was 30% more labile-P_i (H_2O- plus $NaHCO_3$-P_i) in the rhizosphere with PM than in Control. Phosphodiesterase activity increased with PM application. Furthermore, acid phosphomonoesterase, alkaline phosphomonoesterase, and phosphodiesterase activities were all higher in the rhizosphere than in bulk soil at week 16 with PM, indicating that increased labile-P_i was due primarily to stimulation of soil phosphatases to mineralize NaOH-P_{oe}. Soil pH increased with PM application and plant growth, and may have promoted P availability by decreasing sorption of Al- and Fe-associated inorganic and organic phosphates. These results demonstrate that whereas PM application may initially increase NaOH and HCl-P_i, these fractions can be readily changed into labile-P and do not necessarily accumulate as stable or recalcitrant P in soil.

Keywords Ryegrass · Rhizosphere · P availability · Phosphorus · Sequential fractionation · Phosphatase activity

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Introduction

Availability of phosphorus (P) following application of animal manure can be influenced by microbial and chemical properties of the soil, manure composition, and rhizosphere processes. The specific interrelationships between these components have proven to be complex and, despite continued study, a thorough understanding of the interactions among plant roots, manure P, and P solubility has yet to be achieved. In-depth knowledge of factors affecting P availability is important to achieve optimum nutrient levels when manure is applied. Manure application has been reported to increase soil concentrations of both total and soluble P, as well as concentrations of specific P forms, including stable organic P moieties (Erich et al. 2002; Ylivainio et al. 2008; Waldrip-Dail et al. 2009). Furthermore, availability of manure P has been shown to be related to particular P forms present and how they interact with the soil matrix (Tiessen et al. 1984; Guo et al. 2000; He et al. 2004, 2006a).

As organic P forms must be mineralized via phosphatases into inorganic P prior to plant uptake, soil biological activity and enzyme production related to organic P hydrolysis will affect P cycling (Magid et al. 1996). Three

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commonly studied extracellular phosphatases involved in soil P cycling are acid phosphomonoesterase (ACP, EC 3.1.3.2), alkaline phosphomonoesterase (ALP, 3.1.3.1), and phosphodiesterase (PDE, EC 3.1.4). Acid and alkaline phosphomonoesterases are relatively non-specific and can hydrolyze a range of low molecular weight P compounds, including mononucleotides, phytate, sugar phosphates, and polyphosphates; whereas phosphodiesterase catalyzes the hydrolysis of nucleic acids and phospholipids (He and Honeycutt 2001; Turner et al. 2002). As early as 1978, Nannipieri et al. recognized that higher phosphatase activity coincided with increased microbial populations in soil under conditions of P deficiency; however, this relationship was decoupled when soil was amended with KH_2PO_4. While both root exudates and manure can stimulate phosphatase activity by providing soil microorganisms with sources of C, N, and P (Juma and Tabatabai 1977; Tarafdar and Jungk 1987), a negative-feedback mechanism partially controls phosphomonoesterase activity, and enzyme inhibition can occur when high levels of inorganic P are present (Nannipieri et al. 1978, 1990; Olander and Vitousek 2000; Colvan et al. 2001). Phosphatases can also affect environmental quality following mismanagement of manure, as P in surface runoff is related to organic P content and phosphatase activity (Yu et al. 2006).

Sequential fractionation procedures have been used to characterize lability of soil and manure P based on susceptibility to extraction by a series of chemical compounds (Negassa and Leinweber 2009; Toth et al. 2011). A fairly standardized designation is separation of extracted P into labile-P (H_2O-, resin-, and 0.5 M $NaHCO_3$-P_i and P_o), moderately labile-P (0.1 M NaOH-P_i and P_o), and stable-P (HCl-P and residual-P) fractions (Hedley et al. 1982; Cross and Schlesinger 1995; Negassa and Leinweber 2009). In addition to quantifying P lability, this method lends itself well to further separation of P into inorganic and organic forms, as well as estimation of the proportion of organic P that is susceptible to hydrolysis by incubation of the extract with an excess of P-mineralizing enzymes (He and Honeycutt 2001; He et al. 2003).

Sequential fractionation has revealed that poultry manure contains proportionally more HCl-P than other animal manures, constituting 22% to 58% of total P (Codling 2006; He et al. 2006b; Dail et al. 2007). Furthermore, much of the HCl-P of poultry manure is in organic forms, with potential for mineralization into plant-available P (He et al. 2006c, 2008; Ajiboye et al. 2007; Waldrip-Dail et al. 2009). In soil, the HCl-P fraction is considered to represent stable or slow-turnover P and generally contains only small amounts of organic P (Cross and Schlesinger 1995; He et al. 2006c; Negassa and Leinweber 2009). However, repeated applications of poultry manure can increase levels of specific HCl-P forms that are less plant available and accumulate in the soil (Sato et al. 2005). In a previous laboratory incubation study, we observed a transformation between HCl-P in poultry manure and labile P in soil (Waldrip-Dail et al. 2009), indicating that in addition to labile- and NaOH-P, HCl-P could also act as a short-term source of plant-available P.

Ryegrass species are considered to be P-efficient due to a high root/shoot ratio, with an external P requirement of only 50 mg P kg^{-1} (Fohse et al. 1988). Annual and perennial ryegrasses are often used to remediate high P soils, as plant yield and P uptake increase with increasing P application rate (Tunney and Pommel 1987; Butler et al. 2007; Szogi et al. 2010). Phosphorus solubility and phosphatase activity within the rhizosphere of ryegrass and other plant species can be affected by plant P uptake, soil pH and metal content, and in response to root processes (Lindsay 1979; Jungk 1996; Hinsinger 2001).

The primary objective of this study was to evaluate manure-induced changes in soil P distribution and phosphatase activities over time as affected by plant growth. We hypothesized that (1) less labile P forms in poultry manure would provide a source of replenishment for readily labile P following plant uptake rather than accumulate as stable P that was not plant available, (2) poultry manure and rhizosphere processes would enhance phosphatase activity, and (3) the distribution of labile- and HCl-P following PM amendment would change due to both rhizosphere processes and phosphatase activity.

Materials and methods

Soil and manure

Soil was collected from the USDA–ARS research site at Newport, Maine (0–20 cm, Bangor silt loam, coarse-loamy, mixed, frigid, Typic Haplorthod), sieved (6 mm) while still field moist, and air dried. Selected soil properties were determined by the Maine Agricultural and Forestry Experiment Station (MAFES) Soil Testing Laboratory using standard methods (Gee and Bauder 1986; Hoskins 1997). Specific soil properties are as follows: 42% sand, 6% clay, 4.4% organic matter, 2.52% total C, and 5 mg kg^{-1} modified Morgan P (modified Morgan) and 122 mg kg^{-1} modified Morgan K. The soil contained 2 g kg^{-1} total N, with 55% of total N as NH_4^+ and 44% as organic N. Soil pH was 5.2 (1:1 soil to water), and CEC was 4.8 cmol (+) kg^{-1}. Poultry manure was collected from a commercial layer hen/egg production facility in central Maine and stored in an airtight container at 4°C for approximately 1 year until use. The material was obtained from underneath battery cages and was primarily excreta (no bedding) but did contain some feathers and dropped feed. Selected manure properties are shown

in Table 1 and determined as follows. Soil P and K, and manure P, Fe, K, Al, Mn, Ca, and Mg were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) following microwave-assisted HNO_3/HCl digestion (EPA Method 3051). Manure total N was measured following Kjeldahl digestion, and ammonia-N determined colorimetrically following distillation (Scarf 1998). Soil N was determined by a Leco CN-2000 (Leco Corp., St. Joseph, MI, USA). Phosphorus in sequential extracts was determined by extraction of 0.25 g manure with 25 ml each of H_2O, 0.5 M $NaHCO_3$ (pH 8.5), 0.1 M NaOH, and 1 M HCl for 2 h (H_2O) or 16 h (all others) (Dail et al. 2007). Inorganic orthophosphate P (P_i) concentration was quantified by a molybdate blue method (He and Honeycutt 2005). Total P (P_t) of extracts was determined by ICP-OES as previously described and organic P (P_o) estimated as the difference between P_i and P_t.

Greenhouse experiment

This study was conducted from March to July, 2009, in a greenhouse using natural lighting conditions. To increase aeration, soil was mixed with sand at a mass ratio of three parts soil to one part sand (dry weight) using a small cement mixer. Unprocessed poultry manure was then added at rates of 0 (Con) and 112 mg P kg^{-1} (PM), corresponding to an approximate field application rate of 42.6 Mg manure ha^{-1} (calculation made by assuming soil depth of 20 cm and a bulk density of 1.2 Mg m^{-3}). The specific manure application rate chosen was intended to provide P above the recommended rate for optimal ryegrass growth in our soil (22 mg P kg^{-1}, MAFES Soil Testing Laboratory), while maintaining N concentrations were not excessive or potentially inhibitory to plant growth. To ensure that N and K were not limiting nutrients, we amended Con with potassium chloride (KCl) and ammonium nitrate (NH_4NO_3) at rates equivalent to those in the PM treatment (156 mg N kg^{-1}; 197 mg K kg^{-1}), using the assumption that all of the manure-derived K and NH_4^+-N were plant available, and that 25% of the manure organic N would be mineralized during the short study period (Griffin 2008).

Aliquots of 1.5 kg (soil/sand plus appropriate manure and chemical amendment) were placed in 6-in.-diameter pots, gravimetrically moistened to 60% water-filled pore space with distilled water, and packed to a bulk density of approximately 1.2 Mg m^{-3}. One pot per treatment contained a soil probe connected to a Watch Dog 450 data logger (Spectrum Technologies, Inc, Plainfield, IL, USA) for monitoring soil water tension, air temperature, and relative humidity. Daily air temperature in the greenhouse ranged from 19 to 35°C, with a considerable rise around mid-June. Relative humidity ranged from 20% to 78%. Pots were loosely covered with plastic, gallon-size freezer bags and pre-incubated for 7 days on a greenhouse bench with aeration every 3 days. Any emerging weeds were removed using forceps. Following pre-incubation, the pots were gravimetrically brought up to 45% water-filled pore space with distilled water.

After pre-incubation, 12 pre-germinated ryegrass (*Lolium perenne* L. cv. 'Fiesta 3') seedlings were planted at a depth of 0.5 in. Unplanted pots were included as control treatments. Upon emergence, seedlings were thinned and transplanted to ensure six evenly spaced, healthy plants per pot. Soil moisture was maintained gravimetrically to 45% water-filled pore space during the first few weeks of the experiment; however, when plant biomass became significant, it was more practical to use soil probe measurements to maintain near-field capacity (10 kPa). Contents of pots were destructively sampled at 4, 8, and 16 weeks after planting (week 4, 8, and 16, respectively). There were four replicate pots per treatment at each sampling time. At each sampling, soil and plant material were gently tipped out of pots, and plant material was manually removed from the bulk soil. Rhizosphere soil

Table 1 Selected manure properties and inorganic (P_i) and organic P (P_o) distribution in sequentially extracted fractions

Parameter	Value
Solids (%)	30.3
pH (1:1)	6.8
Nutrient content (g kg^{-1})	
Total N	40.3
NH_4^+-N	22.4
NO_3^--N	2.5
P	19.5
Fe	1.1
K	34.0
Al	0.5
Mn	0.3
Ca	153
Mg	6.9
C	335
P in sequential extracts (g kg^{-1})	
H_2O-P_i	1.08
H_2O-P_o[a]	0.39
$NaHCO_3$-P_i	1.71
$NaHCO_3$-P_o	0.95
NaOH-P_i	0.31
NaOH-P_o	0.85
HCl-P_i	3.26
HCl-P_o	1.93

[a] P_o is estimated as the difference between P_i and total P from the specific fraction

was considered to be material which was attached to the roots, and was collected with a small brush and by thumb and forefinger manipulation of root and associated soil aggregates. Both bulk and rhizosphere soils were sieved (2 mm) and stored at 4°C in plastic bags until analysis, which was usually less than a week. Soils were referred to as "unplanted" (soil from pots containing no ryegrass), "bulk" (pots with ryegrass; soil not root-associated), and "rhizosphere" (pots with ryegrass; soil root-associated). Soil total P and N were determined as described above.

Plant root and shoot material (shoots and leaves) were separated by cutting the plant at soil level. Roots were washed to remove any adhering soil particles, and both root and shoot material oven dried at 65°C for 48 h to determine dry biomass. Plant material was ground in a Wiley mill and analyzed for P content by ICP-OES following low temperature dry ashing and ash dissolution (Kalra and Maynard 1991). Soil pH was determined using a 1:1 ratio of water/soil and an Ag/AgCl combination electrode.

Phosphorus fractionation

Phosphorus fractions were determined by a modified Hedley fractionation method as described by Waldrip-Dail et al. (2009). Briefly, 1.0 g soil was sequentially extracted with 25 ml deionized H_2O for 2 h, followed by 0.5 M $NaHCO_3$ (pH 8.5), 0.1 M NaOH, and 1.0 M HCl for 16 h each at 22°C on an orbital shaker (250 rpm). Extracts were centrifuged at 23,700×*g* for 30 min at 4°C and supernatants passed through a 0.45-μm filter. Samples were then diluted and adjusted to pH 5.0. Inorganic orthophosphate (P_i) concentration of extracts was quantified by a molybdate blue method (He and Honeycutt 2005).

To determine the portion of organic P that was enzymatically hydrolyzable (P_{oe}), 0.3 ml of diluted, pH-adjusted soil extract was incubated with 0.2 ml of a buffer/enzyme solution containing 0.25 units each of acid phosphatase type IV-S from potato (EC 3.1.3.2) and 3-phytase from *Aspergillus ficcum* (EC 3.1.3.8) (both from Sigma, St. Louis, MO, USA) at 37°C for 1 h on a shaker-incubator at 250 rpm (He et al. 2006b). Controls were included with omission of either the enzymes or soil extracts. Following incubation, P_{oe} was estimated as the difference in P_i between samples with and without enzyme addition. All incubations were performed in triplicate. Concentration of P_{oe} was not determined for H_2O extracts, as P concentrations were too low for an accurate analysis.

Phosphatase activities

Acid phosphomonoesterase (ACP), alkaline phosphomonoesterase (ALP), and phosphodiesterase (PDE) activities were determined using a modification of the methods of Tabatabai and Bremner (1969). Specifically, 0.1 g field-moist soil (<2 mm sieve) was incubated with 0.4 ml modified universal buffer (MUB) containing 6 μl of toluene at either pH 6.5 (ACP) or pH 11.0 (ALP) and 0.1 ml of 0.05 M *p*-nitrophenol phosphate solution for 1 h at 37°C on an incubator-shaker at 250 rpm. All incubations were performed in triplicate. Following incubation, 0.1 ml of 0.5 M $CaCl_2$ and 0.4 ml of 0.5 M NaOH were added and the resulting suspension centrifuged at 14,000×*g* for 2 min. The supernatant was then removed with a pipette and analyzed by UV–Vis spectroscopy for *p*-nitrophenol released (*p*-NP) at a wavelength of 400 nm. Soil- and substrate-free controls were included. Activity of PDE was determined in a similar manner at pH 8.0, using *bis-p*-nitrophenol phosphate (*bis-p*-NP) as the substrate.

Statistical analysis

The experiment was arranged in a completely randomized design with four replications. Single factor analysis of variance (ANOVA) with a pairwise *t* test was used to examine the effects of manure treatment, soil type (unplanted, bulk, rhizosphere), and time using Systat version 13.0 (Systat Software, Inc., Chicago, IL, USA). Significance of relationships between variables was determined using Pearson product-moment correlation coefficients.

Results

Plant biomass and P uptake

Poultry manure amendment (PM) increased aboveground biomass of young plants, as at week 8 there was more than twice as much shoot biomass in treatments receiving PM than in the unamended pots (Table 2). By week 16, however, there was no statistical difference between treatments in shoot biomass, with an average of 9.4 g pot^{-1}. In contrast to the increase in shoot biomass at week 8 with PM, root biomass did not statistically differ between treatments at any time.

Final P concentration of root material was 37% higher with PM than Con; however, no effect was observed on P concentration of shoots (Table 2). Both root and shoot P concentrations were higher at week 8 than week 16, indicating that P concentrations became more dilute as biomass increased. The shoot P concentrations at the termination of the study were 1.84 mg g^{-1} and 2.57 mg g^{-1} for Con and PM, respectively. Both root and shoot P uptake were higher with PM at weeks 8 and 16.

Table 2 Biomass production, P concentration, and P uptake of root and shoot material in unamended soil (Con) or with poultry manure applied at a P rate of 112 mg kg^{-1} soil (PM) at weeks 8 and 16 of ryegrass growth

	Shoot biomass yield (g)[a,b]	Root biomass yield (g)	Shoot P conc. (mg g^{-1})	Root P conc. (mg g^{-1})	Shoot P uptake (mg)	Root P uptake (mg)	Total P uptake (mg)
Week 8							
Con	1.28b	1.70	2.81	1.90b	3.77b	3.30b	7.1b
PM	2.70a	3.15	3.14	2.73a	8.33a	8.57a	16.9a
Week 16							
Con	8.58	12.1	1.84	1.26b	15.7b	15.2b	30.9b
PM	10.20	13.1	2.57	1.73a	26.2a	22.7a	49.0a

[a] Biomass and uptake values expressed as mass per pot. Each pot contained six ryegrass plants and 1.5 kg soil

[b] Within columns and time periods, means followed by different letters are significantly different ($p \leq 0.05$)

Soil pH, total P, and total N

Soil pH was affected by both PM and the presence of growing plants. The addition of PM increased soil pH in unplanted pots at week 4 from pH 4.9 to 5.7 (Table 3). Soil pH in unplanted pots either did not change significantly from week 4 to week 16 or changes were relatively small, but pH of both bulk and rhizosphere soils from ryegrass-planted pots increased 1 pH unit or more from week 4 to week 16 under both fertilization regimes. In addition, rhizosphere pH at week 8 was higher than that of bulk soil with PM, but not Con. This same trend was not seen at week 16; however, at this point the roots were fully dispersed within the pots and the spatial difference between bulk and rhizosphere soils was small, and probably the primary factor behind the chemical similarity between the bulk and rhizosphere soils at this time. There were significant correlations between rhizosphere soil pH and plant yields at week 8 (Table 4). There was also a positive relationship between rhizosphere soil pH and root P concentration at week 8, whereas the relationship was negative at week 16 (Table 4).

Poultry manure increased soil P in unplanted pots at all time points and in rhizosphere soil at weeks 8 and 16. Whereas there was no statistically significant change over time in total soil P in unplanted pots and bulk soils, total soil P within the rhizosphere decreased from week 4 to week 16 for both amended and control soils. At week 8, shoot and root biomass, root P concentration, total P uptake, and rhizosphere soil pH were all positively correlated to total soil P in the rhizosphere (Table 4). We did not observe the same relationships at week 16 (Table 4).

Soil N contents decreased from week 4 to 16 in the presence of ryegrass and were largely unaffected by poultry manure amendment (Table 3). Overall, there was no significant difference in final N concentration between bulk and rhizosphere soil, or between PM and Con treatments.

Table 3 Soil pH, total P, and total N content of unamended soil (Con) and with poultry manure applied at a P rate of 112 mg kg^{-1} soil (PM) at 4, 8, and 16 weeks of ryegrass growth or incubation (unplanted)

	Soil pH[a]			Soil P (mg kg^{-1})			Soil N (mg kg^{-1})	
	W4	W8	W16	W4	W8	W16	W4	W16
Con								
unplanted[b]	4.9B	5.0Bb	5.1Bb	975B	891Bb	911B	1,748	1,820a
bulk	5.1	5.2Bab	6.7a*	1,128	1,043a	1,066	1,710	1,468b*
rhiz	5.2B	5.5Ba	6.7a*	1,132	1,053Ba	941B*	1,743	1,483b*
PM								
unplanted	5.7A	6.0Ab*	5.5Ab	1,113A	1,199A	1,065A	1,750b	1,745a
bulk	5.5	6.0Ab*	6.4a*	1,084	1,137	1,034	1,803b	1,460b*
rhiz	5.7A	6.5Aa*	6.6a*	1,254	1,280A	990A*	2,163a	1,488b*

*$p \leq 0.05$, difference from W4 across rows and within soil type

[a] Means followed by the same uppercase letter are not different ($p \leq 0.05$) between treatments (Con, PM) within columns and soil types; means followed by the same lowercase letter are not different ($p \leq 0.05$) between soil types (unplanted, bulk, rhiz) within columns and treatments

[b] *unplanted*, *bulk*, *rhiz* soil from pots without ryegrass, and bulk and rhizosphere soil from pots with ryegrass

Table 4 Relationships between rhizosphere P fractions, P utilization by plant material, and activities of acid phosphomonoesterase (ACP), alkaline phosphomonoesterase (ALP), and phosphodiesterase (PDE) in rhizosphere at week 8 and week 16 of ryegrass growth

Rhizosphere soil parameter	Shoot biomass	Root biomass	Shoot P conc.	Root P conc.	Total P uptake	Rhizosphere pH	ACP	ALP	PDE
Week 8									
Rhizosphere pH	0.726**	0.690*	0.440	0.635*	0.568	1	0.560	0.339	0.778**
Total P	0.796**	0.651*	0.037	0.762**	0.718**	0.634*	0.158	0.533	0.372
Labile-P_i	0.785**	0.697*	0.379	0.705*	0.730**	0.849***	0.742*	0.437	0.579*
NaOH-P_i	0.667*	0.458	−0.044	0.225	0.240	0.633*	0.035	−0.081	0.217
HCl-P_i	0.513	0.569	0.551	0.768**	0.665*	0.596*	0.437	0.691*	0.418
Labile-P_{oe}	0.002	−0.009	0.618*	0.333	0.176	0.200	0.282	0.059	−0.029
NaOH-P_{oe}	−0.237	−0.167	0.207	0.445	0.011	0.082	0.163	0.367	−0.005
HCl-P_{oe}	−0.146	−0.301	−0.417	−0.451	−0.197	−0.299	−0.228	−0.192	−0.242
Week 16									
Rhizosphere pH	−0.179	0.013	−0.393	−0.635*	−0.414	1	−0.316	−0.235	−0.326
Total P	−0.024	0.286	0.411	0.268	0.073	−0.361	−0.487	−0.401	0.118
Labile-P_i	0.147	0.045	0.649*	0.751**	0.563	−0.555	−0.487	0.401	0.497
NaOH-P_i	0.994***	−0.463	0.151	0.330	0.001	0.211	−0.500	−0.407	0.141
HCl-P_i	−0.040	−0.048	−0.127	−0.292	−0.048	0.606*	−0.272	−0.238	−0.197
Labile-P_{oe}	−0.021	0.800**	0.356	−0.033	0.455	0.385	−0.382	−0.564	−0.212
NaOH-P_{oe}	−0.269	0.635*	−0.401	−0.413	−0.310	0.275	−0.162	−0.236	−0.390
HCl-P_{oe}	−0.183	0.085	−0.063	0.016	−0.003	−0.333	0.276	0.385	−0.013

Total P uptake=P uptake (mg) from both roots and shoots

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, significant correlation coefficients, $n=12$

Soil P fractionation

At week 4, the majority (~65%) of the extractable inorganic P was present in the NaOH fraction, followed by about 23% as HCl-P_i, and ~11% as labile-P_i (sum of H_2O and $NaHCO_3$-P_i) (Table 5). Labile P_i was not affected by PM at week 4; however, it did increase from week 4 to 8 in unplanted and rhizosphere soil with PM, but not in Con. Furthermore, at week 8, there was more labile-P_i in the rhizosphere than in the corresponding bulk soil with PM. Labile-P_i in the rhizosphere was positively correlated to plant biomass, root P concentration, total P uptake, and rhizosphere soil pH at week 8, and to P concentrations in both root and shoot material at week 16 (Table 4).

Amendment with PM resulted in 40% higher levels of NaOH-P_i in unplanted pots at week 4 (Table 5), which is significantly higher than the quantity of NaOH-P_i supplied by the manure itself (~1.8 mg NaOH-P_i kg^{-1} soil), implying transformation from other P fractions had occurred prior to our week 4 sampling. We did not see the same increase in ryegrass-planted pots at week 4. There was little difference in NaOH-P_i over time with the exception of an increase in rhizosphere NaOH-P_i from week 4 to week 16 with PM. NaOH-P_i in the rhizosphere was positively correlated to shoot biomass at both week 8 and 16, and rhizosphere soil pH at week 8 (Table 4).

The results for HCl-P_i were very similar to NaOH-P_i, with PM causing higher concentrations in unplanted pots at week 4, but not when ryegrass was present, implying that plants had taken up this fraction or it had been converted into other P forms (Table 5). Despite the high proportion of HCl-P_i in our poultry manure (Table 1), there was no increase in HCl-P_i due to PM at weeks 8 or 16, other than an increase in rhizosphere soil at week 8. Rhizosphere HCl-P_i was positively correlated to root P concentration and total P uptake at week 8 (Table 4), and rhizosphere soil pH at both week 8 and 16.

The data for hydrolyzable organic P (P_{oe}) followed the same general extraction pattern as that of inorganic P, with 57–68% as NaOH-P_{oe}, 16–27% as HCl-P_{oe}, and 4–26% as labile-P_{oe} (Table 5). Organic P forms were affected by PM at week 4, with unplanted amended soils having 78% less NaOH- and 85% less HCl-P_{oe} than Con. It appears that poultry manure promoted mineralization of organic P within the first 4 weeks of the incubation. In the rhizosphere, NaOH-P_{oe} concentration doubled from week 4 to week 8 with PM. Furthermore, the rhizosphere concentration of NaOH-P_{oe} with PM was nearly twice that of bulk soil at week 8. Although there was a large decrease in rhizosphere NaOH-P_{oe} from weeks 8 to 16, levels remained elevated over bulk soil. The labile-P_{oe} fraction in the rhizosphere was positively correlated to shoot P concentrations (week 8) and

Table 5 Extractable inorganic (P_i) and enzymatically hydrolyzable organic P (P_{oe}) fractions in unamended soil (Con) and with poultry manure at a rate of 112 mg P kg^{-1} soil (PM) at 4, 8, and 16 weeks of ryegrass growth or incubation (unplanted)

	Labile-P[a,b] (mg kg^{-1})			NaOH-P (mg kg^{-1})			HCl-P (mg kg^{-1})		
	W4	W8	W16	W4	W8	W16	W4	W8	W16
Extractable inorganic P (P_i) fractions									
Con									
unplanted[c]	89	96	45Bb*	437B	426	449	154Bb	166	247*
bulk	83	95	60a*	527	452	553	194a	164*	219
rhiz	79	102B	53Bab*	530	463	537	199ab	152B	276
PM									
unplanted	95	120ab*	67A*	614A	444	548	202A	161	234
bulk	98	114b	68*	550	514	509	214	200	240
rhiz	82	135Aa*	69A	505	512	557*	206	218A	240
Extractable enzymatically hydrolyzable organic P (P_{oe}) fractions									
Con									
unplanted[c]	13b	18	17	186A	86	107	74Aa	76	61A
bulk	22ab	16	24	164	75	72	17b	80*	54*
rhiz	22a	10	14	46	104	64	39ab	54	59
PM									
unplanted	28	15	16	61B	141ab	73a	17B	40	3B
bulk	19	11	16	102	69b	17b	26	67	41
rhiz	14	20	12	62	132a*	38a	38	34	53

*$p \leq 0.05$, difference from W4 across rows within soil type and fractions

[a] Labile P=P from H_2O and $NaHCO_3$ extracts (P_i) or from $NaHCO_3$ only (P_{oe}), as P_{oe} from H_2O extracts was too low for detection

[b] Means followed by the same uppercase letter are not different ($p \leq 0.05$) between treatments within columns and soil types, means followed by the same lowercase letter are not different ($p \leq 0.05$) between soil types within columns and treatments

[c] *unplanted*, *bulk*, *rhiz* soil from pots without ryegrass, bulk soil, and rhizosphere soil

root biomass (week 16) (Table 4). We also saw a positive relationship between rhizosphere NaOH-P_{oe} and root biomass at week 16 (Table 4).

Phosphatase activities

Activity of acid phosphomonoesterase (ACP) was the highest of all the three enzymes tested, averaging 323 mg *p*-nitrophenol kg^{-1} h^{-1} across all treatments and time points (Fig. 1a). Manure had very little effect on ACP activity, with the exception of a slightly higher activity of rhizosphere ACP with PM than Con at week 8. There was no difference between bulk and rhizosphere soil in the activity of ACP until week 8, where there was higher activity in rhizosphere soil with PM than unplanted. In addition, at week 16, bulk soil activity with PM decreased to a level that was 43% less than that of rhizosphere soil and 19% less than activity in unplanted pots. Few correlations were seen between ACP activity and P fractions, but there was a positive relationship between rhizosphere labile-P_i and ACP activity in the rhizosphere at week 8 (Table 4).

Alkaline phosphatase (ALP) activity at week 4 ranged from 65 to 185 mg*p*-NP kg^{-1} h^{-1} (Fig. 1b). At week 4, ALP activity in unamended, unplanted soil was twice that of PM-amended unplanted soil. In addition, ALP activity in unplanted pots was negatively correlated to total soil P at this time point ($r=-0.734$, $p \leq 0.01$, data not shown). At weeks 8 and 16, there was no difference between Con and PM in unplanted pots.

In contrast to the results from unplanted pots, when ryegrass was present PM had little overall effect on ALP activity. Although we saw a manure-related increase of about 1 pH unit at week 8, there was not much difference in ALP between the treatments with the exception of rhizosphere soil: at week 8 rhizosphere pH was higher with PM than Con (Table 3) and coincided with greater ALP activity. At weeks 8 and 16, unplanted pots had a pH significantly lower than that of rhizosphere soil; however, ALP activity did not differ in the two soil types at these time points. The data suggests that, while pH may influence ALP to some extent, other factors are also involved in controlling ALP activity. Similar to the finding for ACP, we saw slightly higher ALP activity in the rhizosphere than bulk

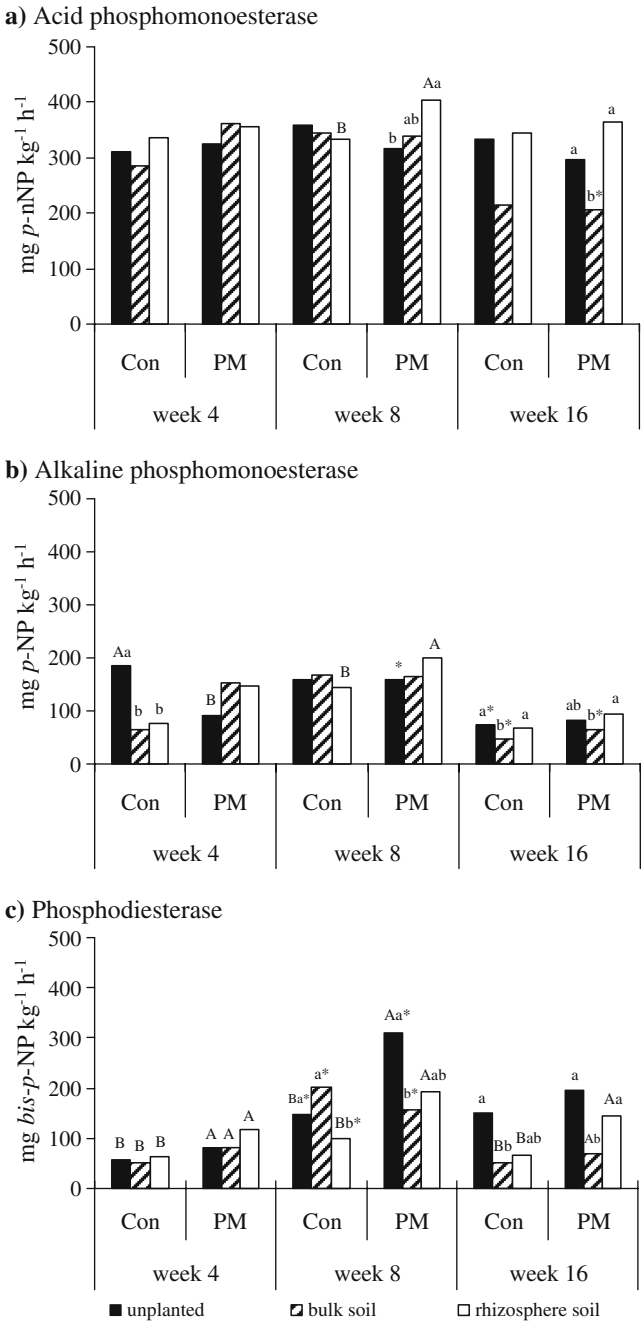

Fig. 1 Activities of **a** acid phosphomonoesterase, **b** alkaline phosphomonoesterase, and **c** phosphodiesterase in unamended soil (Con) or with poultry manure added at 112 mg P kg^{-1} soil (PM). Different *uppercase letters* within a soil type and sampling period indicate significant difference ($p \leq 0.05$) between treatments, *lowercase letters* indicate significant difference ($p \leq 0.05$) between soil types within treatments for each sampling period. *$p \leq 0.05$, difference from W4 for that particular soil type and treatment

soil at week 16 with both treatments. Rhizosphere ALP was positively correlated to rhizosphere HCl-P_i at week 8, and was accompanied by higher concentrations of this P fraction with PM (Tables 4 and 5).

Of the three phosphatases under investigation, phosphodiesterase (PDE) activity was the most responsive to PM amendment (Fig. 1c). At week 4, PDE activity of unplanted soil was 43% higher with PM than Con, with activities of 56 (Con) and 80 mg*bis-p*-NP kg^{-1} h^{-1} (PM). Activities of PDE in bulk and rhizosphere soil were also higher at week 4 with PM than Con. There was a large increase in enzyme activity from week 4 to week 8, which in rhizosphere soil was correlated with soil pH (Table 4). At week 16, however, PDE activity in all soils decreased, returning to activities similar to those at week 4. As with the other enzymes investigated, PDE activity was lower in the bulk soil than the rhizosphere at week 16.

Discussion

This study showed that poultry manure promoted higher root and shoot P uptake and increased root P concentrations. Our data for shoot P uptake were comparable to that reported for perennial ryegrass by other researchers (Chen et al. 2002; Ylivainio et al. 2008). Increased P uptake by ryegrass accompanying higher fertilizer P is common, including when P is derived from animal manures (Gaston et al. 2003; Butler et al. 2007; Szogi et al. 2010); however, most studies have focused on aboveground biomass and did not examine the P content of root material. Investigations have been conducted on P uptake by ryegrass roots (Chen et al. 2002; Pederson et al. 2002), but to our knowledge, the current work is one of the first studies to compare perennial ryegrass root biomass and P content in unamended soil to that following poultry manure amendment. In the current study, P was partitioned roughly even into roots and shoots, as root material constituted an average of 57% of total biomass and contained 46–49% of the P content of the plant (Table 2).

Higher levels of labile-P_i in the rhizosphere than in the corresponding bulk soil with PM at week 8 suggest that poultry manure provides a source for replenishment of soil solution P. This data when coupled with significant correlations between rhizosphere labile-P_i and plant biomass and P uptake data emphasize the importance of this fraction on plant growth and P utilization. Other studies have reported that P uptake is related to water-extractable P and total P concentrations of soils and amendments (Gaston et al. 2003; Zvomuya et al. 2006; Vu et al. 2008). Data from the current study supports these findings.

In our study, we saw that NaOH-P_i in the rhizosphere increased over time in soil amended with PM. In general, we would expect the rhizosphere to become depleted in inorganic P due to plant uptake, and depletion of labile- and NaOH-P_i has been observed up to 3 mm from the root surface of perennial ryegrass (Chen et al. 2002). However, there have also been reports of a buildup of specific P forms

around the root vicinity (Tarafdar and Jungk 1987; Hinsinger and Gilkes 1996; Hinsinger 2001). Hinsinger and Gilkes (1996) saw an increase in the NaOH-P fraction of ryegrass up to 2.5 mm from the root surface, most likely due to dissolution of soil P forms by root exudates. In the current work, we saw neither depletion nor accumulation of NaOH-P in the rhizosphere as compared to bulk soil. Correlation between NaOH-P_i in the rhizosphere, shoot biomass, and rhizosphere soil pH indicate that higher levels of NaOH-P_i promote plant growth or plant roots are increasing NaOH-P extractability, and pH may be influencing solubility of this fraction.

Despite the high proportion of HCl-P_i in PM, we saw no accumulation of this fraction following amendment. Whereas long-term, repeated applications of animal manure with a high proportion of moderately labile- and stable-P can result in higher HCl-P_i, that may not be true following a single application or when the material is applied to meet plant P requirements (Sato et al. 2005; He et al. 2008; Ylivainio et al. 2008). Sato et al. (2005) used P K-edge X-ray absorption near-edge structure spectroscopy on soils with a history of poultry manure application and found that short-term manuring yielded both Fe-associated phosphates and soluble Ca–P forms, such as dibasic and amorphous calcium phosphates, whereas longer-term manure application resulted in transformation of manure P into more stable Ca–P forms, such as beta-tricalcium phosphate. Thus, our single application of manure and the short duration of this experiment could have precluded the conversion of manure P to stable, less plant-available P forms that would accumulate in the soil.

The large changes we observed in soil pH due to both manure amendment and growing ryegrass could have influenced extractability and bioavailability of NaOH- and HCl-P in this study. Plants can alter soil pH by releasing root exudates, such as organic acid anions, to enhance mineral nutrient solubility, as well as liberation of H^+ and OH^- (or HCO_3^- resulting from OH^- carbonation) in order to counterbalance cations or anions entering the roots (Walker 1960; Nye 1981; Hinsinger 1998, 2001, 2003). Decomposition of organic acid anions can also increase soil pH due to proton consumption in the decarboxylation process (Barekzai and Mengel 1993). In addition, the direction and magnitude of pH changes can be influenced by the particular form of N taken up by the plants, as studies have shown that nitrate uptake (NO_3^-) can result in increased rhizosphere pH, while ammonium (NH_4^+) uptake can decrease pH (Youssef and Chino 1988; Gahoonia et al. 1992; Hinsinger 1998). Nitrate fertilization to ryegrass can result in an increase in soil pH of up to 0.6 units at the root surface (Gahoonia et al. 1992). Roughly half the N in the PM used in this study was NH_4^+-N, with the remainder as NO_3^- and organic N (Table 1). Although the supplemental NH_4NO_3 fertilizer added to Con supplied equal parts NH_4^+ and NO_3^-, apparently the ryegrass took up more NO_3^- than NH_4^+ while excreting OH^- or HCO_3^- to maintain ionic balance, which increased soil pH (Nye 1981; Hinsinger 2003).

Soil pH is important in controlling P speciation, as well as precipitation–dissolution and adsorption–desorption reactions, and thus P solubility and availability to plants (Hinsinger 2001). Iron- and Al-oxides, which sorb P and are partially solubilized by NaOH, exhibit highest P sorption potential at around pH 5.0, with very low sorption occurring above pH 7.0 (Gahoonia et al. 1992; Stevenson and Cole 1999): close to the values found in our ryegrass-planted soils. In addition, Ca-phosphates have decreasing solubility with increasing pH (up to pH 8.0) (Hinsinger 2001). Gahoonia et al. (1992) found that nitrate-induced pH increases to rhizosphere soil mobilized P, most likely by reducing the strength of specific adsorption to Fe or Al surfaces. We observed positive correlations between soil pH and all inorganic P fractions at week 8 in our study (Table 4), suggesting that the higher levels of HCl-P_i in PM amended rhizosphere soil at week 8 as compared to Con (Table 5), coupled with a pH increase (Table 3), could be the rationale behind the higher levels of labile-P_i in the rhizosphere of PM amended soil at both week 8 and 16 than unamended.

We observed significantly less NaOH- and HCl-P_{oe} extracted from soils amended with PM compared to those without manure in unplanted soil at week 4, suggesting rapid mineralization had occurred. Singh et al. (2009) also found a rapid mineralization of total P_o when poultry litter was applied to field soils in a rice–wheat rotation, with the majority of P release occurring within the first 20 days of application. In addition to changes in total P_o, transformation of NaOH- and HCl-extractable P_o to other forms has been reported following application of animal manure to soil (He et al. 2006c, 2008; Negassa and Leinweber 2009; Waldrip-Dail et al. 2009). He et al. (2006c, 2008) found that 20 years of repeated poultry litter applications at an annual rate of 27 Mg ha^{-1} did not result in accumulation of hydrolyzable organic HCl- and NaOH-P in an Alabama sandy loam soil, but did increase levels of labile and stable inorganic P, suggesting that a transformation occurred between the fractions. In addition, in a previous study we found that short-term (108 days) incubation of soil with poultry manure containing 31% of total P as HCl-P did not increase organic P in any fraction when applied at a rate of 200 mg P kg^{-1} (Waldrip-Dail et al. 2009). Thus, it appears that HCl-P from poultry manure is available within the short term. In a study of ryegrass fertilized with fox manure, which has 65–89% of its P in the HCl fraction, 54% of the P was immediately plant available and within 3 years the P bioavailability increased up to 87% (Ylivainio et al. 2008). In addition, while fox manure resulted in initially higher levels of extractable P_i and P_o, after 3 years

HCl-P was not any higher than in soil that received no manure. Given that labile P fractions exist in equilibrium with moderately labile P fractions, and these two P pools are sensitive to change due to fertilization or nutrient depletion (Schmidt et al. 1997), transformation between stable and labile P forms from manure could be an important mechanism for maintaining soil P balance, particularly in response to plant P uptake.

Acid phosphomonoesterase, alkaline phosphomonoesterase, and phosphodiesterase activities were within the range reported by other research groups (Colvan et al. 2001; Chen et al. 2002; Acosta-Martinez and Harmel 2006; Yu et al. 2006). As in the current study, Chen et al. (2004) also found that ACP activity predominates over ALP activity when soil pH is <7.0. Under conditions of low available P, both plants and microbes can produce phosphatases in order to meet nutrient demands (Nannipieri et al. 1990); however, plant roots have been found to have the ability to produce only acid phosphatase, not alkaline phosphatase (Tarafdar and Claassen 1998; Colvan et al 2001). Lower ACP activity in bulk soil than in the rhizosphere at week 16 suggests that P depletion following plant uptake stimulated the production or activity of root-borne acid phosphatases and could be related to the drop in rhizosphere labile-P_i concentrations from week 8 to week 16. Although there were few correlations between ACP activity and P fractions, there was a positive relationship between rhizosphere labile-P_i and ACP activity in the rhizosphere at week 8 (Table 4). When coupled with the increase in labile-P_i from week 4 to week 8 (Table 5), this suggests that ACP activity may be involved in maintaining the balance of soil solution P in the rhizosphere in this particular soil.

Lower activity of ALP in unplanted soil with PM at week 4 suggests that manure, or the P added with the manure, is inhibiting ALP activity. It is generally believed that ALP is not excreted by plant roots (Juma and Tabatabai 1988; Li et al. 2004), thus this result suggests inhibition of production or activity of bacterial or fungal ALP. Higher ALP and PDE activities in the rhizosphere than in bulk soil at week 16 could be due to pH changes, as rhizosphere pH was slightly higher than bulk soil with PM. Activity of ALP has been linked to soil pH, with higher activities following manure application ascribed to a more alkaline soil pH (Eivazi and Tabatabai 1977; Acosta-Martinez and Harmel 2006). This data also supports the findings of other studies reporting that PDE can be produced by plant roots as well as microorganisms and is affected by rhizosphere processes (Tarafdar and Claassen 1998; Chen et al. 2002).

Orthophosphate monoesters have been proposed to predominate organic P extractable by 0.1 M NaOH, whereas diesters represent P that is more labile and extractable with 0.5 M $NaHCO_3$ (Negassa and Leinweber 2009). We propose that root-derived ACP is largely responsible for the hydrolysis of accumulated rhizosphere NaOH-P_{oe} in this study, resulting in higher levels of labile-P_i at week 8 and promoting higher P uptake of ryegrass plants grown in soil amended with poultry manure. The overall lack of significance in correlations between phosphatase activity and specific P fractions is not surprising, as reports on the relationships between soil P and phosphatase activity have not been consistent, indicating that the interactions are complex and that factors other than soil P concentration are involved in controlling enzyme activity and P cycling (Colvan et al. 2001; Chen et al. 2002, 2004; Acosta-Martinez and Harmel 2006). Chen et al. (2002) investigated relationships between enzyme activities (ACP, ALP, PDE) and NaOH-P_o under ryegrass in two soils contrasting in organic matter, nutrient, and clay content, but with similar pH, finding significant negative correlations in soil with higher nutrient concentrations and proportion of clay, while no relationship was noted in a sandier, less-fertile soil. There have also been differences noted in relationships between specific phosphatase activities and extractable soil P. Colvan et al. (2001) found that although long-term (100 years) application of farm-yard manure increased levels of extractable P and activity of all three common phosphatases on a managed grassland, there was no relationship between ACP activity and extractable P, whereas there was a positive correlation with ALP and PDE activities. It appears that phosphatase activity is tightly coupled to P concentrations only when the soil P level is limiting or excessive, and under conditions of sufficient P (as is the case in our study) that the relationship is uncoupled. Furthermore, any feedback mechanism that results in inhibition of enzyme production by roots or soil biota is a longer-term process (Olander and Vitousek 2000), and may not have been a factor during our short study period.

In addition, the standard phosphatase assay does not discriminate between enzymes of different origins. Phosphatase assays detect activity of recently produced root and microbial enzymes, as well as those from dead cells or immobilized on soil clay or humic colloids (Burns 1982; Sinsabaugh 1994). Furthermore, root phosphatases are sensitive to high concentrations of soil P_i, whereas soil phosphatases and immobilized extracellular phosphatases are probably not controlled by P_i levels (Nannipieri et al 1990; Adams and Pate 1992). Tarafdar and Jungk (1987) reported that ACP and ALP activities were higher in the rhizosphere of four plant species than in the bulk soil, and this corresponded with higher fungal and bacterial populations. It is therefore not easy to determine if higher phosphatase activity is related to an increase in the production of root phosphatases or a result of root exudates enhancing the rhizosphere for microbial growth. The inability to separate root-derived activity from that of microbial phosphatase or to separate activity of newly produced enzymes from that of older, stabilized extracellu-

lar phosphatases makes full interpretation of the results difficult. Further studies of this type, particularly when paired with molecular techniques to quantitate extracellular enzymes in soil based on ancestry and distance from the root surface, are warranted in order to fully elucidate the interactions between P forms in soil and manure, phosphatase activity, and rhizosphere processes.

Conclusions

Incorporation of poultry manure into soil promoted transformation and mineralization of less-labile inorganic and organic P into labile-P_i in the rhizosphere, which resulted in higher root P concentrations and total P uptake by perennial ryegrass. Soil acid phosphomonoesterase activity predominated over the other enzymes investigated, with activities twice those of alkaline phosphomonoesterase and phosphodiesterase. Poultry manure application resulted in higher activities of acid phosphomonoesterase, alkaline phosphomonoesterase, and phosphodiesterase in rhizosphere soil at week 8 as compared to unamended rhizosphere soil. Based on soil pH and phosphatase activity measurements, we proposed that enhancement of NaOH and HCl-P availability by PM was due to (1) stimulation of root phosphatase production, (2) enrichment of the surrounding soil for microbial phosphatase production or activity, and (3) increased solubility of Al- and Fe-associated phosphates.

This study demonstrated that although PM may temporarily increase NaOH and HCl-P_i, in soil these P fractions may be readily transformed into plant-available, labile-P; therefore, PM application to a non-calcareous soil does not necessarily lead to accumulation of stable, HCl-P. The stimulation of phosphatase activity by both PM application and rhizosphere processes is important in mineralization of organic P from manure and in controlling the balance between soil P forms. Further study on the effects of PM application on the interactions between phosphatase activity, specific P fractions, and soil chemical properties under field conditions is warranted to verify our greenhouse pot observations and to better understand the impacts of PM on soil P cycling.

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