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Large variations in readily-available phosphorus in casts of eight earthworm species are linked to cast properties

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Abstract

Phosphorus (P) is an important nutrient for plant growth. However, P is often poorly available for uptake by roots because it strongly adsorbs to the soil mineral phase. Recent research shows that earthworms can temporally and locally increase P availability to plants. However, the pathways through which they do so are not fully understood, and it remains unclear to what extent this capacity varies among earthworm species. Here we study the variation among earthworm species with respect to readily-available P in casts as well as other physico-chemical cast properties, in a greenhouse pot experiment using a soil with a low P status. The earthworms belong to eight commonly occurring earthworm species in the Netherlands: two epigeic species (a mixture of the compost earthworms *Dendrobaena veneta/Eisenia fetida; Lumbricus rubellus*); four endogeic species (*Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Aporrectodea rosea*, *Octolasion lacteum*); and two anecic species (*Aporrectodea longa; Lumbricus terrestris*). For all species, the pH in water extracts of earthworm cast (pH=7.4-8.2) was significantly higher ($p<0.001$) than for the control bulk soil (pH=6.6) and differed significantly ($p=0.003$) among earthworm species. Similarly, the dissolved organic carbon (DOC) concentration in the same water extracts was an order of magnitude higher in earthworm cast compared to the control bulk soil and varied among species ($p<0.001$). The size of the total pool of reversibly adsorbed P in earthworm cast was greater than in the control bulk soil, but no significant differences were found among earthworm species. Differences among species were present for the readily-available P pools extracted from casts, including P-Olsen and water-extractable ortho-P. Water-extractable ortho-P concentrations were much higher in the casts of all species as compared to the control bulk soil (0.9-6.8 vs 0.06 mg l^{-1} or to 9.0-68 vs 0.6 mg kg^{-1}). Highest ortho-P levels were measured in *L. rubellus* casts and the lowest in casts of *A. chlorotica*. A positive correlation between the concentrations of DOC and ortho-P was observed ($R^2=0.72$, $p<0.001$). The observed variation in all measured physico-chemical cast properties could not be explained by conventional ecological earthworm classifications. Our results show that the nature and magnitude of earthworm-induced increased P availability differs dramatically among earthworm species. This strongly suggests that, apart from its size, species composition of the earthworm community is key to optimizing P availability to plants.

Keywords: earthworms, phosphorus, physico-chemical cast properties, community composition

1. Introduction

The availability of nutrients in soils for uptake by plants is crucial for the production and quality of food, feed and fibre. For phosphorus (P), a plant-essential macronutrient, availability in soils is often limited. Plants take up P from the soil solution in the inorganic form as orthophosphate (mainly $H_2PO_4^-$ and HPO_4^{2-}; further referred to as ortho-P) (Hawkesford et al., 2012), but the amount of this readily-available P form in soil solution is relatively small (Morel et al., 2000). Between 40% and 70% of the total P content in agricultural soils is made up of inorganic P (Harrison, 1987) - with an average of 57% according to Menezes-Blackburn et al. (2018) - with the remainder being present as organic P. Most inorganic P in fertilised agricultural soils is adsorbed to reactive mineral surfaces including metal (hydr)oxides and oxidic edges of clay minerals, or is present in the form of calcium- or magnesium-phosphate minerals (Hinsinger, 2001; Gérard, 2016). Phosphorus can be present in organic form when it is contained within organic matter (OM). During the growing season, there is a constant release of P from the soil solid phase to the soil solution through desorption of adsorbed P or phosphate mineral dissolution and mineralisation of organic P to supplement P uptake by plants. These processes of adsorption and desorption are very diverse and are largely influenced by biological factors like enzymes or organic acids that can be excreted by the microbial community or plant roots (Rodriguez and Fraga, 1999). Because of the constant release,, soil available P has been defined as the sum of P present in soil solution and the amount of P reversibly adsorbed to the soil solid phase or present in soluble phosphate minerals (Nawara et al., 2017). Organic P could be added to this definition of soil available P.

When the P supplementing flux from the soil solid phase to the plant roots is unable to satisfy the plant P demand, the availability of P becomes limiting for optimal plant growth (Koopmans et al., 2004). For agricultural soils, this is often ameliorated by the application of P fertiliser in order to avoid P deficiency. However, the global commercial phosphate reserves used for P fertiliser production are limited (Cordell et al., 2009), which causes an increasing need for alternative and more sustainable ways to supply the required P for optimal plant growth.

Over the last decennia there have been several reports indicating that earthworm activity can temporally and locally increase the amount of readily-available P in the soil (see review by Le Bayon and Milleret (2009)). Recent studies (Vos et al., 2014; Ros et al., 2017) have linked this increase in readily-available P in soils to an increase in grass production and grass P uptake.

Earthworms are conventionally classified in three main categories, based on their feeding and burrowing behaviour (Bouché, 1977). These include: (i) *epigeic* earthworms; live in the litter layer and the upper centimetres of the soil and feed on relatively fresh surface litter. Although compost earthworms do not live in the soil, they do belong to this ecological group; (ii) *endogeic* earthworms; live deeper in the soil where they make non-permanent burrows. They feed on soil particles and associated OM, which is relatively old organic material; (iii) *anecic* earthworms; create deep permanent vertical burrows in which they drag down the relatively fresh surface litter they feed on. In a recent meta-analysis (Van Groenigen et al., 2019) these earthworm functional groups have been suggested to differently affect P availability to grass. If the ability to increase the availability of soil P to plants varied among ecological groups, earthworm community composition - rather than only community size - could be hypothesized to affect plant P availability.

At the basis of this hypothesis lie the interspecific differences among earthworms in the physico-chemical properties of casts governing the earthworm-induced effect on readily-available P. The mechanisms underlying the change in these properties, are, however, not yet fully understood. Multiple pathways for earthworm-induced increases in readily-available P have been suggested (see Vos et al. (2014) and Ros et al. (2017) for more details): (i) an increase in the concentration of ortho-P in solution as a consequence of the elevated pH in earthworm cast; (ii) desorption of ortho-P from reactive mineral surfaces due to competitive adsorption with elevated concentrations of dissolved organic carbon (DOC) in casts (Geelhoed et al., 1998; Ros et al., 2017); (iii) increased mineralisation of organic P through stimulated microbial activity inside the earthworm gut

(Tiunov and Scheu, 2000; Drake and Horn, 2007); (iv) selective feeding of earthworms on OM or fine particles including metal (hydr)oxides and clay minerals with a higher P content compared to the bulk soil, leading to increased intake of P (Sharpley and Syers, 1976; Curry and Schmidt, 2007).

The reported effects of earthworms on physico-chemical properties affecting readily-available P in casts differ strongly throughout literature. Multiple studies have observed elevated pH levels in earthworm casts compared to the bulk soil, but these increases vary between 0.5-2.0 pH units (Sharpley and Syers, 1976; Barois and Lavelle, 1986; Basker et al., 1994; Kuczak et al., 2006; Briones et al., 2008; Vos et al., 2014; Van Groenigen et al., 2019). According to the pathways specified above, next to pH, DOC in the soil solution can affect the concentration of ortho-P by competing with ortho-P for the same binding sites on reactive metal (hydr)oxides. Reported DOC concentrations are several orders of magnitude higher in earthworm cast compared to the bulk soil (Bolan and Baskaran, 1996; Ros et al., 2017) and differences among earthworm species have been observed (Vos et al., 2014). The magnitude of an earthworm-induced increase in the P content of earthworm casts varies dramatically throughout literature. According to the meta-analysis of Van Groenigen et al. (2018), total P content in casts varied between a ~50% reduction and a 3-4 fold increase compared to the bulk soil and soil P tests which are commonly used to measure plant-available P, including P-Olsen, P-Bray I and II, P-Mehlich, and P-Truog, varied between a ~30% reduction and a 8-9 fold increase. The increase in cast P content when measured with water, dilute salt solutions or resin extractable P, varies from a factor 2 or 3 compared to the bulk soil (Sharpley and Syers, 1976; Kuczak et al., 2006) to several hundreds of times (Vos et al., 2014; Ros et al., 2017). The large variation in the extent to which cast P content increases may be largely ascribed to methodological differences among studies in the extraction of P, while differences in physico-chemical soil properties and earthworm species possibly play a role as well. In the aforementioned meta-analysis (Van Groenigen et al., 2019), no conclusive explanation was found for the mechanisms behind the increase in available P, and the contribution of each of the pathways mentioned previously still remains elusive. A systematic study to the different controlling factors of P availability in earthworm cast is still lacking, as well as a comparison between different earthworm species under comparable experimental conditions. The latter is important since different earthworm species vary strongly in their feeding and burrowing behaviour, resulting in possible different effects on P availability.

Here we study the differences in earthworm-induced increased P availability for eight earthworm species that are common to the Netherlands. We focus on three P pools that differ in their availability to grass. The objectives of this study are (i) to assess the effect of different earthworm species on the amount of readily-available P in casts; (ii) to quantify the variation in cast physico-chemical properties that can affect the availability of P, so as to explore the pathways that govern the earthworm-induced effect on readily-available P; and (iii) to relate our findings to current knowledge on earthworm ecology.

2. Materials and methods

2.1 Soil collection and physico-chemical soil properties

For this experiment an acidic sandy soil from the top layer (0-25 cm) of an extensively managed pasture with low soil P status was collected in Achterberg, the Netherlands (51°59′32″N, 5°35′2″E) in November 2016. The soil can be classified as a plaggic podzol (IUSS Working Group WRB, 2015). For the determination of relevant physico-chemical properties, soil was air-dried and sieved (2 mm). Soil particle size distribution was determined by the sieve and pipette method (Houba et al., 1997) and the OM content was estimated from loss-on-ignition in a muffle furnace (550 °C). Soil pH was measured in a 0.01 M $CaCl_2$ extract, which was obtained at a soil to solution ratio of 1:10 (w:v) and shaken for 2 h on a linear shaker at 180 strokes min^{-1} (spm) (Houba et al., 2000).

To determine the soil P status, an extraction with acetic acid and ammonium lactate was carried out (P-AL) (Egnér et al., 1960). With this method, P contained within the soil solution plus a part of P that is reversible adsorbed to the surface of metal (hydr)oxides is extracted (Schoumans and Groenendijk, 2000). In the Netherlands, P-AL is routinely used as a soil extraction method in agricultural practice to determine the soil P status for the P fertiliser recommendation system of grassland (Reijneveld et al., 2010). The total amount of P reversibly adsorbed to reactive surfaces of metal (hydr)oxides (P_{ox}) and the amounts of reactive Fe- and Al-(hydr)oxides were determined by extraction with 0.2 M acid ammonium oxalate (Schwertmann, 1964). The P, Fe, and Al concentrations in the extracts were measured with inductively coupled plasma - atomic emission spectroscopy (ICP-AES; Varian Vista Pro). Further analytical details on the measurement of P_{ox} are given in section 2.4. Based on these data, the P loading of reactive metal (hydr)oxides (α) was calculated according to Van der Zee and Van Riemsdijk (1988):

$$\alpha = P_{ox} / (Al_{ox} + Fe_{ox}) \qquad (1)$$

where P_{ox}, Al_{ox} and Fe_{ox} are the concentrations of P-, Al- and Fe- (hydr)oxides expressed in mmol kg^{-1}. Total and organic soil P were determined according to Kuo (1996). In short, inorganic soil P was determined by digestion of soil with 0.5 M H_2SO_4, followed by measuring ortho-P according to the molybdenum blue method (Murphy and Riley, 1962) using segmented flow analysis (SFA; Skalar, SAN^{++}). Consequently, OM was ignited in a muffle furnace, after which the soil was digested again with 0.5 M H_2SO_4 and ortho-P was determined to obtain total soil P. Organic P was calculated as the difference between total and inorganic soil P.

2.2 Experimental setup

Our pot experiment was set up as a fully randomized block design with earthworm species (nine experimental treatments: seven single species, one obligatory mixture of two species and a control without earthworms) as treatment. Each treatment was replicated three times, resulting in a total of 27 pots. The replicates were distributed over three blocks and both the location of the blocks and the setup of the pots within the blocks were randomized weekly. We used Mitscherlich pots (diameter: 20 cm; height: 23 cm) filled with 7 kg air-dried, sieved (5 mm) and homogenised soil. The soil was fertilised with N (70 kg ha^{-1} equally divided over N-NH_4 and N-NO_3 as $(NH_4)_2SO_4$, NH_4Cl, KNO_3 and $Ca(NO_3)_2$), K (70 kg ha^{-1} as KNO_3), S-SO_4 (12 kg ha^{-1} as $(NH_4)SO_4$), Ca (50 kg ha^{-1} as $Ca(NO_3)_2$ and $CaCl_2$), and trace elements (2.5 μg B pot^{-1}, 2.5 μg Mn pot^{-1}, 0.1 μg Cu pot^{-1}, 0.25 μg Zn pot^{-1} and 0.05 μg Mo pot^{-1}). De-ionised water was added to obtain a water holding capacity (WHC) of 60%. After the pots were filled with the fertilised soil, a small unfertilised seedbed of 0.5 kg was added on top, in which 4 g of perennial ryegrass (*Lolium perenne*) seeds were sown. The grass was pre-grown for a period of 35 days before the earthworms were added to the pots. During the pre-growing period of the grass, it was cut twice 5 cm above the soil surface (two weeks after sowing and one day prior to the addition of the earthworms). The cuttings were shredded and left on the soil surface. Pots were watered with de-ionised water every other day through a watering pipe in the centre of the pots. Once a week, the soil moisture content was adjusted to exactly 60% of WHC by watering the pots gravimetrically. After the addition of the earthworms, all pots were covered with elevated nylon nettings, which permitted light entry and grass growth, but prevented the earthworms from escaping.

The earthworm treatments consisted of eight species commonly occurring in the Netherlands: two epigeic species: (i) a mixture of the compost earthworms *Dendrobaena veneta* (Rosa, 1886) and *Eisenia fetida* (Savigny, 1826) (these earthworms could only be purchased in a mixture and it was impossible to separate them alive), (ii) *Lumbricus rubellus* Hoffmeister, 1843; four endogeic species: (iii) *Allolobophora chlorotica (*Savigny, 1826) (both the green and the pink morph), (iv) *Aporrectodea caliginosa* (Savigny, 1826), (v) *Aporectodea rosea* (Savigny, 1826), and (vi) *Octolasion lacteum* Örley, 1881; and two anecic species: (vii) *Aporrectodea longa* (Ude, 1895) and (viii) *Lumbricus terrestris* Linnaeus, 1758, as well as (ix) a control without earthworms (Table S1). Earthworm density for the different treatments was (i) 47 (1496), (ii) 25 (796), (iii) 25 (796), (iv) 48 (1528), (v) 12 (382), (vi) 36 (1146), (vii) 16 (509), (viii) 10 (318) and (ix) 0 earthworms pot^{-1} (earthworms m^{-2}) respectively. Preliminary experiments were conducted to obtain estimates for the cast production of the different species at densities (all species combined) that are commonly observed in pastures in the Netherlands (Didden, 2001). When this estimate proved to be sufficient for chemical analysis, a comparable earthworm density was used in the experiment, i.e., species (vii) and (viii). For the other species (i, ii, iii, iv, and vi) higher densities were used because normal field densities were expected to yield insufficient cast material. This was also expected for species (v), but collection of this species was difficult and we added all earthworms we could find.

Earthworms were collected from fields and park areas in and near Wageningen, the Netherlands. Exceptions were the mixture of compost earthworms (purchased from Berendsen Wormenkwekerij, Markelo, the Netherlands), *O. lacteum* (collected in orchards in Galicia, Spain) and *L. terrestris* (purchased from Star Food Holland BV, Barneveld, the Netherlands). Before their addition to the pots, earthworms were kept in dark mesocosms under controlled conditions (16 °C) with poplar (*Populus* ssp L.) leaves as feed. Only adult specimens were used for our pot experiment. After their intestines had been voided for 48 h according to the wet filter paper method of Dalby et al. (1996), the earthworms were added to the pots.

2.3 Sample collection

Five days after the earthworms had been added to the pots, they were collected. For this purpose, the soil was removed from each pot while limiting damage to the rooting system of the grass as much as possible, followed by the separation of the earthworms from the soil by hand. Afterwards, the soil with the rooting system and grass was carefully placed back in the pot. Earthworm casts were collected according to Vos et al. (2014). In short: earthworms were rinsed with water to remove any adhering soil particles and the casts used for chemical analysis were excreted during the next 48 h when the earthworms were left in 100 mL polyethylene cups. Twice a day the cups were gently shaken by hand to activate the earthworms. The cast material was collected in porcelain crucibles, which were subsequently sealed airtight and stored at 3 °C to prevent desiccation of the cast. The earthworms were reintroduced in the pots and the described procedures of earthworm and cast collection were repeated five days later. Within this same time interval, however, casts had to be collected for a third time for the smallest earthworm species, *A. rosea* (i.e., species (v)), in order to obtain as much cast as possible for chemical analysis. Earthworms from this species were therefore collected two days after they had been reintroduced into the pots, followed by cast collection. They were again reintroduced for a third time, one day before the final collection of all earthworm species. The collected casts were added to those previously stored and mixed thoroughly before chemical analysis.

Samples of moist bulk soil from the control pots were homogenized and stored at 3 °C until all cast material was collected and chemical analysis of all samples was conducted at the same time.

2.4 Chemical analyses

We used three soil P tests to determine different P pools in the control bulk soil and earthworm casts: (i) P extractable with acid ammonium oxalate (P_{ox}) to quantify the total pool of reversibly adsorbed P (Hiemstra et al., 2010); (ii) a modified P-Olsen based method, which represents P in soil solution plus a part of P reversibly adsorbed to reactive mineral surfaces (Hiemstra et al., 2010) and (iii) water-extractable ortho-P as a proxy of the amount of readily-available P in the soil solution (Torrent and Delgado, 2001; Koopmans et al., 2006).

Subsamples of the moist bulk soil from the control pots and earthworm cast of all species were dried for 24 h at 40 °C and used to determine the moisture content. Next, these subsamples were grinded to pass a 2 mm sieve and homogenised.

The amounts of P_{ox}, Fe_{ox} and Al_{ox} were determined according to Schwertmann (1964) by adding dried material to a solution of 0.2 M acid ammonium oxalate (pH=3.0) at a solid to solution ratio of 1:20 (w:v). The suspensions were horizontally shaken for 2 h at 180 spm in a dark room at 20 °C. Subsequently, the suspensions were centrifuged for 10 minutes at 3000 rotations min^{-1} (rpm) and the P, Fe, and Al concentrations in the supernatants were measured with ICP-AES.

The P-Olsen method (Olsen et al., 1954) was modified according to Hiemstra et al. (2010): 0.2 g dried material and 0.2 g washed powdered active carbon were added to 0.5 M $NaHCO_3$ (pH=8.5) at a solid to solution ratio of 1:50 (w:v). The suspensions were shaken for 255 h (~10 days) at 30 spm on a horizontal shaker at 20 °C. After centrifuging for 10 min at 3000 rpm, the suspensions were filtered over a 0.45 µm filter (NC 45, Whatman), acidified with HCl to pH=2.0, and put in an ultrasonic bath to remove the released CO_2. The ortho-P concentration was subsequently measured by SFA using the molybdenum blue method (Murphy and Riley, 1962).

In order to differentiate between the feeding sources of the earthworms, the isotopic signature of the ingested carbon (C) was analysed. Therefore a part of the dried subsamples was sent to the stable isotope facility of UC Davis (CA, USA) to determine the total C and $\delta^{13}C$ content of the soil and cast material. Samples were combusted at 1080 °C in a reactor packed with tungsten (VI) oxide and copper oxide. Subsequently, oxides were removed in a reduction reactor, after which the He carrier flowed through a water trap of $Mg(ClO_4)_2$. N_2 and CO_2 were separated with a molecular sieve adsorption trap before measurement with a micro cube elemental analyser (Elementar Analysensysteme GmbH) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd.) (Stable Isotope Facility UC Davis).

All other analyses were performed with moist material since drying and rewetting of soil has been shown to increase the release of P and DOC in water and weak salt extracts (Koopmans et al., 2006; Koopmans and Groenenberg, 2011). In order to create a water extract as a proxy of the soil solution, ultra-pure water (UPW) was added to the moist soil and cast material at a solid to solution ratio of 1:10 (w:v; based on dry weight) after a correction had been made for the moisture content at 40 °C. The suspensions were shaken horizontally for 24 h with 75 spm at 20 °C. Subsequently, they were centrifuged for 10 min at 3750 rpm and the supernatants were filtered through a 0.45 µm filter (NC45, Whatman). The ortho-P concentration in these water extracts was measured according to the molybdenum blue method (Murphy and Riley, 1962) by SFA. The pH and electrical conductivity (EC) were measured after which EC was converted to ionic strength (I) according to Griffin and Jurinak (1973). Concentrations of DOC were calculated as the difference between total dissolved carbon (TC) and inorganic carbon (IC), which were both measured by SFA by infrared detection of the produced CO_2 after acidification with 0.38 M H_3PO_4 (IC) or after persulphate and tetraborate oxidation under ultraviolet light followed by acidification with 0.45 M H_2SO_4 (TC) (Houba et al., 2000). The aromaticity of DOC was assessed by measuring light absorbance at 254 nm (A_{254}) as described in Vos et al. (2014). Since ferric iron absorbs light at the same wavelength as DOC (Weishaar et al., 2003), Fe concentrations in the water extracts were measured using high resolution - inductively coupled plasma - mass spectrometry (Thermo Scientific Element 2) and used to correct the value of A_{254} to $A_{254\text{-cor}}$:

$$A_{254\text{-}cor} = A_{254} - 0.085\, Fe + 0.0007 \qquad (2)$$

with A_{254} and $A_{254\text{-cor}}$ in cm^{-1} and Fe in mg l^{-1}. Subsequently the degree of aromaticity of DOC (% aromatic DOC of total DOC) was estimated (Weishaar et al., 2003):

$$Aromaticity = 3.63 + 6.52 \times (100 \times A_{254\text{-}cor} / DOC) \quad (3)$$

with DOC in mg C l^{-1}. The concentration of NH_4^+ in the water extracts was determined by SFA by a combination of Gas Dialysis and the Berthelot method (Ros et al., 2011).

2.5 Statistical analyses

Analysis of variance (ANOVA) was performed in IBM SPSS statistics version 23 after testing the data for normality and homogeneity of variance of the residuals. Earthworm species was used as an independent factor and block as a random factor in order to determine significant differences at $p<0.05$. When significant differences were observed, Tukey’s honestly significant difference test was carried out as a post-hoc test. Statistical test were performed either among all earthworm species (excluding soil) or between an earthworm species and the control bulk soil.

3. Results

3.1 Physico-chemical soil properties and earthworm survival

The physico-chemical properties of the soil used in this study are shown in Table 1. The soil is an acidic sandy soil with 1.6% clay and 4.2% OM. The P loading of the reactive metal (hydr)oxides was relatively low and based on the P-AL value, the P status of the soil can be classified as 'low' (Reijneveld et al., 2010).

Earthworm survival differed per earthworm species, but was overall 83% after the final earthworm collection (results not shown). Even though casts of *A. rosea* were collected three times, all replicates had to be pooled for chemical analysis. Therefore, replicates were no longer present and this species could not be included in any statistical test. Because the collected amount of cast was still insufficient to measure all variables, only those measurements that required a small amount of material or were measured in the same sample as another variable (P_{ox}, Al_{ox}, Fe_{ox}, pH, moisture content, ionic strength, C content and $\delta^{13}C$) could be measured.

3.2 Physico-chemical properties of bulk soil and earthworm casts

The casts of all earthworm species had a significantly higher P_{ox} content than the control bulk soil (Fig. 1a). There was, however, no significant variation among the earthworm species. Since the total content of reactive metal (hydr)oxides measured as Fe_{ox} (Fig. S1f) and Al_{ox} (Fig. S1g) was for none of the earthworm species significantly different from that of the control bulk soil, this leads to a significantly higher value of the molar P_{ox} / (Fe_{ox} + Al_{ox}) ratio (P loading of reactive metal (hydr)oxides, α) in earthworm casts than in the control bulk soil (Fig. S1h).

For P-Olsen and water-extractable ortho-P, the amounts of readily-available P extracted from earthworm casts were higher than those extracted from the control bulk soil. Differences among earthworm species were present for P-Olsen (Fig. 1b; $p<0.001$), but these differences were even more pronounced for water-extractable ortho-P (Fig 1c; $p<0.001$). The highest ortho-P concentrations were measured in the water extracts of the casts of *L. rubellus* (6.21 ± 0.53 mg l^{-1} or to 62.1 ± 5.3 mg kg^{-1}), which were two orders of magnitude higher than those of the control bulk soil (0.06 ± 0.005 mg l^{-1} or 0.6 ± 0.05 mg kg^{-1}). This indicates that in the case of *L. rubellus* more than 14% of P_{ox} was present as water-extractable ortho-P, while in the control bulk soil this was only 0.2%. The lowest ortho-P concentrations in cast were observed in the water extracts of the casts of *A. chlorotica* (0.95 ± 0.05 mg l^{-1} or 9.5 ± 0.5 mg kg^{-1}), but these ortho-P concentrations were still more than 15 times higher than those in the control bulk soil and consisted of more than 2.5% of P_{ox}.

The pH of the water extracts of earthworm casts was for all species significantly higher than for the control bulk soil (Fig. 2a) and varied significantly among species (p=0.003). Similarly, the DOC concentrations in the water extracts of the casts of different earthworm species were one order of magnitude higher compared to those of the control bulk soil (Fig. 2b) and there was significant variation in the DOC concentrations among the earthworm species (p<0.001). The pattern of variation of the DOC concentrations resembled that of the variation in the ortho-P concentrations (Fig. 1c and 2b). According to Fig. 3, DOC and ortho-P are linearly related with a R^2 of 0.72 (p<0.001).

Significant variation among earthworm species was observed for the total C content of the earthworm casts (p<0.001). However, only for *A. chlorotica* the total C content of the casts was significantly higher than that of the control bulk soil (Fig. 2c). The isotopic signature of the egested C, $\delta^{13}C$, differed significantly among earthworm species (p<0.001), with the cast of endogeic species and compost worms generally enriched in ^{13}C, and those of the two anecic species and the epigeic species *L. rubellus* depleted in ^{13}C compared to the control bulk soil (Fig. 2d).

For moisture content, ionic strength, NH_4^+ and IC, significant variation among earthworm species was present as well. With the exception of *O. lacteum*, earthworm casts contained more moisture than the control bulk soil (Fig. S1a). The ionic strength and the NH_4^+ and IC concentrations in the water extracts of the casts were for all earthworm species significantly higher than for the control bulk soil (Fig. S1b, S1c and S1d). No significant variation among earthworm species was observed for the aromaticity of DOC in the water extracts of the casts, but the percentage of aromatic DOC was for all earthworm species significantly lower than for the control bulk soil (Fig. S1e).

4. Discussion

4.1 Phosphorus in earthworm casts

The low P status (P-AL) of the soil used in our pot experiment is the result of low or absent P application rates over the last three decades. Other soil properties such as pH and the contents of OM and Fe- and Al-(hydr)oxides are within the range found by Koopmans et al. (2006) for a large series of agricultural acidic sandy topsoils in the Netherlands.

For all studied earthworm species, the levels of P_{ox} in the earthworm casts are significantly higher compared to the control bulk soil, but the extent to which P_{ox} increased in casts did not differ significantly among the earthworm species (Fig. 1a). This suggests that an elevated P_{ox} content is a common trait among the earthworm species that were studied here.

An increased amount of P_{ox} can lead to elevated levels of both pools of readily-available P (i.e., P-Olsen and water-extractable P) because of buffering of these readily-available P forms via desorption of reversibly adsorbed P. It can therefore explain (part of) the increase of ortho-P in water extracts of earthworm casts. However, the modelling study of Ros et al. (2017) has shown that the contribution of the increase in P_{ox} to the elevated levels of water-extractable P from earthworm casts is relatively small. These modelling results therefore suggest that multiple pathways will contribute to earthworm-induced increased P availability.

In the present study, P-Olsen values in the casts of *L. rubellus* increase to a larger extent than those in the casts of the other earthworm species (Fig. 1b), while the concentrations of water-extractable ortho-P are even more variable among the studied earthworms (Fig. 1c). Both the absolute value of the ortho-P concentrations that have been measured in the water extracts of casts, and the corresponding earthworm species, when placed in order of decreasing ortho-P concentrations (i.e., *L. rubellus* > *L. terrestris* > *A. caliginosa*), are in agreement with previous findings (Vos et al., 2014; Ros et al., 2017). The observed variation among earthworm species in the amounts of readily-available P as measured with P-Olsen and water-extractable ortho-P clearly demonstrate that earthworm species differ in their ability to alter the extent to which P_{ox} is present in readily-available P forms.

In summary, our results show that P availability is elevated in earthworm casts compared to the control bulk soil. However, the magnitude of this earthworm-induced effect on P availability differs for the P pool considered and the variation in the amounts of readily-available P (i.e., water-extractable ortho-P and P-Olsen) is much larger than for P that is reversibly adsorbed to reactive surfaces of metal (hydr)oxides and therefore only potentially available to plants (i.e., P_{ox}).

4.2 Pathways for earthworm-induced increased P availability

An earthworm-induced increase of P availability can have multiple contributing mechanisms. These will be discussed according to the pathways mentioned in section 1.

- Elevated pH: The pH may directly affect the concentration of ortho-P in the water extracts (pathway (i) in section 1). Our results show a significantly higher pH in earthworm casts compared to the control bulk soil (Fig. 2a), which is in agreement with literature (Sharpley and Syers, 1976; Barois and Lavelle, 1986; Basker et al., 1994; Kuczak et al., 2006; Vos et al., 2014; Ros et al., 2017; Van Groenigen et al., 2019). A pH increase in earthworm casts may be due to mineralisation of organic N to NH_4^+ and the accompanying consumption of protons (Basker et al., 1994). Mineralisation also results in the release of HCO_3^- which increases the pH as well.

Very few studies reported pH differences in casts of different earthworm species under similar experimental conditions, because mostly they did not study multiple species. However, the observed variation among earthworm species in our experiment agrees with the results of Basker et al. (1994) who reported a significantly higher pH in casts of *L. rubellus* than of *A. caliginosa*.

The increased pH contributes to an increase in the dissolved ortho-P concentration that is in equilibrium with the adsorbed P pool of the casts. This increase is mainly due to a change in the speciation of ortho-P, as can be illustrated by writing the overall adsorption equilibrium reaction as: Soil + $H_2PO_4^-$ (aq) ⇔ Soil-H_2PO_4. In the water extracts of the control bulk soil (pH~6.6), ortho-P is predominantly present as $H_2PO_4^-$, but at an increase of the pH (on average pH~7.7 in earthworm casts), the concentration of the species $H_2PO_4^-$ decreases due to proton dissociation: $H_2PO_4^-$ (aq) ⇔ HPO_4^{2-} + H^+ (aq) ($pK_c = 6.9$ at I=0.01 M). This leads to desorption of ortho-P in an attempt to maintain the above adsorption equilibrium. Consequently, the total dissolved ortho-P concentration increases strongly and at a change of the pH by 1 unit this increase can be a factor 10. However, the interaction of Ca and ortho-P for binding to soil should be considered as well. An increase of pH will increase the binding of Ca to soil (Rietra et al., 2001), which will bring positive charge to the soil surface and thereby stimulate the binding of ortho-P, which contributes to a decrease of the ortho-P concentration in solution. The overall change in the ortho-P concentration will be the result of both opposing effects.

Although the increase in the ortho-P concentration as a result of the elevated pH in earthworm casts can potentially be large (up to a factor 10), even such an increase is not enough to explain the observed increase in the ortho-P concentrations in this study (factor 16 to 105, Fig. 1c). This agrees with the results of Ros et al. (2017), who showed by modelling their results of a pot experiment, that an increase in pH similar to that measured in our study, will have only a limited effect on ortho-P solubility. Their modelling results are supported by a weak correlation between ortho-P and pH for our data (R^2=0.32; p=0.0046). The large increase of the ortho-P concentration observed in our study can therefore not be understood from the change in pH alone.

- Competitive adsorption of DOC: The observed increase in DOC concentrations in the water extracts of the earthworm casts compared to the control bulk soil (Fig. 2b) is in agreement with literature (Bolan and Baskaran, 1996; Vos et al., 2014; Ros et al., 2017). Elevated levels of DOC can be caused by stimulated mineralisation inside the earthworm gut or excretion of DOC-rich substances by the earthworms (e.g. mucus) (Lopez-Hernandez et al., 1993). Following pathway (ii) in section 1, DOC can compete with ortho-P for binding sites on reactive mineral surfaces, which results in desorption of ortho-P to the solution. A strong correlation between the concentrations of DOC and ortho-P in the water extracts of the earthworm casts and control bulk soil is found (Fig. 3), which emphasizes the importance of the link between both variables. The results of our study thereby corroborate the findings of Ros et al. (2017), who suggested that competitive desorption of DOC and ortho-P may be an important pathway for earthworm-induced increased P availability in earthworm casts.

Contradictory to the quantity of DOC in water extracts of earthworm casts, the quality of DOC was found to be similar for all earthworm species when the percentage of aromatic DOC is considered (Fig. S1e). For all species DOC in the water extracts of the earthworm casts is less aromatic compared to the control bulk soil. A lower aromaticity of cast DOC is in line with literature, although the magnitude of this effect varies (Vos et al., 2014). Such a lower percentage of aromatic DOC has been related to a larger contribution of hydrophobic neutral and hydrophilic compounds (which have a lower molecular weight) to the total DOC concentration (Swietlik and Sikorska, 2006). These smaller and more aliphatic compounds increase the susceptibility of DOC in earthworm casts towards mineralisation compared to the DOC of the control bulk soil.

- <u>Stimulated mineralisation of organic P:</u> Le Bayon and Milleret (2009) suggested stimulated mineralisation of organic P as a possible explanation for the observed increase of the levels of P_{ox} in earthworm casts compared to the control bulk soil. Increased mineralisation rates in earthworm casts have been observed in literature by the measurement of an increased activity of phosphatases, the enzymes that catalyse the mineralisation process of organic to inorganic P (Satchell and Martin, 1984). The present study did not yield sufficient cast material to measure the activity of phosphatases or otherwise assess the mineralisation rate in earthworm casts directly. Such measurements would provide valuable information on the importance of this pathway (pathway (iii) in section 1) to earthworm-induced increased P availability. Therefore, we recommend that direct measurements of the mineralisation rate in earthworm casts, like measuring the activity of phosphatases, will be addressed in future studies.

It is likely that stimulated mineralisation occurred in our study. The conditions inside the earthworm gut are strongly favourable for a high mineralisation rate: (i) a large microbial community (Tiunov and Scheu, 2000; Drake and Horn, 2007); (ii) a large availability of moisture: we measured a higher moisture content in fresh earthworm casts than in the control bulk soil (Fig. S1a) which is often encountered in literature and related to the saturation of fresh casts with mucus (Clause et al., 2014). It cannot be ruled out for this study that a high excretion of mucus is due to stressful experimental conditions, especially in the case of *A. rosea*; and (iii) a large availability of organic substrates: earthworms feed on organic C-rich material which was provided by the OM present in the soil (Table 1), but also amply available through the growing grass and the decomposing grass cuttings that were left on the soil surface after previous grass cuts. One may expect that much of the ingested organic C is used for the metabolism of the earthworms, since the organic C content of the egested cast material was for most earthworm species not significantly different from that of the bulk soil (Fig. 2c).

Mineralisation can be illustrated by the following representative reaction equation:

$$(CH_2O)_mNH_3 + m\ O_2 \Leftrightarrow NH_4^+ + HCO_3^- + (m\text{-}1)CO_2 + (m\text{-}1)H_2O \qquad (4)$$

This reaction equation shows that stimulated mineralisation of OM inside the gut of earthworms results in increased excretion of NH_4^+. A strong increase in the concentration of this monovalent cation is found in the water extracts of casts compared to the control bulk soil (Fig. S1c), which is also reported in literature (Ros et al., 2017). The production of NH_4^+ as a result of stimulated mineralisation leads to an increase in the ionic strength by one order of magnitude (Fig. S1b) and a strong correlation (R^2=0.98; p<0.001) is found between the ionic strength and the NH_4^+ concentration.

Following equation (4), another consequence of stimulated mineralisation is the increased production of inorganic carbon (IC). Our data show that the IC concentrations are enhanced in the water extracts of earthworm casts (Fig. S1d). At the observed pH level, IC is mainly present as HCO_3^- (Weng et al., 2011). Although the bicarbonate anion may also compete with ortho-P for binding sites on reactive metal (hydr)oxides, its competitive strength is relatively low compared to DOC (Weng et al., 2012). As IC contributes only 8-23% to the TC concentration in the water extracts of earthworm casts, its direct contribution to the release of ortho-P through a competition effect will be limited.

The mineralisation products NH_4^+ and HCO_3^- are produced according to a 1:1 molar ratio (equation (4)). Our data show that both ions are indeed well correlated ($R^2=0.83$; $p<0.001$), indicating their simultaneous production and corroborating the suggestion of stimulated mineralisation. However, NH_4^+ and HCO_3^- are not produced in the expected ratio, because the production of NH_4^+ is about twice the production of HCO_3^- (Fig. S1c and S1d). This might be due to partial neutralization of HCO_3^- by acid from the soil that was ingested by the earthworms, as the relatively low pH of the soil (pH=6.0) can cause buffering of HCO_3^- by protons that are bound to the cation exchange complex of the soil.

Although our data suggest that stimulated mineralisation of OM did occur in the present study - which will lead to an increase in P_{ox} - this pathway most likely does not explain the entire increase in P_{ox} in the earthworm casts. If soil OM would be the sole source of this extra P and the molar C/P ratio of the decomposed OM equals our experimental value (Table 1; with a C mass fraction of 0.58, 42 g OM kg^{-1} = 24.4 g C kg^{-1} = 2.0 mol C kg^{-1}; P_{org} = 99 mg P kg^{-1} = 3.2 mmol P kg^{-1}; so the molar C/P ratio = 635), decomposition of approximately 50 g OM kg^{-1} soil is required (average ΔP_{ox} = 117 mg P kg^{-1} (Fig. 1a) = 3.8 mmol P kg^{-1}, which according to the calculated molar C/P ratio of the OM yields 2.4 mol C kg^{-1}). This is impossible as our soil contains only 42 g OM kg^{-1} (Table 1). If the amount of organic P in the OM as a whole is not enough to explain the elevated levels of P_{ox}, an additional mechanism that contributed to the increase of P_{ox} must have been present.

- <u>Selective feeding:</u> The observed increase in P_{ox} in earthworm casts compared to the control bulk soil can also be caused by an increased intake of P due to selective feeding of the earthworms (pathway (iv) in section 1). Selective feeding can occur on different sources of organic C, which both result in an elevated intake of P: (i) a real external source of OM in the form of growing grass (roots) and organic residues, or (ii) a soil-related source of organic C in a matrix of OM with embedded metal (hydr)oxide nanoparticles (Hiemstra et al., 2010) of different sizes. In the latter case, no external source of OM is required for explaining a higher P intake by the earthworms, if the earthworms preferentially feed on soil OM containing relatively small oxide particles. Calculations show that, at the same Fe_{ox} and Al_{ox} content of the control bulk soil and ingested soil (Fig. S1f and S1g), only a subtle difference in particle size can explain the experimentally observed large difference in the amount of P_{ox} between the control bulk soil and earthworm casts (see Fig. S2 for a more detailed discussion). This corresponds with our results that show that the ratio $\alpha = P_{ox} / (Fe_{ox} + Al_{ox})$ was indeed significantly higher for the earthworm casts than for the control bulk soil (Fig. S1h).

Selective feeding on fine soil particles was already mentioned by Sharpley and Syers (1976), who observed increased proportions of fine soil particles (<4 μm) in earthworm casts in a silt loam soil. At the same time, these authors observed elevated amounts of both inorganic and organic P in earthworm casts as compared to the surrounding bulk soil. Sharpley and Syers (1976) attributed this to incorporation of P from the soil, plants or dung, into the earthworm casts. Following our above given explanation of smaller metal (hydr)oxide particles having a higher P loading than larger metal (hydr)oxide particles, it is also likely that these smaller metal (hydr)oxide particles are responsible for the P enrichment of earthworm casts measured by Sharpley and Syers (1976). However, these authors did not measure particle size down to nano-size which could have supported this hypothesis.

Since the earthworms used in this study belong to different functional groups and are therefore assumed to feed on different food sources, we measured the isotopic signature of the egested C (Fig. 2d) in order to determine on which C source selective feeding occurred. Fresh OM contains more of the lighter ^{12}C and has therefore a more negative value of $\delta^{13}C$, while older OM is relatively enriched in the more heavy ^{13}C (Bernoux et al., 1998). Such older OM is often associated to soil particles and is therefore the expected form of OM in which metal (hydr)oxide nanoparticles are embedded. All of the studied endogeic earthworm species produced casts that are enriched in ^{13}C with respect to the control bulk soil, which agrees with feeding on relatively old, soil-associated OM. In order to reach the measured amount of P_{ox} in earthworm casts, these earthworms should have fed selectively on the smaller (hydr)oxide particles according to mechanism (ii) of selective feeding described previously. For the anecic species and the epigeic *L. rubellus*, ^{13}C contents that are lower than the ^{13}C content of the control bulk soil were found, indicating that these earthworms fed on fresh OM (e.g. growing grass (roots) and organic residues). P_{ox} in their casts is likely increased according to the aforementioned mechanism (i) of selective feeding. Our data therefore show that the earthworms in our study selectively feed on different C sources, including either an external source of organic C or a soil-related source of organic C in a matrix of OM with embedded metal (hydr)oxide nanoparticles. Since the levels of P_{ox} are not significantly different among the earthworm species, selective feeding on both these C sources results in an equal contribution to P_{ox}.

In summary, our study clearly shows that there is a large variation among earthworm species in many of the physico-chemical properties of their casts which can affect the availability of P. Changes in solution conditions (pH and DOC) influence the ortho-P binding process directly, which also applies for the ortho-P loading of the cast surfaces (higher P_{ox} and α) as the result of selective feeding or P release by mineralisation. Earthworm-induced increased P availability is therefore the result of multiple pathways: (i) elevated pH; (ii) competitive adsorption of DOC; (iii) stimulated mineralisation of organic P; and (iv) an increased intake of P due to selective feeding of the earthworms. In order to semi-quantify our results we carried out multiple linear regression in R statistical software version 3.2.1 (R core team 2015; packages usdm, MASS and relaimpo; both forward and backward selection of variables; lmg metric). To assess how much of the total variance in ortho-P could be accounted for by the different pathways we used the following variables as proxies: (i) elevated pH: pH, (ii) competitive adsorption: DOC and IC (iii) stimulated mineralisation: C content and (iv) selective feeding: P_{ox}. Of these five parameters, pH showed a high level of collinearity (VIF>4), leading to its removal from te model. The model that best described our data was:

$$\text{Ortho-}P = 0.048 * DOC + 0.013 * P_{ox} - 0.35 * C\ content - 0.10 * IC + 2.77 \qquad (5)$$

which explained R^2=84% of the total variance observed in ortho-P. The partial contributions of each of the included parameters were 42% for DOC, 28% for P_{ox}, 8% for C content and 6% for IC. This calculation therefore agrees with the modelling study of Ros et al. (2017) and confirms the important role of competitive adsorption between DOC and ortho-P for P availability.

However, based on the results of our study, no conclusive evidence on the exact importance of each of the pathways can be given. This means that further research to this topic is required, which should especially focus on the importance of each of the discussed pathways.

4.3 Classification of earthworm species

The isotopic signature of the egested organic C can be considered as a proxy for the type of OM the earthworms feed on. As discussed in section 4.2, Fig. 2d indicates that all of the studied endogeic earthworm species ingested relatively old, soil-associated OM and the anecic species and the epigeic *L. rubellus* fed on fresh OM. This is in agreement with the feeding habit of these earthworm functional groups as mentioned by Bouché (1977). The only exception is the value of $\delta^{13}C$ of the casts of the mixture of compost earthworms. Since compost worms are classified as epigeic, a relatively low value of $\delta^{13}C$ would be expected based on this classification, but the opposite was measured. However, during the experiment, the compost earthworms lived in a habitat that is unnatural for these species (soil instead of compost) and it is therefore possible that they have shown unnatural behaviour.

While the isotopic C signature matches with the conventional classification of earthworms into functional groups, the different P pools and other physico-chemical properties of earthworm cast that are measured in this experiment, do not. With the exception of P_{ox} and the aromaticity of DOC, all measured physico-chemical properties vary among the studied earthworm species, but the pattern of variation does not correspond with that of their functional groups. This agrees with the meta-analysis of Van Groenigen et al. (2019) that concludes that conventional earthworm classification in three main functional groups does not affect any of the soil fertility properties that they considered. The only exception was a marginally significant effect of functional groups on the amount of available P that was measured as P-Olsen, P-Bray or comparable soil P tests. However, this could not be explained satisfactorily with the collected database, especially since the results were compiled for a large variety of experimental conditions and a suite of different soil P tests. In our present study, which followed a much more standardized approach with respect to experimental conditions and soil chemical analyses, we do not find an effect of earthworm functional groups on readily-available P, indicating that the observed effect in the meta-analysis is likely an artefact. We conclude that, based on the most comprehensive experimental study to date, conventional ecological earthworm classification in functional groups is not able to explain the observed differences in readily-available P or any other of the measured physico-chemical cast properties. However, readily-available P and many other physico-chemical cast properties vary strongly among earthworm species, which implies that the composition of the earthworm community, rather than only its size, is key to optimizing P availability to plants. Future studies should therefore also consider the effect of combinations of earthworm species (which could possibly stimulate each other’s effect on P availability) in the search of using to the fullest the capacity of earthworms to increase P availability to plants.

5. Conclusions

All studied earthworm species similarly increased the size of the oxalate extractable P pool. However, differences among species were observed in the more readily-available pools of P-Olsen and water-extractable ortho-P. The measured variation among earthworm species clearly demonstrates that earthworm species differ in their ability to alter the extent to which P_{ox} is present in readily-available P forms.

Earthworm-induced increased P availability is the result of multiple pathways: : (i) elevated pH; (ii) competitive adsorption of DOC; (iii) stimulated mineralisation of organic P; and (iv) an increased intake of P due to selective feeding. No conclusive evidence on the exact importance of these pathways could be given here, and therefore further research is required.

Conventional ecological earthworm classifications do not explain the observed large variation in readily-available P and other measured physico-chemical cast properties. This implies that earthworm community composition, rather than only its size, is key to optimizing P availability to plants.

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Figure captions

Figure 1. Physico-chemical parameters in bulk soil and earthworm cast: P_{ox} (mg kg^{-1}; a), P-Olsen (mg kg^{-1}; b) and water-extractable ortho-P (mg kg^{-1} on the primary y-axis and in mg l^{-1} on the secondary y-axis; c). Letters indicate significant differences among earthworm species and asterisks show the level of significance between earthworm casts and bulk soil. Error bars are denoted as SE and colours indicate earthworm functional groups (light grey: epigeic; grey: endogeic; dark grey: anecic).

Figure 2. Physico-chemical parameters in bulk soil and earthworm cast: pH (a), DOC (mg l^{-1}; b), C content (mg kg^{-1}; c) and δ^{13}C (‰; d). Letters indicate significant differences among earthworm species and asterisks show the level of significance between earthworm casts and bulk soil. Error bars are denoted as SE and colours indicate earthworm functional groups (light grey: epigeic; grey: endogeic; dark grey: anecic).

Figure 3. Correlation between the concentrations of DOC (mg l^{-1}) and ortho-P (mg l^{-1}) in the water extracts of the cast and bulk soil samples.

Table 1. Physico-chemical properties of the soil used in this study.

	Soil
Sand [%]	87.7
Silt [%]	6.0
Clay [%]	1.6
OM [g kg^{-1}]	42
pH - $CaCl_2$	6.0
P-AL [mg P_2O_5 100 g^{-1}]	24
Total P [mg kg^{-1}]	343
Organic P [mg kg^{-1}]	99
P_{ox} [mg kg^{-1}]	272
Fe_{ox} [mmol kg^{-1}]	11.3
Al_{ox} [mmol kg^{-1}]	60.6
α*	0.12

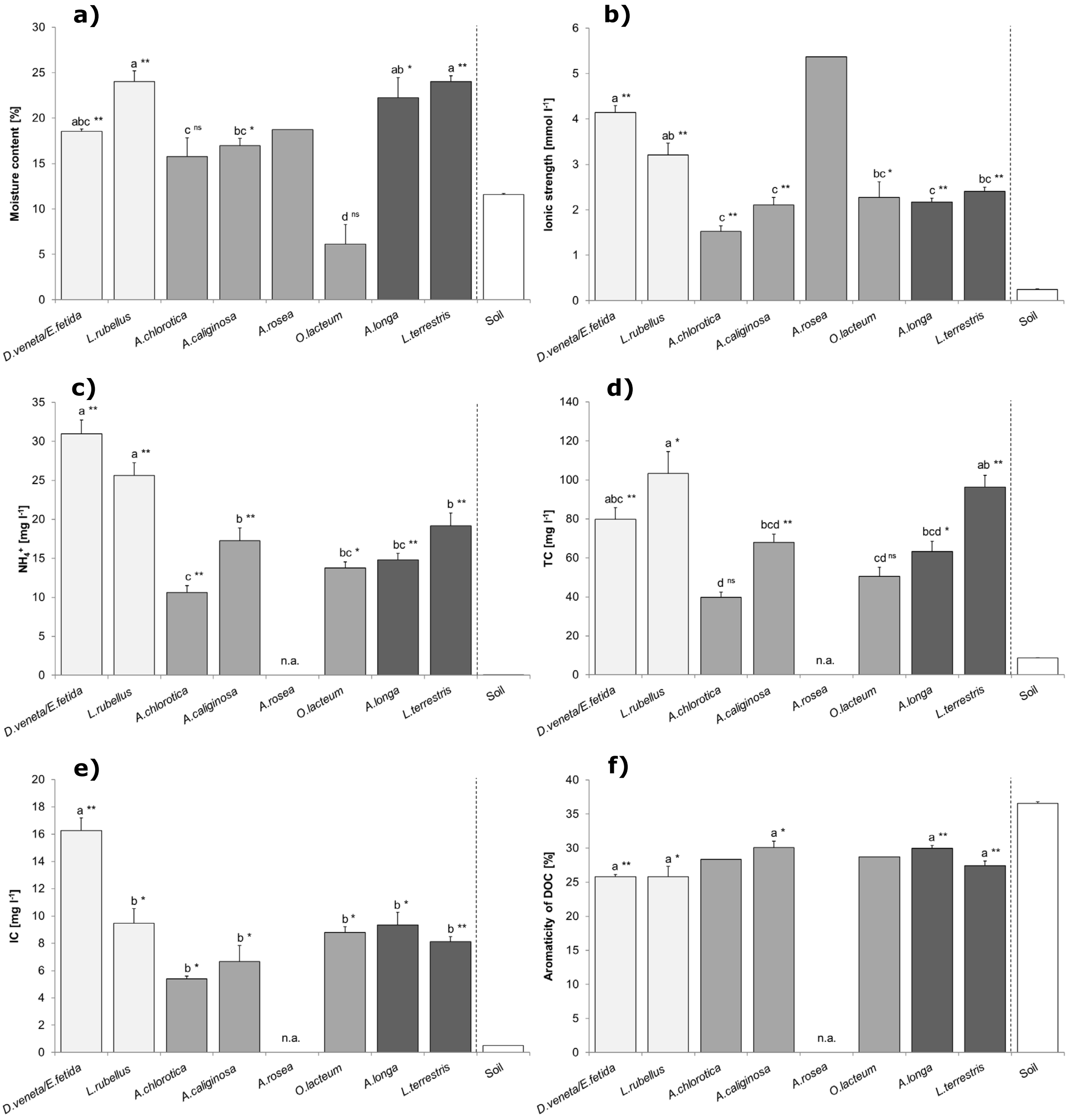
* P loading of reactive metal (hydr)oxides

Supporting Information

Table S1. Description of the earthworm species used in this study (information based on Sims and Gerard (1985)).

Functional group	**Earthworm species**	**Length (mm)**	**Diameter (mm)**	**Colour**	**Habitat**
Epigeic (compost)	*Dendrobaena veneta*	50-155	4-8	Pink with transverse dark stripes	Compost, decaying leaves, manure, rich organic soils
Epigeic (compost)	*Eisenia fetida*	60-120	3-6	Pink with transverse dark stripes	Compost, rotting vegetation, manure, rich organic soils
Epigeic	*Lumbricus rubellus*	60-130	3-4	Brownish / purplish red	Wide range of habitats with high organic content; typical for grasslands
Endogeic	*Allolobophora chlorotica*	30-80	3-7	Green and pink morph	Pastures, gardens, cultivated land, near roots
Endogeic	*Aporrectodea caliginosa*	40-180	3.5-7	Pale pink / whitish grey	Pastures, gardens, cultivated land
Endogeic	*Aporrectodea rosea*	25-85	2-6	Rosy pink with orange clitellum	Pastures, gardens, woodlands
Endogeic	*Octolasium lacteum*	35-160	2.5-6	Whitish grey, yellow spot on tail	Pastures, gardens, orchards
Anecic	*Aporrectodea longa*	90-170	4-9	Purplish black head, pale grey tail	Gardens, pastures, woodland
Anecic	*Lumbricus terrestris*	90-350	6-10	Brownish / purplish red head, orange grey tail	Undisturbed grasslands and orchards

Figure S1. Physico-chemical parameters in bulk soil and earthworm cast: moisture content (%; a), ionic strength (mmol l^{-1}; b), NH_4^+ (mg l^{-1}; c), TC (mg l^{-1}; d), IC (mg l^{-1}; e) aromaticity of DOC (%; f), content of Fe-(hydr)oxides (mg kg^{-1}; g), content of Al-(hydr)oxides (mg kg^{-1}; h) and the P loading of metal (hydr)oxides (α) (-; i). Letters indicate significant differences among earthworm species and asterisks show the level of significance between earthworm casts and bulk soil. Error bars are denoted as SE and colours indicate earthworm functional groups (light grey: epigeic; grey: endogeic; dark grey: anecic).



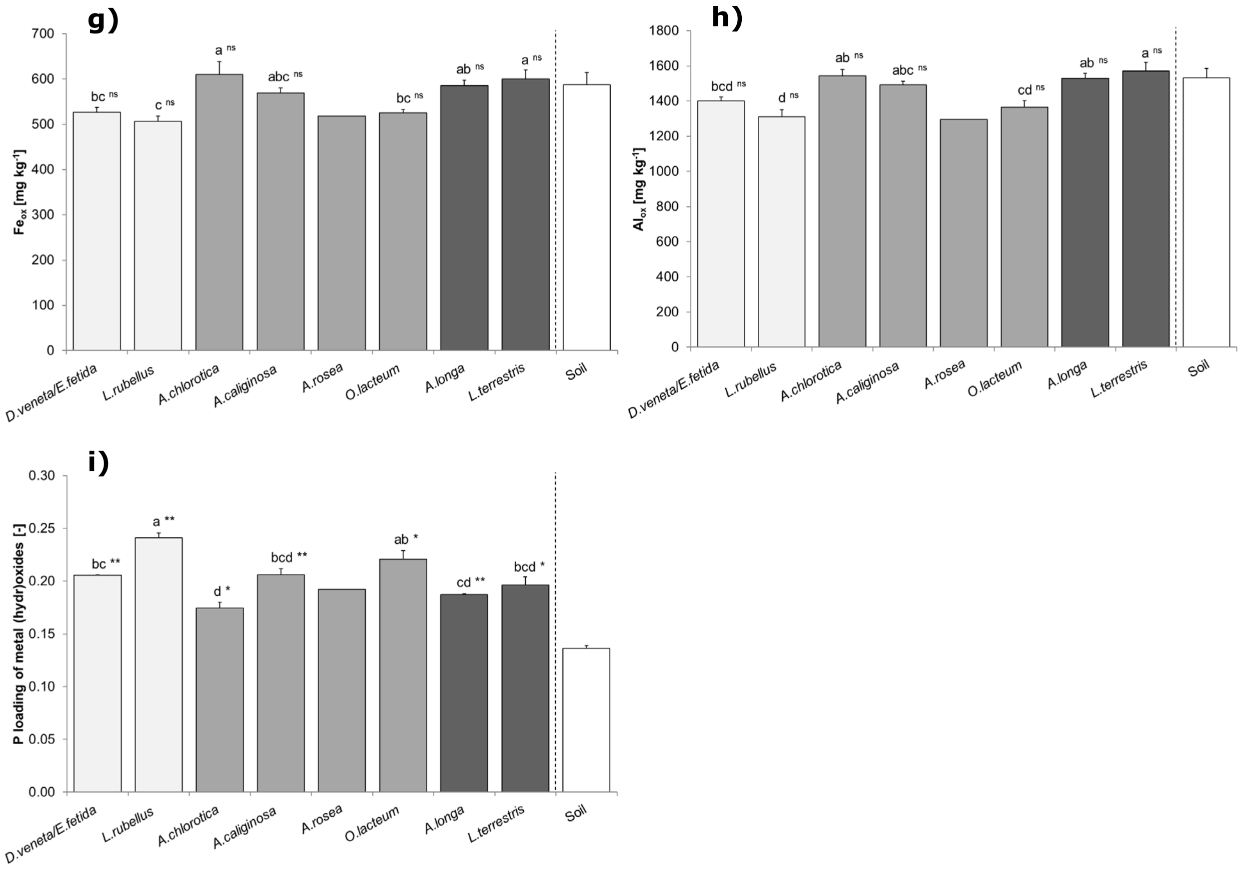
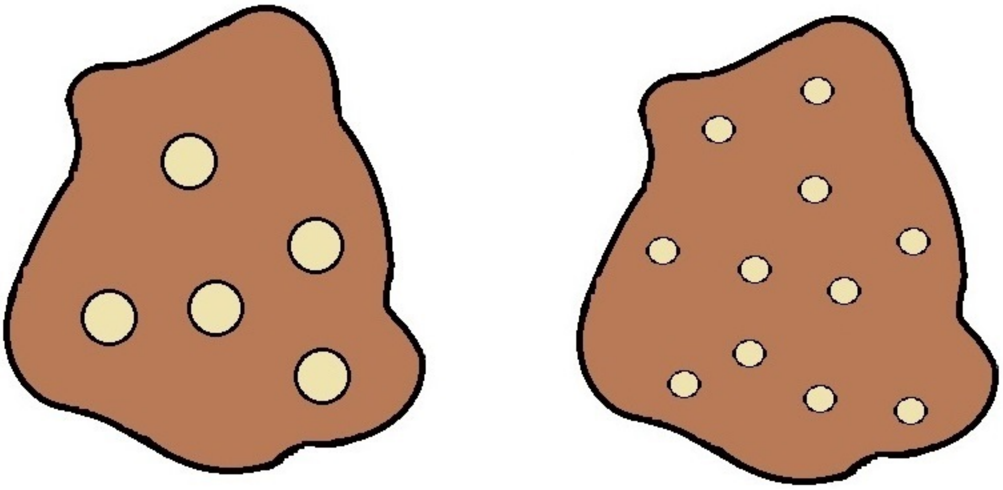

g)
Fe$_{ox}$ [mg kg^{-1}]
0
100
200
300
400
500
600
700
bc ns
c ns
a ns
abc ns
bc ns
ab ns
a ns
h)
Al$_{ox}$ [mg kg^{-1}]
0
200
400
600
800
1000
1200
1400
1600
1800
bcd ns
d ns
ab ns
abc ns
cd ns
ab ns
a ns
i)
P loading of metal (hydr)oxides [-]
0.00
0.05
0.10
0.15
0.20
0.25
0.30
bc **
a **
d *
bcd **
ab *
cd **
bcd *
D.veneta/E.fetida
L.rubellus
A.chlorotica
A.caliginosa
A.rosea
O.lacteum
A.longa
L.terrestris
Soil

Figure S2. Two matrices of OM with embedded metal (hydr)oxide nanoparticles representing the average soil (left) and earthworm cast (right). In a heterogeneous soil, part of the material may include aggregates containing smaller oxide particles than the mean. If earthworms feed selectively on these smaller particles, the specific surface area (A_{mean}) of the metal (hydr)oxide particles they ingest will be higher, allowing these particles to adsorb more P per unit mass. As the same equilibrium P concentration in solution applies to both systems (average soil and the part of the soil the earthworms feed on, both considered at the moment of ingestion), the particles will have the same P adsorption density expressed as the amount of P adsorbed per unit m^2 reactive surface of metal (hydr)oxide (Γ). In the calculations used for this figure, the chosen value of Γ = 2.3 µmol m^{-2} is within the range of Γ values observed for a large series of agricultural top soils (Hiemstra et al., 2010). As our data show the same oxide content (Fe_{ox} and Al_{ox} in µmol g^{-1}) in both systems, our calculations demonstrate that even a subtle difference in the mean particle size of the metal (hydr)oxides in two matrices (d_{mean}=2.5 nm for the average soil and d_{mean}=2.0 nm for earthworm cast) can already explain the significant difference in the amount of P reversibly bound per unit oxide mass (ɑ value) measured in this study. Hence, selective feeding of the earthworms on specific soil fractions with smaller oxide particles can therefore result in a higher P_{ox} content of earthworm casts.



	Average soil	Earthworm cast
d_{mean} [nm]	2.5	2.0
A_{mean} [m^2 g^{-1}]	640	850
Γ [µmol PO_4 m^{-2}]	2.3	2.3
α* [-]	0.14	0.20

* $\alpha = P_{ox} / (Fe_{ox} + Al_{ox}) \approx (\Gamma * A_{mean}) / (Fe_{ox} + Al_{ox})$

Numbers are based on calculations according to the Supporting Information of Hiemstra (2018)