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Phosphatase activity in the drilosphere and its link to phosphorus uptake by grass

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ABSTRACT

Earthworms can increase the solubility of phosphorus (P) in soil, an effect which to a large extent is controlled by mineralisation of organic P. Phosphatases, a class of hydrolytic enzymes, catalyse this mineralisation process. However, a consistent comparison of their activity among earthworm species and for different soil spheres is still missing. Here we aim to better understand the activity of phosphatases in relation to earthworm-enhanced P-availability, and specifically that of phosphomonoesterases (PME) which directly liberate orthophosphate. We conducted a greenhouse pot experiment with five different earthworm species. The PME activity was assessed in earthworm casts, burrows and bulk soil for both single species and mixed communities. Analyses were performed at both the pH-H_2O of the bulk soil (6.5) and of the casts (7.5). The PME activity measured at both pH values was highly correlated ($R^2 = 0.98$; $p = 2.2x10^{-16}$) and was strongly elevated by earthworm activity in the order cast > burrows > bulk soil. The PME activity was particularly high in the casts of *A. longa* relative to casts of other species. Large variation in PME activity in the drilosphere was observed among earthworm species, but this variation was not related to earthworm ecological categories. Our data also indicate that an elevated P concentration in grass shoots could result from increased PME activity via greater hydrolysis of organic P.

Phosphorus (P) is an essential nutrient for plant growth taken up by roots from the soil solution as orthophosphate (ortho-P). However, the concentration of ortho-P in soil solution is limited because of its strong binding to the mineral phase (Hesterberg, 2010; Morel et al., 2000). Earthworms are able to increase the ortho-P concentration in the soil solution (Le Bayon and Milleret, 2009). Many of the mechanisms by which earthworms enhance P-availability are triggered by the mineralisation of organic P in the earthworm's gut, which a) results in a direct contribution to the total pool of inorganic P; b) when organic carbon is mineralised along with P, this leads to a pH increase to the dominant pH in earthworm cast (i.e., pH≈7.5, at which the majority of ortho-P is present as HPO_4^{2-}) thereby decreasing ortho-P adsorption and increasing soluble ortho-P; and c) initiates particle growth of Fe-(hydr)oxides in casts, lowering the reactive surface area of Fe-(hydr)oxide-dominated soils and decreasing ortho-P adsorption (Vos et al., 2022a).

The predominant form of organic P in most soils is monoester P compounds, especially phytate (Koopmans et al., 2007; Magid et al., 1996; Turner et al., 2002). The mineralisation of organic P is catalysed by phosphatases, a class of hydrolytic enzymes which occur in different forms in soil and soil organisms (Kiss et al., 1975). Extracellular phosphatases are produced by plant roots and microorganisms when relatively little of soil P is available for uptake (Olander and Vitousek, 2000; Tadano et al., 1993; Toor et al., 2003). As phosphodiesterases strictly only result in (monoester) organic P, it is the activity of phosphomonoesterases (PME) that directly governs the contribution of organic P mineralisation to P-availability because it results in the release of ortho-P. In this study we therefore focus on the activity of PME.

Besides the production of phosphatases by plant roots and microorganisms, these enzymes can be excreted by earthworms and their gut microorganisms (Satchell and Martin, 1984). The activity of phosphatases in the drilosphere, the part of the soil influenced by earthworms via egestion or burrowing (Brown et al., 2000), has often been found to be strongly elevated compared to the bulk soil (e.g., Hoang et al., 2016; Le Bayon and Binet, 2006; Satchell and Martin, 1984; Sharpley and Syers, 1976; Wan and Wong, 2004). However, large variation among earthworm species has also been observed (Satchell and Martin, 1984), which

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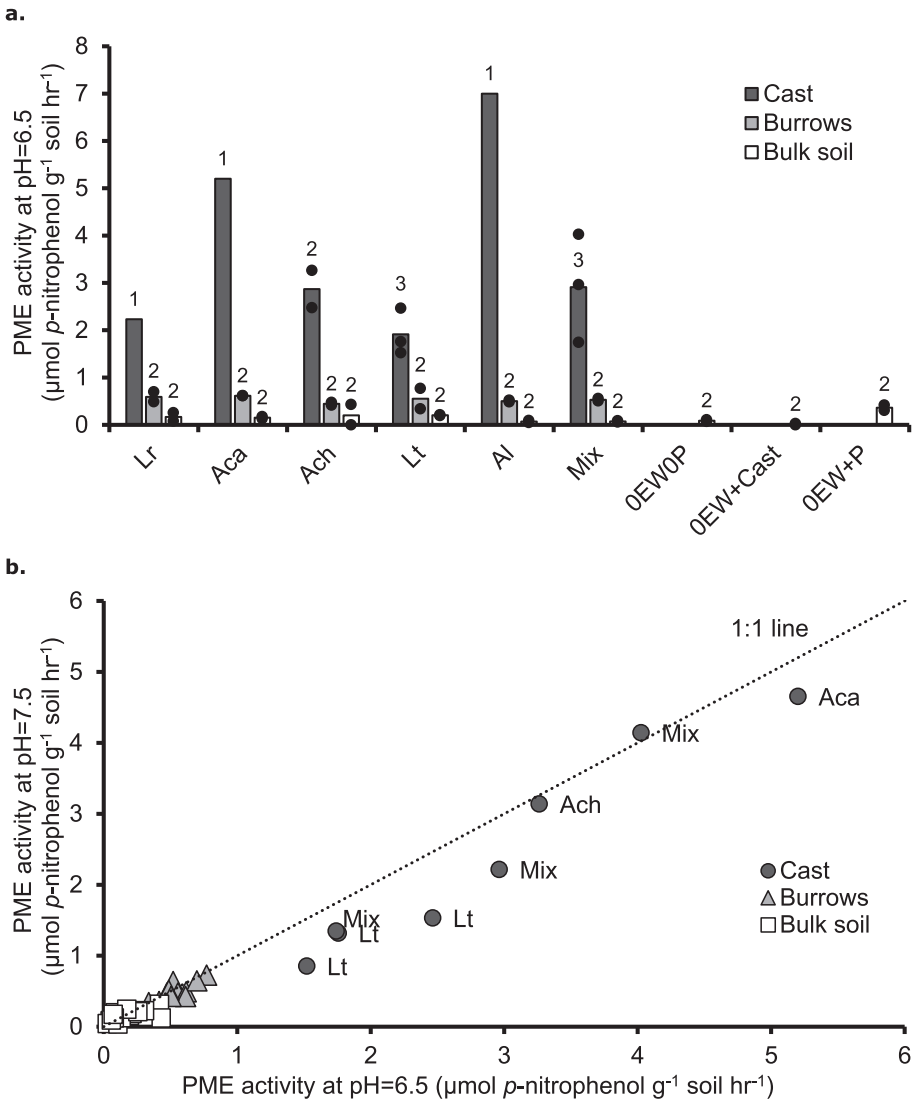

Fig. 1. The PME activity for the various soil spheres and treatments as measured at pH=6.5 (a), and the correlation between PME activity as measured at pH=6.5 and pH=7.5 with respect to the 1:1 line (b). In panel (a), data collected at pH=6.5 are presented; treatments with low sample mass could only be measured for PME activity at one pH value. Dots indicate the PME activity of each assay replicate (n=2 for burrows and bulk soil). Due to limited sample mass, the number of replicates for PME activity measurements of casts differed by treatment. For all measured samples the number of experimental replicates (n) is indicated above the graph.

might be related to different feeding behaviour of each ecological category (e.g., litter-dwelling epigeic earthworms, soil-dwelling endogeic earthworms, and vertically burrowing anecic earthworms; Bouché (1977)). Although various earthworm species and different soil spheres have been evaluated, these studies often used varying methods to determine phosphatase activity or collect (surface) casts. A comprehensive comparison of the phosphatase activity among multiple earthworm species for different soil spheres under standardized conditions would therefore improve our understanding of the effect of earthworms on P-availability. This study aims to create an overview of the variation among earthworm species and soil spheres to fill that research void. Furthermore, measurements of phosphatase activity are often conducted at a buffered pH which is deemed to be optimal for enzyme activity (i.e., pH=6.5). However, these methods were originally developed on only eight bulk soils (Tabatabai and Bremner, 1969) while phosphatase pH optima are now recognized to be highly variable and not related to the pH of the soil (Herbien and Neal, 1990; Wade et al., 2021). As there may be earthworm-specific phosphatase pH optima, future research could evaluate this by constructing pH activity curves for casts from specific species, as has been done for soils (Halstead, 1964; Herbien and Neal, 1990; Tabatabai and Bremner, 1969; Wade et al., 2021). As a first step, however, enzyme assays are required at the pH that reflects the *in-situ* pH (i.e., of the corresponding soil sphere) in what some have termed "natural assays", as this can give more insight into actual soil functioning (Burns et al., 2013). Assaying PME activity at the pH of the drilosphere will therefore improve our understanding of potential *in-situ* mineralisation rates among earthworm species.

In order to assess PME activity in earthworm casts relative to bulk soil, we conducted a greenhouse pot experiment using the top layer of the acidic sandy soil (pH-H_2O=6.5) with a low agronomic soil P-status described by Vos et al. (2019). The soil organic matter content of this soil was 42 g kg^{-1} and the total P content amounted to 343 mg kg^{-1}, 29 % of which was organic (i.e., 99 mg kg^{-1}). Details on the used materials and methods can be found in SI 1. The pots were sown with perennial ryegrass (*Lolium perenne*) and five earthworm species common in the Netherlands were added 21 days after seeding as single-species treatments representing the three main ecological categories: (i) *Lumbricus rubellus* Hoffmeister, 1843 (Lr; epigeic); (ii) *Aporrectodea caliginosa* (Savigny, 1826) (Aca; endogeic); (iii) *Allolobophora chlorotica* (Savigny, 1826) (pink morphotype; Ach; endogeic); (iv) *Lumbricus terrestris* Linnaeus, 1758 (Lt; anecic); (v) *Aporrectodea longa* (Ude, 1895) (Al; anecic). A treatment was included with a mixture of all five species in which the

total earthworm mass was distributed equally over all species (Mix). Additionally, control treatments without earthworms (e.g., to compare PME activity in the absence and presence of earthworms) were established for soil that was either unamended (0EW0P) or amended with mineral P (0EW+P). Finally, a treatment was included in which weekly *L. terrestris* casts from non-experimental pots undergoing the same conditions as the experimental pots were applied on the soil surface (0EW+Cast), in order to distinguish between the effects of earthworms on added cast fertility and earthworm activity. Earthworm densities in all treatments were approximately 286 g fresh (empty gut) earthworm m^{-2}, which is in the upper range observed in grasslands in the Netherlands (Van Vliet et al., 2007). All treatments had four replicates and the pots were randomized weekly.

To reflect the harvesting frequency of intensively managed grasslands in the Netherlands, grass was cut every three weeks at 5 cm above the soil surface resulting in a total of five harvests of which the fresh and dry yields were recorded. The experiment was ended 84 days after the earthworms had been added to the pots. The P and nitrogen (N) contents of the grass shoots of the final harvest (n=4) were determined according to Novozamsky et al. (1983). Samples were collected from three different soil spheres: cast, burrows (here together referred to as the drilosphere) and bulk soil. Cast samples were collected from polyethylene cups to which the earthworms were added to empty their guts after removal from the pots following Vos et al. (2019) (cast). Additional samples were collected by scraping the earthworm burrow walls (burrows) and by sampling the remaining soil (bulk soil). Subsequently, PME activity was measured according to Tabatabai and Bremner (1969) on fresh samples of all casts for which sufficient material was present. Two fresh replicates of each treatment were analysed for the other two soil spheres. In short, 0.115 M *p*-nitrophenyl phosphate solution in modified universal buffer was provided as a substrate and PME activity was determined by colorimetric measurement of the enzyme-hydrolysed product of this substrate (*p*-nitrophenol). As this method was challenged by the production of sufficient cast material for measurement, the method was downscaled to measure small amounts of cast material (see SI 2 for the used scaled-down procedure). For all soil spheres, PME activity was measured at pH=6.5, which equals the pH-H_2O of our soil, and at pH=7.5 to approximate the pH-H_2O prevailing in earthworm casts irrespective of the earthworm species used (Vos et al., 2022a; Vos et al., 2019). Considering the changes in pH over the digestive tract of earthworms, as well as the consequences of such changes for enzyme activity and gut microbiota, was outside the scope of this study. Ortho-P was measured in 1:10 (w:v; based on dry weight) water extracts as a proxy for soil solution for casts, burrows and bulk soil (Torrent and Delgado, 2001) following Vos et al. (2019). Statistical analyses are described in SI 3.

Our results demonstrate large differences in PME activity among earthworm species (Fig. 1a). The PME activity at pH=6.5 was on average 5.8 times higher ($p = 2.45x10^{-5}$) in casts than in burrows, which was, in turn, on average 4.0 times higher ($p = 1.65x10^{-6}$) than in bulk soil. This agrees well with both the general observation of elevated PME activity in the drilosphere compared to the bulk soil (Hoang et al., 2016; Satchell and Martin, 1984; Sharpley and Syers, 1976; Wan and Wong, 2004), as well as with previously observed differences between casts, burrows and bulk soil (Le Bayon and Binet, 2006). For the single-species treatments of Lr, Aca and Al, insufficient cast mass prohibited replicated PME activity measurements and thereby statistical analysis. Replicated assays of PME activity in Ach and Lt casts showed that the maximum variation within a treatment was 1.6 (max/min), whereas across all casts this variation was almost three times larger (4.6). This corroborates previously observed differences in PME activity among earthworm species and their gut microbiota (Satchell and Martin, 1984; Wan and Wong, 2004).

The observed variation among species did not clearly relate to earthworm ecological categories. For instance, the two anecic species demonstrate both the lowest (Lt) and the highest (Al) measured PME activity in casts. Meanwhile, the PME activity in earthworm casts showed an additive effect, as the mixture had the average PME activity of all five treatments with single species. This suggests that the composition of an earthworm community (i.e., which species occur in what ratio) is key to overall cast PME activity.

A strong correlation between PME activity in all soil spheres measured at both pH values was observed ($R^2 = 0.98$; $p = 2.2x10^{-16}$; $PME_{pH=7.5} = 0.87 \times PME_{pH=6.5}$; Fig. 1b). The assay pH did therefore not affect the relative differences in PME activity between treatments. However, the slope of the linear relationship is lower than one and most data points in Fig. 1b are situated below the 1:1 line (especially at higher enzyme activities), which suggests a pH optimum of PME in this soil below pH=7.5. Consequently, measuring PME activity at the oft-assumed optimum of pH=6.5 (Tabatabai and Bremner, 1969) would overestimate the potential PME activity in casts, as these have a pH-H_2O of around 7.5 (Vos et al., 2022a; Vos et al., 2019).

Since PME production by plants and microorganisms is regulated by P demand relative to the supply of soil-available P (Olander and Vitousek, 2000; Tadano et al., 1993; Toor et al., 2003), an inverse relation between the water-extractable ortho-P concentration and PME activity is expected. However, this inverse relationship was not observed in the bulk soil of the control treatments with differing P fertilisation (0EW0P, 0EW+Cast, 0EW+P; Fig. S1). The PME activities measured in the bulk soil in our study were relatively low (e.g., Le Bayon and Binet, 2006; Tabatabai and Bremner, 1969), but the 0EW+P treatment had an elevated PME activity and also the highest water-extractable ortho-P concentration due to fertilisation with inorganic P. As we did not observe any indications of an effect of surface-applied casts on PME activity and water-extractable ortho-P in the bulk soil (i.e., PME activities and soluble ortho-P concentrations in the bulk soil of the 0EW0P and 0EW+Cast treatments were both low), this led to a trend in which water-extractable ortho-P increased with PME activity. Interpreting this relationship is complex, as it involves comparing a (maximum potential) production rate (i.e., PME activity) with a static pool size (i.e., water-extractable ortho-P), without information on possible sinks. It could be that for the 0EW+P treatment the activity of PME is still high in case of an elevated water-extractable ortho-P pool if the ortho-P flux through that static pool is relatively low, e.g., if consumption of ortho-P by microorganisms is low. This is a general challenge to the interpretation of enzyme activities with nutrient availability due to opposite and co-operating negative and positive feedbacks via product inhibition and mineralisation, respectively.

We did not observe significant differences in the ortho-P concentrations between earthworm burrows and bulk soil ($p = 0.15$), although the average ortho-P concentration in burrows (0.085 ± 0.021 mg P/L) was slightly higher than in bulk soil (0.077 ± 0.014 mg P/L). As mentioned above, this may be attributed to the direct use of ortho-P produced in burrows from PME-induced hydrolysis of organic P by microorganisms (Le Bayon and Binet, 2006). Unfortunately, only the casts from two species (11.2 (Lr) and 5.9 (Lt) mg P/L) could be analysed for water-extractable ortho-P due to insufficient cast material, which impedes assessing the relation of this P pool with PME activity. However, as these measured ortho-P concentrations related equally (55–57% - i.e., same fraction, which allowed us to assume that the trend among earthworm species in ortho-P concentrations from cast is similar) to those in Vos et al. (2019) who used the same earthworm species, soil, and experimental conditions, we linked PME activities from the earthworm species in the current study to those ortho-P concentrations. The PME activity in casts was negatively related with water-extractable ortho-P concentrations in Vos et al. (2019), in agreement with the expected inverse relationship between PME activity and water-extractable ortho-P. For instance, Lr casts had a high soluble ortho-P concentration and low PME activity, whereas Al casts had a low soluble ortho-P concentration and high PME activity. Substantially (23 times) higher PME activity ($p = 3.26x10^{-8}$) for cast compared to bulk soil likely indicates that mineralisation directly contributes to the increased level of water-

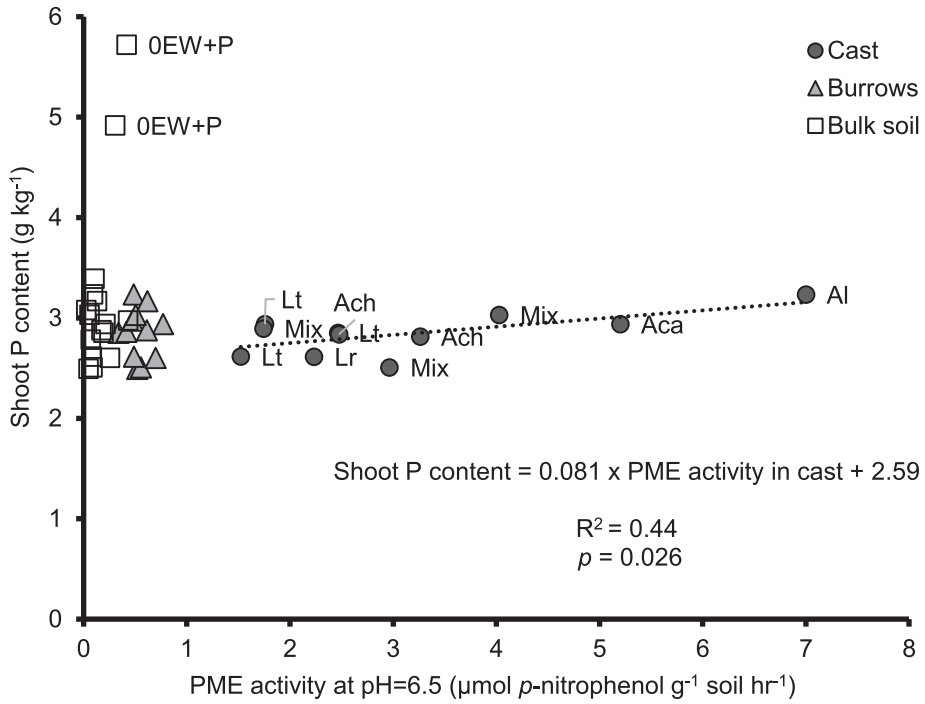

Fig. 2. Correlation between PME activity in different soil spheres measured at pH=6.5 and P content of grass shoots for the samples of which PME activity was measured. For the bulk soil sphere, data include the control treatments without earthworms (0EW0P, 0EW+Cast, 0EW+P). The dotted line represents the correlation observed in earthworm casts.

extractable ortho-P in cast compared to the corresponding bulk soil (Vos et al., 2019).

As soil–plant conditions were very similar in all treatments (same soil, same grass species, similar grass yield – data not shown), we did not evaluate possible contributing effects of arbuscular mycorrhizal fungi (AMF) and plant root exudates on PME activity, because these would be expected to be constant across treatments. Therefore, the contribution to the measured PME activity in burrows and bulk soil of AMF and roots remains unclear. We observed a positive correlation between PME activity in casts at pH=6.5 and shoot P content in the present experiment ($R^2 = 0.44$; $p = 0.026$; Fig. 2). A similar relationship was present for PME activity at pH=7.5, but because of the lower number of datapoints this relation was nonsignificant ($R^2 = 0.19$; $p = 0.28$). Although these results should be interpretated with care given that grass growth was not P-limited (nor N-limited; Koerselman and Meuleman (1996); Fig. S2) and that there was little variation in shoot P content (2.5–3.4 g kg^{-1}), they indicate that an elevated P concentration in grass shoots could be a consequence of an increase in PME activity resulting in more hydrolysis of organic P. As neither P nor N limited grass growth, higher P uptake might be explained by luxury P consumption, which is a common plant response (Chapin, 1980; Oyarzabal and Oesterheld, 2009). At the same time, however, the P content measured in the grass shoots is not consistent with *L. perenne* luxury P consumption, because P contents are in the adequate range (2.0–5.5 g kg^{-1}) for this grass species (Reuter and Robinson, 1997). As Al casts showed both the highest PME activity and the highest shoot P content, this species is likely the most instrumental of the studied species to affect P-availability via increasing PME activity. This matches with observations from a field experiment on the same acidic sandy soil with a low P-status in which Al most contributed to P uptake by perennial ryegrass (Vos et al., 2022b). However, the increase in P uptake (i.e., final P content of the shoots × dry biomass of the final harvest) in our experiment varies only between a 3% decrease and a 17% increase in mg P pot^{-1} with respect to the control without P fertilisation (0EW0P) for the various earthworm species, while P uptake increased on average 91% in the pots that received mineral P fertilisation (see SI 6). Therefore, the earthworm-induced effect on grass P uptake through mineralisation of organic P is subtle and shoot P content does not increase to the same level as by mineral P fertilisation (Fig. 2).

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Co-author is chair of the editors-in-chief of Geoderma (JWvG); Co-author is associate editor of Geoderma (AJM).

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2023.116690.

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