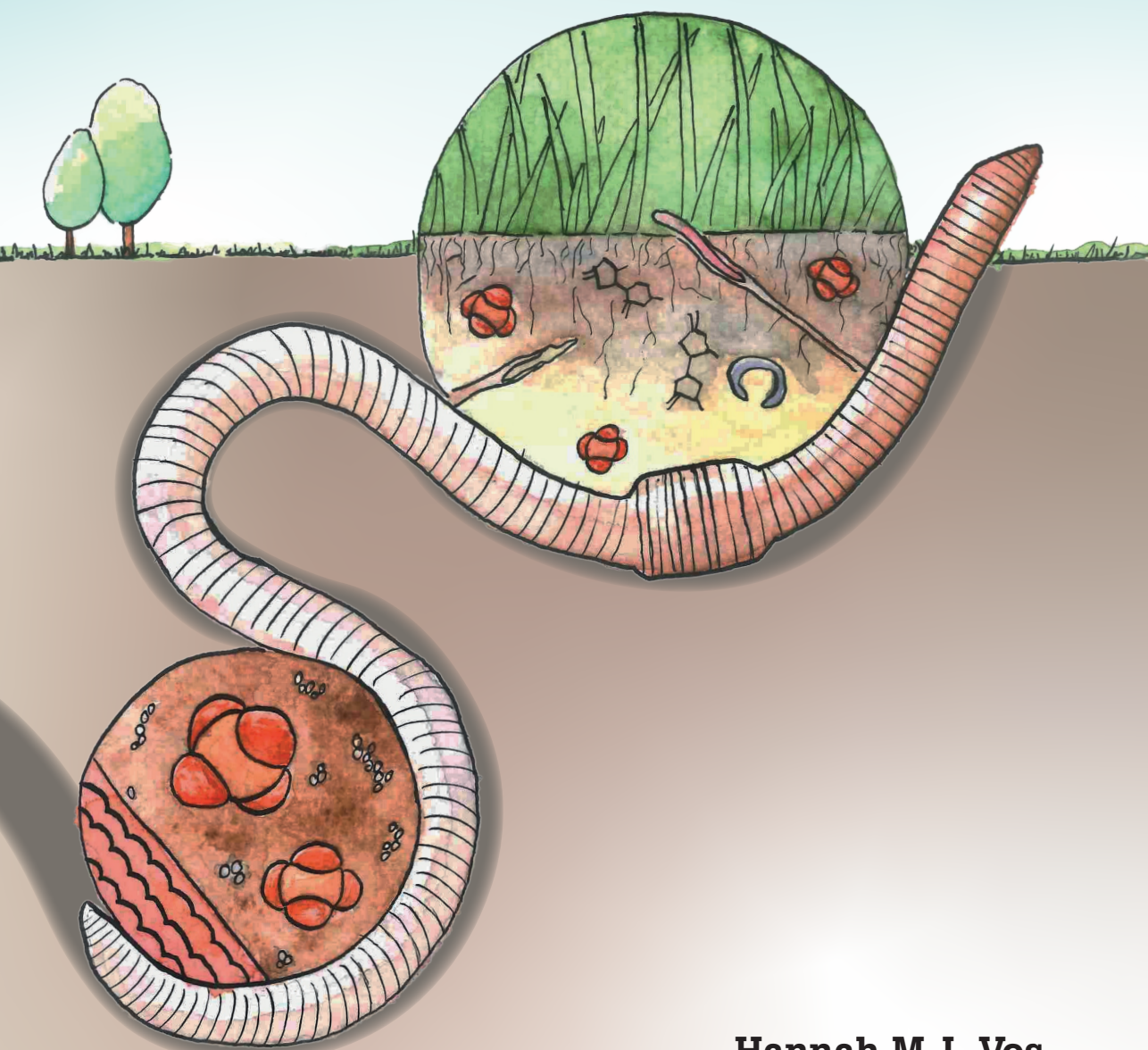


Earthworm-enhanced phosphorus availability in soil

from unravelling the mechanisms to assessing its significance



Hannah M.J. Vos

Propositions

1. Mineralisation inside the gut drives earthworm-enhanced phosphorus availability both directly and indirectly.
(this thesis)
2. Distinguishing between classical earthworm feeding guilds has no additive value in understanding the effect of earthworms on phosphorus availability.
(this thesis)
3. Progressive insight is both a driver of science and a barrier to publish its advances.
4. Optimal conditions for fundamental scientific research contradict with the current demand for fast results.
5. The physical properties of hydrogen make it an ineffective energy carrier for ships.
6. To combat climate change, emission quotas must be adopted for leisure activities.
7. Scientists are like cats: it takes long periods of patience interspersed with moments of quick action to catch results.

Propositions belonging to the thesis, entitled

*Earthworm-enhanced phosphorus availability in soil:
from unravelling the mechanisms to assessing its significance*

Hannah M.J. Vos
Wageningen, 24 June 2022

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Thesis

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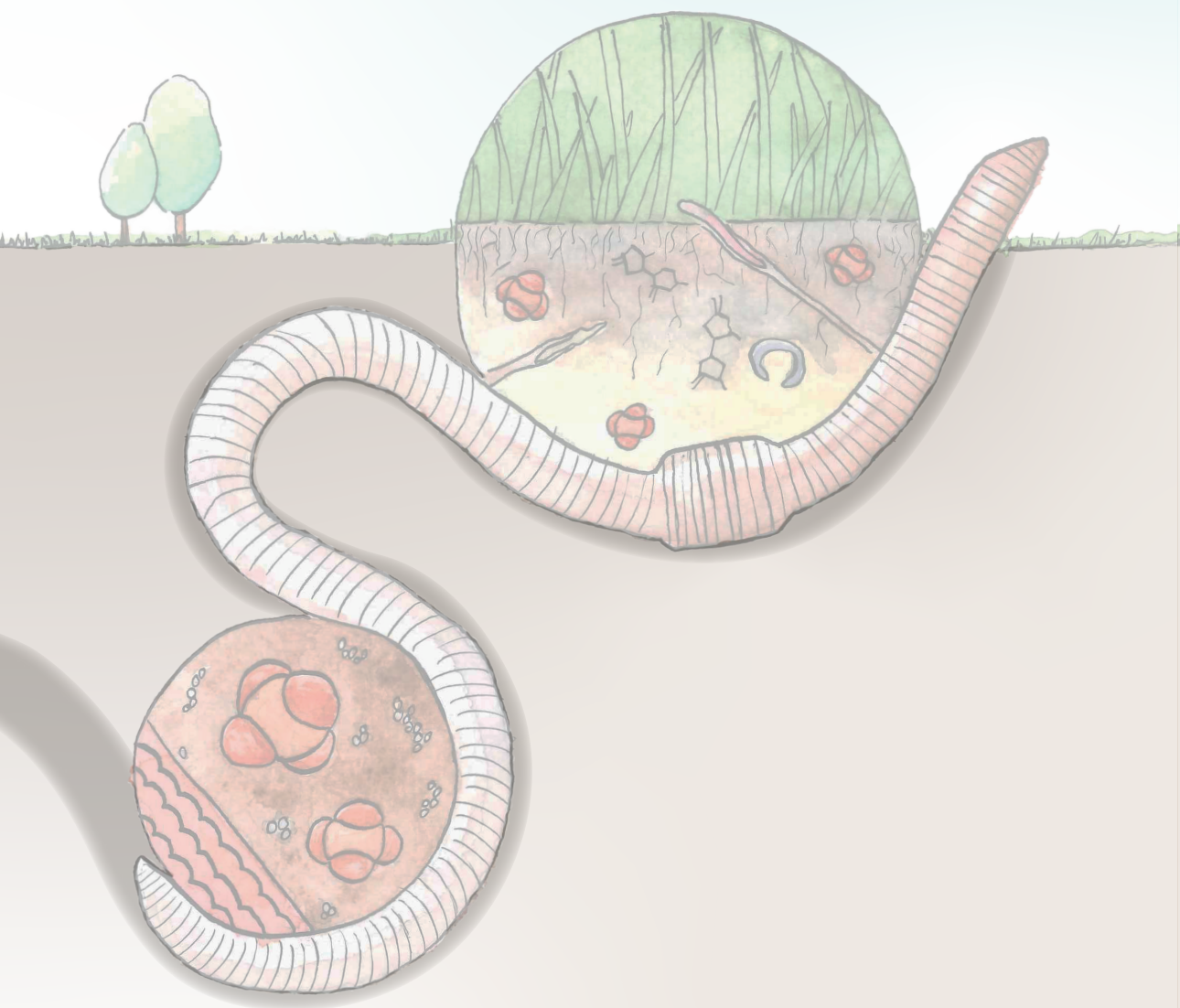
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Chapter 1

General introduction

Hannah M.J. Vos

1.1 Introduction

Phosphorus (P) is **fundamental to life on earth**: it is in our DNA, our cell membranes and bones, and it is an important constituent for the transport of energy through our bodies (Hawkesford et al., 2012; Hesterberg, 2010; Meharg and Marschner, 2012; Vance et al., 2003). Because of this, we need to ingest sufficient amounts of P through our food, and the first step for the entrance of P into our food chain is when it is taken up from soil by arable crops and animal feed. With the current increase of the world population and the changing human diet towards an increasing consumption of animal products, food production needs to grow, and thereby our need for P will increase (Godfray et al., 2010; Sattari et al., 2012; Tilman et al., 2011). Since P cannot be manufactured or substituted by any other element in the many different biomolecules in living organisms, we have to rely completely on P already present on earth (Ashley et al., 2011).

Generally P is not rare in soils – it is the 11th most common element on earth (CRC Handbook of Chemistry and Physics, 2016) – but similar to the ancient Greek myth of Tantalus who was punished by Zeus to suffer from eternal hunger and thirst while standing in a pool of water almost within reach of overhanging branches of a fruit tree, most of the P in soil is beyond the reach of plant roots and soil biota. The availability of P in soil for plant uptake can be very low, as P binds strongly to mineral soil constituents (Hesterberg, 2010; Morel et al., 2000). The resulting P-deficiency can be a serious restriction for plant growth in many soils, which is often ameliorated by the addition of animal manure or mineral P fertiliser. In agriculture, these external P inputs are essential to counterbalance the removal of P by produce, achieve or maintain adequate yields, and support a sustainable intensification of agriculture (Sattari et al., 2016). However, animal manure and mineral P fertiliser are not always available or financially affordable in sufficient amounts. Moreover, mineral P fertilisers are obtained from phosphate rock and the reserves of the economically extractable part of this resource are declining. Although the predictions of the lifetime of phosphate rock reserves vary strongly, this resource is finite and will become depleted in the future (Cordell et al., 2009; Koppelaar and Weikard, 2013; Sattari et al., 2012; Syers et al., 2008; Van Vuuren et al., 2010). This is considered as one of the most **serious threats to global food security**.

Despite declining reserves, P fertiliser is not always used optimally in agriculture (Ros et al., 2020; Shepherd et al., 2016). Excessive or inappropriate use of P can result in P losses to the environment, which can pose high environmental risks of eutrophication of water bodies and loss of biodiversity (Buda et al., 2012; Carpenter et al., 1998; Sawyer, 1966; Schoumans and Groenendijk, 2000). The safe **planetary boundaries** for P use are already crossed and human influence on the P cycle may substantially destabilise the planet (Steffen et al., 2015).

The current challenges associated with agricultural P use enforce us to search for more sustainable forms of plant P nutrition. To realise this, agricultural practices should focus on reducing external P inputs by better utilising soil P with a yet limited availability

to plants (Rowe et al., 2016; Sattari et al., 2012). Many mechanisms to **increase the sustainability of P use** are currently explored. A hitherto little studied mechanism is based on the many ecosystem services provided by soil fauna, especially earthworms, although it is known that these soil invertebrates can largely affect P cycling (Le Bayon and Milleret, 2009; Ros et al., 2017). In this thesis, I will further explore how earthworms can contribute to a more sustainable form of P use by sustaining optimal P acquisition for plants, thereby reducing the need for external P fertiliser inputs through increasing the level of readily plant-available P in soil. In my work, I will use a combination of experiments at different scales (i.e., greenhouse pot and field scale) and surface complexation modelling, which will be focussed on the Netherlands. In this first chapter, I will review scientific literature, outline existing knowledge gaps and present the aims of my thesis. Much of the information presented here is based on an explorative study by Vos et al. (2014), which findings were an important motivation to study this topic more in depth.

1.2 Phosphorus

1.2.1 Phosphorus in soils

The **global distribution of total soil phosphorus** varies largely from 35 to 5300 mg P kg⁻¹ soil, with a median value of 800 mg P kg⁻¹ soil (Hesterberg, 2010). Approximately 30-65% of this P is present in organic form and is a part of soil organic matter, present in microbial biomass, or in organic molecules like phytic acid, phospholipids or DNA (Hesterberg, 2010; Turner et al., 2002) (Fig. 1.1). The remainder of soil P (35-70%) is present as inorganic P (Hesterberg, 2010), and Menezes-Blackburn et al. (2018) reported a global average value of 57±7% for the inorganic P fraction in 258 arable soils and pastures. Inorganic P constitutes P bearing minerals as well as P adsorbed to e.g., soil metal-(hydr)oxides or clay minerals. The ratio between organic and inorganic P can be affected by P fertilisation. In soils that have received long-term inputs of mineral P fertiliser or animal manure the fraction of inorganic P is usually increased (Dou et al., 2009; Koopmans et al., 2007; Lehmann et al., 2005). In the Netherlands, the focus area of my research, the pool of total soil P increased during the 20th century because of heavy animal manure applications as a consequence of agricultural intensification. This resulted in an average P accumulation in Dutch agricultural soils of approximately 2050 kg P ha⁻¹ in the top 0.5 m (Schoumans and Chardon, 2015). The average global pool of total soil P is estimated at 1762 kg P ha⁻¹ in the first 15 cm, however this value is based on soils in North America, Europe and Oceania, and the much smaller values reported for soils in South America, Africa and Asia were excluded due to their small sample size (Menezes-Blackburn et al., 2018).

The total amount of P in a soil can be divided into different **P pools** (Fig. 1.1). Most of these P pools are not readily-available for plant uptake, but via several processes P can be converted from one pool to another. Such processes can involve soil formation and

weathering in which P minerals like calcium (Ca) phosphates dissolve (reverse process: precipitation). As the phosphate anion has a pH-dependent charge which is negative in the natural pH range of most soils, it can bind strongly to positively charged soil metal-(hydr)oxides like iron (Fe) or aluminium (Al) (hydr)oxides, oxidic edges of clay minerals, Ca carbonates, and negatively charged ligands of soil organic matter via polyvalent metal cation (e.g., Al^{3+} and Fe^{3+}) bridging (Gérard, 2016; Gerke, 2010; Hinsinger, 2001; Mendez et al., 2020; Schoumans, 2014; Weng et al., 2011).

Soils are strongly heterogenous and contain of a wide variety of interacting constituents that affect the solubility of P. Dissolved organic matter (DOM) in the soil solution, which has a pH-dependent negative charge, can compete with phosphate for binding sites on metal-(hydr)oxides, and therefore the presence of DOM in solution results in an elevated solubility of phosphate (Geelhoed et al., 1998; Hiemstra et al., 2010b; Hiemstra et al., 2013; Nagarajah et al., 1970; Weng et al., 2012). Cations like Ca, however, can facilitate the adsorption of P onto metal-(hydr)oxides, resulting in a decrease of the phosphate concentration in solution (Mendez and Hiemstra, 2020; Rietra et al., 2001; Weng et al., 2012). Moreover, adsorption processes are strongly pH-dependent. Although

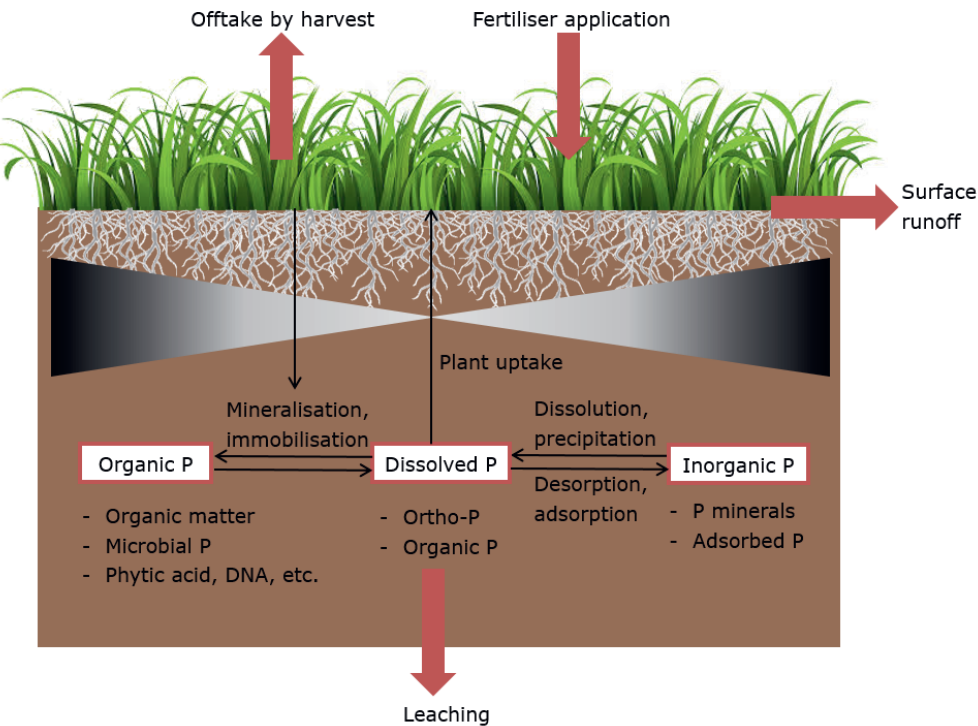


Figure 1.1 General overview of the different P pools (boxes) in the soil. Red arrows are the main inputs and outputs of P to the soil system while the black arrows signify flows of P. The triangular gradient shows the size of each P pool (the higher, the larger) and its availability to plant uptake (the lighter in colour, the more readily plant-available).

the maximum phosphate concentration in soil solution is often estimated at pH 6.5 (Lindsay, 1979; Price, 2006), the true maximum differs per soil and is the result of the combination of many soil factors (Barrow, 2017) (e.g., Weng et al. (2011) observed a minimum in P solubility at neutral pH for six agricultural topsoils from the Netherlands). Because of the many interactions between P and other soil constituents, as well as the strong dependency of P solubility on factors like soil pH and ionic strength, P chemistry in soils is complex and the outcome varies largely between soils.

Many of the factors influencing soil P chemistry differ among **soil types**, and therefore the type of soil has a large effect on dissolved P in the soil solution. As the soil map of the Netherlands displays many different soil types (Soil Atlas of Europe, 2005), large differences in the natural dissolved P concentration in soils can be expected. This natural dissolved P concentration can be estimated from the P concentration of deep groundwater (>13 m) as this is supposedly unaffected by agricultural practices due to the high P adsorption of the overlying soil (Schoumans and Chardon, 2015). In the Netherlands, the P concentration of deep groundwater varies strongly with soil type: the highest soluble P concentrations occur in calcareous clay soils (>1.0 mg P L⁻¹) and the lowest in non-calcareous sands (<0.1 mg P L⁻¹) (Schoumans and Chardon, 2015).

1.2.2 Phosphorus uptake by plants

Phosphorus is a key nutrient for many functions in plants (e.g., energy generation, photosynthesis and membrane synthesis) (Ashley et al., 2011; Hawkesford et al., 2012; Hesterberg, 2010; Meharg and Marschner, 2012; Vance et al., 2003). Plant roots take up P in the form of HPO_4^{2-} and H_2PO_4^- (orthophosphate, hereafter referred to as 'ortho-P') from the small pool of soil P present in dissolved form in the **soil solution** (Hawkesford et al., 2012; Hesterberg, 2010). However, as P binds strongly to the soil mineral phase, the amount of P in the soil solution of the top 30 to 60 cm of the soil, which is readily-available for plant uptake, is in general only 0.5% - 1.5% of the amount required by plants during a growing season (Hesterberg, 2010; Morel et al., 2000). The remainder is supplied by the soil solid phase via buffering processes, such as desorption or dissolution of P (section 1.2.1) over the course of the growing season (Nawara et al., 2017; Syers et al., 2008). Furthermore, mineralisation of soil organic matter, microbial biomass and plant residues can contribute to plant P uptake (Hesterberg, 2010; McLaughlin et al., 1988). A low ability of the soil to buffer the solubility of ortho-P at a sufficiently high level can therefore result in P-limiting growth conditions for plants (Sattari et al., 2012). Plant P-availability thus depends on both the ortho-P concentration in soil solution and the rate at which the soil can replenish ortho-P after roots have depleted its concentration in soil solution (soil P buffering capacity) (Nawara et al., 2017).

Plants have evolved their own adaptations to P-limited soil conditions. These **P acquisition strategies of plants** are diverse and comprise the entire 'root economic space' (Bergmann et al., 2020). They can encompass changes in root architecture (e.g., root

(hair) length and root/shoot ratio), as this affects the rooted soil volume exploited by plants in their search for P (Hill et al., 2006; Lynch, 1995; Lynch, 2007; Marschner et al., 2006; Ramaekers et al., 2010). Additionally, increased P uptake by plants can be realised when plants form a symbiosis with arbuscular mycorrhizal fungi (AMF) in which plant-produced carbon (C) from photosynthesis is 'traded' for P taken up by these fungi from the larger exploited soil volume of the combined plant-AMF system compared to the soil volume exploited by plants (Parniske, 2008; Richardson et al., 2009). Furthermore, plant roots can excrete organic acids (which can increase the solubility of soil P by inducing competition for adsorption sites for P, or lower the rhizosphere pH and increase dissolution of phosphate-bearing minerals), excrete phosphatases (enzymes catalysing the mineralisation of organic P to readily plant-available P), or stimulate plant growth-promoting microorganisms (Marschner et al., 2006; Ryan et al., 2001; Wang and Lambers, 2020).

1.2.3 Characterization of soil phosphorus

For characterizing the different forms and quantities of P in soil, a suit of approaches can be used. In order to assess the agronomic P-status of a soil, soil P tests are traditionally used to chemically extract the fraction of the total P pool from soil which is available for plant uptake. Based on the result of such soil P tests, the P fertility level of the soil can be classified, and P fertiliser recommendations can be given. Many countries and regions have their own soil P test and classification system, due to differences in soil type, climate, fertilisers and crops, as well as economic and technical analytical constraints (Jordan-Meille et al., 2012; Pierzynski, 2000; Ros et al., 2020; Sharpley et al., 2012). In addition to agronomic soil P tests, methods are available for the selective chemical extraction of reactive soil constituents like metal-(hydr)oxides and P associated with these oxides. Additionally, the size of specific P pools in soil, like P solubility in 0.01 M CaCl₂ soil extracts, can be quantified with surface complexation modelling (SCM) (Hiemstra et al., 2013; Weng et al., 2011). Furthermore, spectroscopic techniques like solution ³¹P Nuclear Magnetic Resonance spectroscopy (³¹P-NMR) and X-ray Absorption Near Edge Structure spectroscopy (XANES) can be used for identifying inorganic and organic P forms in soil (Koopmans et al., 2003; Koopmans et al., 2007; Schmieder et al., 2018). The presence of enzymatic phosphatase activity can be used to reflect biological processes in soil, as these enzymes are related to mineralisation of organic P (Toor et al., 2003). In my research, I used a selection of these approaches, e.g., various P extraction methods, SCM and enzymatic phosphatase activity analysis, and those will be discussed below.

Chemical extraction methods

Agronomic soil P tests can generally be distinguished in soil P intensity methods and soil P quantity methods (Nawara et al., 2017), although the difference between the two can be ambiguous. The combination of soil P intensity and soil P quantity forms the

potentially available P pool for plant uptake (Nawara et al., 2017). Soil P intensity methods are related to the amount of available P for plant uptake on the short or medium-short (e.g., a growing season) term. Extractions with either water or dilute salt solutions (e.g., 0.01 M CaCl_2) employed at a relatively low solution-to-soil ratio (SSR) can be used as a proxy for the amount of readily plant-available P in the soil solution, as they are intended to mimic soil solution conditions (Houba et al., 2000). In contrast, soil P quantity methods are related to the total (non-occluded) P pool in soil which is responsible for buffering P in soil solution. A variety of soil P tests exist to estimate the soil P quantity-related pool (Pierzynski, 2000), for instance P-Olsen (0.5 M NaHCO_3 ; Olsen et al., 1954), Mehlich III (the extracting solution consists of various components like NH_4F , HNO_3 and EDTA; Mehlich, 1984) and Bray P1 (mixture of HCl and NH_4F ; Bray and Kurtz, 1945). For determining the agronomic soil P status within the context of the Dutch P fertiliser recommendation scheme and the so-called P application standards from the Dutch manure policy (section 1.3.1) in the recent past Pw was used for arable land and P-AL for grassland. The Pw is based on an extraction of soil with water at a high SSR of 60 L kg^{-1} soil (Sissingh, 1971), whereas P-AL is based on a soil extraction with ammonium lactate (Egnér et al., 1960). Nowadays, both the P fertiliser recommendation scheme of arable crops and grassland and the P application standards are based on a combined measurement of 0.01 M CaCl_2 -extractable P as an intensity method and P-AL as a P quantity method as this combination accounts for the effect of soil P buffering (Van Rotterdam et al., 2012).

Methods for the **selective chemical extraction** of metal-(hydr)oxides and associated P include an extraction of soil with dithionite-citrate-bicarbonate (DCB) (Holmgren, 1967; Mehra and Jackson, 1960) or acid ammonium oxalate (AO) (Schwertmann, 1964). While the AO extraction method extracts non-crystalline metal-(hydr)oxides as well as associated P, the DCB extraction method extracts both non-crystalline and crystalline metal-(hydr)oxides from soil as well as the associated P. The P associated with crystalline metal-(hydr)oxides can be considered as largely occluded P, whereas P associated with non-crystalline metal-(hydr)oxides represents reversibly adsorbed P (Mendez et al., 2020; Mendez et al., 2022). With the AO extraction method, Fe (Fe_{ox}), Al (Al_{ox}) and P (P_{ox}) are simultaneously extracted and measured and can be used to calculate the P saturation index (α) as the molar ratio of P_{ox} against $[\text{Fe}+\text{Al}]_{\text{ox}}$ (Van der Zee and Van Riemsdijk, 1988). This parameter α has been developed for non-calcareous sandy soils where P adsorption is dominated by metal-(hydr)oxides and represents the P loading of soil metal-(hydr)oxides. For non-calcareous sandy topsoils with an agricultural land use in the Netherlands, the P loading α is on average 0.22 (Koopmans et al., 2006). The maximum P loading (α_{max}) of soil metal-(hydr)oxides varies between 0.4 and 0.6 (Van der Zee et al., 1988; Van der Zee and Van Riemsdijk, 1988). The value of α_{max} is used for calculating the so-called degree of P saturation (DPS), which is defined as the molar ratio between the amount of P accumulated in soils in the form of P_{ox} to a certain depth and the maximum P sorption capacity to that depth (Schoumans, 2000). Since the DPS is

related to the equilibrium P concentration in soil solution, it can be used as an indicator of the risk of P leaching from soil to ground- and surface waters if a critical threshold (e.g., a general value of 25% of the total P sorption capacity or a soil type specific value) is used (Schoumans and Chardon, 2015).

Surface complexation modelling

Besides chemical P extractions, the size of specific soil P pools can be estimated by surface complexation modelling (SCM). One of the most advanced SCMs today is based on a combination of the Charge Distribution (CD) model (Hiemstra and Van Riemsdijk, 1996) and the MULTiSite Ion Complexation (MUSIC) model (Hiemstra et al., 1996). With the **CD-MUSIC model**, the equilibrium ortho-P concentration in solution can be predicted as a function of the adsorbed P content and soil properties (Hiemstra et al., 2013; Weng et al., 2011). For this model a complete set of model parameters is available for two of the most important Fe-(hydr)oxides present in soils: well-crystallised goethite and non-crystalline (or poorly crystalline) ferrihydrite (Hiemstra et al., 2010a; Hiemstra and Zhao, 2016; Mendez and Hiemstra, 2019; Mendez et al., 2020). In the CD-MUSIC model, the complex behaviour of ion adsorption to mineral surfaces is represented by considering both the chemical composition of the solution phase (pH, ionic strength, ionic composition) and the type and properties of metal-(hydr)oxides (specific surface area (SSA) and site density of the reactive groups of either goethite or ferrihydrite as proxy for soil metal-(hydr)oxides). Additionally, the presence of organic matter at the surface of Fe-(hydr)oxides can be taken into account. As input, CD-MUSIC requires parameters resulting from chemical extractions like the concentrations of ions in solution, pH, ionic strength, SSR, the amount of metal(hydr)oxides and reversibly adsorbed P measured as P_{ox} . Additionally, the model requires the reactive surface area (RSA in $m^2 g^{-1}$ soil) of metal-(hydr)oxides as model input. When the RSA is scaled on the amount of metal-(hydr)oxides involved, it results in the SSA (in $m^2 g^{-1}$ metal-(hydr)oxide). Often, generic values for the SSA are used (e.g., Bonten et al., 2011; Ros et al., 2017) to predict the binding of ions to the non-crystalline or crystalline metal-(hydr)oxide fraction: e.g. 600 (Dzombak and Morel, 1991) and $100 m^2 g^{-1}$ metal-(hydr)oxide (Hiemstra et al., 1989). However, the SSA of metal-(hydr)oxides can vary considerably among soils (Hiemstra et al., 2010a; Mendez et al., 2020; Mendez et al., 2022). Therefore Hiemstra et al. (2010a) developed the probe-ion method, by which the RSA of a soil sample can be determined based on the binding of ortho-P as a probe-ion on the RSA of a soil.

Enzymatic phosphatase activity analysis

The methods discussed so far, all focus on quantifying specific soil P pools that can be linked to P uptake by plants or have an operational definition. To assess the importance of biological processes which are linked to **mineralisation of organic P**, the enzymatic phosphatase activity in soil can be determined. Phosphatase enzymes catalyse the

hydrolysis of organic P into ortho-P and thereby contribute to an increase in the amount of P available for plant uptake. Phosphatase enzymes occur in many forms in soil and soil organisms (Kiss et al., 1975) and are released by plant roots, microorganisms and other soil biota in response to a need for P (Olander and Vitousek, 2000; Satchell and Martin, 1984; Toor et al., 2003). The potential activity of these enzymes can be measured by classical methods, like the method described by Tabatabai and Bremner (1969), in which the enzymatic action of phosphatases present in soil acts on a substrate after which the conversion product (e.g., fluorescent or coloured compounds) can be measured in solution. The actual enzyme activity in soil depends on specific soil conditions like pH, temperature and the availability of organic P as a substrate (Sinsabaugh et al., 2008; Wallenstein and Weintraub, 2008). Various techniques exist to estimate the *in situ* enzyme activity and are based on functional genes in soil DNA, proteomic profiling or the quantification of the products of enzymatic degradation (Wallenstein and Weintraub, 2008).

1.3 Phosphorus use: challenges and possible solutions

1.3.1 Challenges

To overcome or avoid P-limitation for plant growth, farmers can increase the soil P content and availability of P by the application of animal manure or mineral P fertiliser. The latter is produced from **phosphate rock**, which is located and mined in a limited number of countries, especially in Morocco (including Western Sahara), China and the United States (United States Geological Survey, 2021). Since the distribution of phosphate rock reserves is limited mostly to these countries, this might lead to geopolitical tensions in the future. Furthermore, mining phosphate rock in its current form and amount is neither environmentally nor economically sustainable (Cordell et al., 2009) as its excavation and treatment causes environmental pollution and is based on the mining of a non-renewable and finite resource. The reserves of the economically profitable part of phosphate rock are declining, although the predictions of the lifetime of P reserves vary strongly and range from decades to centuries (Cordell et al., 2009; Koppelaar and Weikard, 2013; Sattari et al., 2012; Syers et al., 2008; Van Vuuren et al., 2010). However, all predictions emphasise the finite nature of this resource.

Meanwhile, the (economic) opportunities to apply P fertiliser vary globally as animal manure and mineral P fertiliser are not always available or financially affordable in sufficient amounts. This results in **large global P imbalances**, leading worldwide to both strong deficits and high surpluses of the amount of P required by plants (Bouwman et al., 2013; MacDonald et al., 2011; Sattari et al., 2012; Syers et al., 2008; Tóth et al., 2014; Vitousek et al., 2009). Globally, an estimated 5.7 billion hectares of land are considered deficient in P (Niu et al., 2013), and P deficits cover almost 30% of the global cropland area

(MacDonald et al., 2011). Simultaneously, overapplication of P occurs in other regions, mainly in Western Europe, North America and Oceania, that is largely caused by high animal manure loads from intensive livestock production (Bouwman et al., 2013). Crop uptake in the first year after fertiliser P application is generally only 10-20% (Sattari et al., 2012; Syers et al., 2008) and application of P beyond plant requirements leads to P accumulation in soil and higher environmental P losses. This 'legacy P' (Kleinman et al., 2011) saturates the soil P buffering capacity, analogous to the DPS concept discussed in section 1.2.3. Thereby it increases the risk of (subsurface) leaching and runoff of P to ground- and surface waters and contributes to **eutrophication**. Enrichment of surface waters with P stimulates primary production as freshwater ecosystems are often P-limited. Ultimately, eutrophication results in a decreasing oxygen concentration in the water column and loss of biodiversity (Carpenter et al., 1998; Oenema et al., 2005; Sawyer, 1966; Schoumans and Groenendijk, 2000).

In the Netherlands, the agronomic soil P-status of agricultural land increased steadily during the 20th century to levels considered as 'ample sufficient' to 'high' due to a positive national P surplus as a consequence of agricultural intensification (Reijneveld et al., 2010). The increase varied by soil type and land use, and was larger for arable land than for grassland, and larger for sandy soils than for peat, clay and loess soils. For the last three decades of the 20th century, the agronomic soil P-status of grasslands remained constant, whereas it kept increasing for arable land (Reijneveld et al., 2010). According to Schoumans and Chardon (2015), 43% of the agricultural land is regarded as P-saturated when a soil type specific critical threshold is used for the DPS. Due to the strong increase in the soil P stock and associated negative effects, the Dutch government has implemented more strict **P application limits** by the Dutch manure policy from 1984 onwards (Klootwijk et al., 2016; Reijneveld et al., 2010; Van Middelkoop et al., 2016). These P application limits caused the annual national P surplus to level off (Reijneveld et al., 2010) and were aimed at realising equilibrium P fertilisation of agricultural soils on the long-term, in which P inputs by fertilisation equal P removal by produce (Verloop et al., 2010).

Even though on average the agronomic soil P-status of Dutch agricultural soils is high, still some soils can have a **low plant-available P content**. This is either because the total soil P content is low, translating into a low level of readily plant-available P, or because soils are so-called P-fixing soils where the total soil P content is relatively high but readily plant-available P is low due to the strong P binding properties of these soils (Ramaekers et al., 2010; Schoumans et al., 2004). Even in soils which are considered legacy P soils, P limitation can occur when such soils are mined as a strategy to draw-down the soil P-status for reducing the risk of P leaching. During P mining, the P supply to grass or fast-growing crops can become limiting for an optimal P uptake, even when the total soil P content is still relatively high (Koopmans et al., 2004a; Koopmans et al., 2004b; Smolders et al., 2021).

1.3.2 Possible solutions

The simultaneous deficiency and (over)abundance of P in soil has been labelled the world's 'P paradox' (Shepherd et al., 2016). The challenges this global P imbalance bring us are exacerbated by the challenges of limiting phosphate rock reserves and the risk of (surface) water pollution. In addition, the growing world population and its shift towards a more animal-based (i.e., P rich) diet, poses further challenges by increasing the demand for P resources (Godfray et al., 2010; Sattari et al., 2012; Tilman et al., 2002). These challenges force us to search for more **sustainable forms of plant P nutrition**. In agriculture, P inputs are essential to counterbalance the output of P by its produce and are required to support a sustainable intensification of agriculture (Sattari et al., 2016). To realise this, enhancing the utilisation of soil P by an increased P use efficiency of plants is required, which at the same time would allow to reduce the need for external P fertiliser inputs (Faucon et al., 2015; Rowe et al., 2016; Sattari et al., 2016; Stutter et al., 2012; Syers et al., 2008). To realise this, it is vital to consider alternative approaches to enable plants to increase the utilisation of soil P.

One of these alternative approaches explores the role of soil fauna in the soil P cycle and their effect on plant P acquisition under P-limited conditions. According to several studies from the last decennia, the activity of **earthworms** can temporally and locally increase the concentration of readily plant-available P (see review by Le Bayon and Milleret (2009)), which can explain the observed increase in grass production and grass P uptake (Mackay et al., 1982; Ros et al., 2017; Vos et al., 2014). As earthworms influence many soil properties, which, in turn, affect P solubility, they can alter the amount P which is readily-available for plant uptake. However, it is still largely unknown what underlying mechanisms are responsible for this, and to what extent earthworms can beneficially affect P uptake and plant growth in agricultural systems. Moreover, the relative importance of these mechanisms are likely to differ across soil types and among earthworm species due to soil-specific properties and the large differences in feeding and burrowing behaviour among earthworm species (section 1.4.2). This makes it difficult to determine the conditions under which earthworms may significantly improve plant P supply and increase the sustainability of P nutrition. However, since there are many possible interactions between earthworms and the soil P cycle, furthering the understanding of these earthworm effects can be a starting point to manage earthworm populations in such a way they become a viable strategy to increase soil P utilisation and the sustainability of our agricultural systems.

Up to now, several pathways have been proposed for the effect of earthworms on plant-available P, as shown in Fig. 1.2. Further **elucidating and understanding** these mechanisms both chemically - by understanding the interactions between soil constituents - and ecologically - by understanding the effects of different earthworm species and the interactions between them and the environment - is required to optimally utilise earthworms to increase plant-available P and thereby enhance the sustainability of

agricultural systems. Additionally, earthworms affect nutrient dynamics at various spatial scales (Brussaard et al., 2007) which should all be considered to elucidate the contribution of earthworms to plant-available P. These scales range from the nanoscale involved in the adsorption processes of P, via the greenhouse scale in which the ongoing processes can be studied under controlled conditions, to finally the field scale, where the significance of the earthworm-effect can be assessed under realistic conditions.

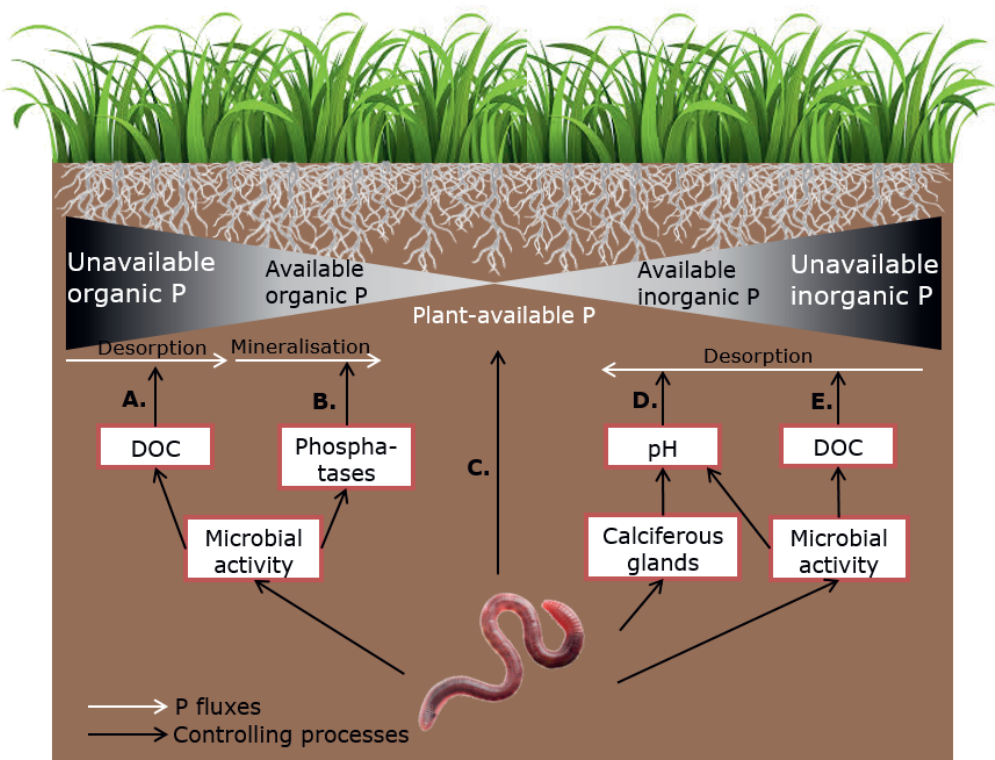


Figure 1.2 Proposed mechanisms (indicated by the letters A-E) by which earthworms may affect plant P-availability:

- **DOC (A+E):** The concentrations of dissolved organic carbon (DOC) are increased in earthworm cast (earthworm faeces) compared to the bulk soil (Barois and Lavelle, 1986; Lopez-Hernandez et al., 1993; Ros et al., 2017; Sizmur et al., 2011; Vos et al., 2014). This difference, which can be more than an order of magnitude, is likely caused by stimulated mineralisation of organic matter by the microbes in the earthworm gut and excretion of DOC-rich mucus from the earthworm body (Lopez-Hernandez et al., 1993; Ros et al., 2017). As DOC compounds can compete with ortho-P for binding sites on the soil metal-(hydr)oxides as described in section 1.2.1, this can result in an increase of the concentration of readily plant-available P in the soil solution (Geelhoed et al., 1998; Vos et al., 2014).

- **Increased mineralisation (B):** The conditions inside the earthworm gut are favourable for microbial activity (Aira et al., 2009; Barois and Lavelle, 1986; Drake and Horn, 2007; Tiunov and Scheu, 2000), which can increase the activity of phosphatase enzymes in earthworm casts and burrows (Hoang et al., 2016; Le Bayon and Binet, 2006; Satchell and Martin, 1984; Tiwari et al., 1989; Wan and Wong, 2004). These enzymes catalyse the mineralisation process of organic into inorganic P, increasing the amount of plant-available P for plant uptake.
- **Selective feeding (C):** Earthworms feed on organic material (including organic acids, DNA and microbial cells), often in combination with soil minerals, and actively select their food source (Curry and Schmidt, 2007; Doube et al., 1997; Lee, 1985; Sharpley and Syers, 1976). Hereby they can accumulate P-rich material from organic substances or fine-textured soil particles rich in metal-(hydr)oxide and clay mineral particles with higher adsorbed P content (Brown et al., 2004; Jiménez et al., 2003; Sharpley and Syers, 1976; Van Groenigen et al., 2019).
- **pH (D):** Earthworms can alter the pH of their casts dramatically and pH increases in casts of up to 2 pH units compared to the bulk soil have been observed (Barois and Lavelle, 1986; Basker et al., 1994; Kuczak et al., 2006; Sharpley and Syers, 1976; Van Groenigen et al., 2019; Vos et al., 2014). A conclusive explanation for this pH increase remains elusive, but some studies suggest the secretion of CaCO_3 from calciferous glands of earthworms which could buffer the pH at these elevated values (Briones et al., 2008; Lambkin et al., 2011). Others attribute this pH increase to the release of alkalinity during the mineralisation process in the earthworm gut (Basker et al., 1994; Ros et al., 2017). As P adsorption to soil metal-(hydr)oxides is strongly pH-dependent, the difference in pH between the bulk soil and earthworm casts affects P solubility: the net positive surface charge of soil metal-(hydr)oxides decreases with an increase of pH, resulting in a decrease of P adsorption and thereby an increase of the P concentration in the soil solution (Geelhoed et al., 1997; Regelink and Koopmans, 2021; Weng et al., 2011; Weng et al., 2012).

(This figure is based on the NWO proposal ALWOP.2015.092, granted to J.W. van Groenigen, and by which this research was funded).

1.4 Earthworms

1.4.1 Earthworms and soil quality

The ancient Greek philosopher Aristotle (384-322 BC) described earthworms as 'the intestines of the earth', although it was only until the 18th century that these soil invertebrates were linked to soil fertility (Rota, 2011). In 1881, Charles Darwin published his last scientific book entitled 'The formation of vegetable mould through the action of worms with observations on their habits', in which he described a variety of topics related

to earthworm activity (Darwin, 1881). Later, earthworms were described as '**ecosystem-engineers**' (Jones et al., 1994) because of their many effects on both biotic and abiotic soil properties. Because of these many effects, earthworm presence in agroecosystems leads to a 23% increase of aboveground biomass and a 25% increase of crop yield according to a meta-analysis of 58 published studies (Van Groenigen et al., 2014). This earthworm effect was largest in non-fertilised systems.

One of the most studied effects of earthworms relate to their influence on soil nutrients. Earthworms can both concentrate nutrients and transform them into more available forms, and casts are therefore hotspots of **soil fertility** (Brown et al., 2004; Van Groenigen et al., 2019). Relative cast fertility (i.e., the enrichment of earthworm casts with respect to the bulk soil) varies between 40 to 48% for the total pools of C, N and P according to a meta-analysis of 405 observations (Van Groenigen et al., 2019). For available P (84%, and determined by various P quantity methods) and mineral N (241%) these numbers are even larger, and general increases in pH (0.5 pH unit) and the cation exchange capacity of the soil (40%) were also observed (Van Groenigen et al., 2019). The increase of cast fertility was largest in the presence of plants or other sources of organic C inputs resulting from residue or animal manure applications.

Besides these large effects on soil fertility, earthworms affect soil structure through their burrowing and casting in a process called '**bioturbation**' (Darwin, 1881; Kutschera and Elliott, 2010; Meysman et al., 2006). Their burrowing creates macropores that increase water infiltration and soil aeration (Bottinelli et al., 2017; Edwards et al., 1992; Schon et al., 2017; Schrader et al., 2007), aggregate stability (Frazão et al., 2019; Marashi and Scullion, 2003; Pulleman et al., 2005; Zangerlé et al., 2011) and create low resistance areas for plant roots to grow (Pitkanen and Nuutinen, 1997). In earthworm guts, casts and burrow walls, microbial activity flourishes (Devliegheer and Verstraete, 1997; Drake and Horn, 2007; Tiunov and Scheu, 2000). Earthworms feed on organic matter in various stages of decay, often in combination with soil minerals, but also living microorganisms and nematodes are ingested (Curry and Schmidt, 2007; Doube et al., 1997; Lee, 1985; Zeibich et al., 2019). Hereby they help to break down and incorporate plant residues in soil, which increases contact between the plant residues and microbes and subsequently increases mineralisation of organic material and release of its nutrients. Alternatively, fragments of undecomposed organic matter may be locked in microaggregates, contributing to C stabilisation (Bossuyt et al., 2005; Frazão et al., 2019; Pulleman et al., 2005; Vidal et al., 2019). However, these long-term effects on C sequestration are in contrast with the short-term effects of earthworm-induced emissions of greenhouse gasses (Lubbers et al., 2013; Rizhiya et al., 2007; Speratti and Whalen, 2008), which complicates the role of earthworms to combat climate change. Because of the large effects these invertebrates have on the soil, the entire sphere influenced by earthworms is called the '**drilosphere**' (Brown et al., 2000; Lavelle, 1988).

1.4.2 Ecological classification

Globally, a wide variety of earthworm species can be found, with sizes ranging from a few cm to 2-3 m and colours from brown, to green, pink or yellow (Lavelle and Spain, 2001). Although at first glance earthworms may look primitive, these soil macrofauna have a circulatory and nerve system and a complex reproductive apparatus (Lavelle and Spain, 2001). Classically earthworm species are categorised in three functional groups based on their feeding and burrowing behaviour (Bouché, 1977). These earthworm functional groups are depicted in Fig. 1.3 and consist of epigeic, endogeic and anecic earthworms.

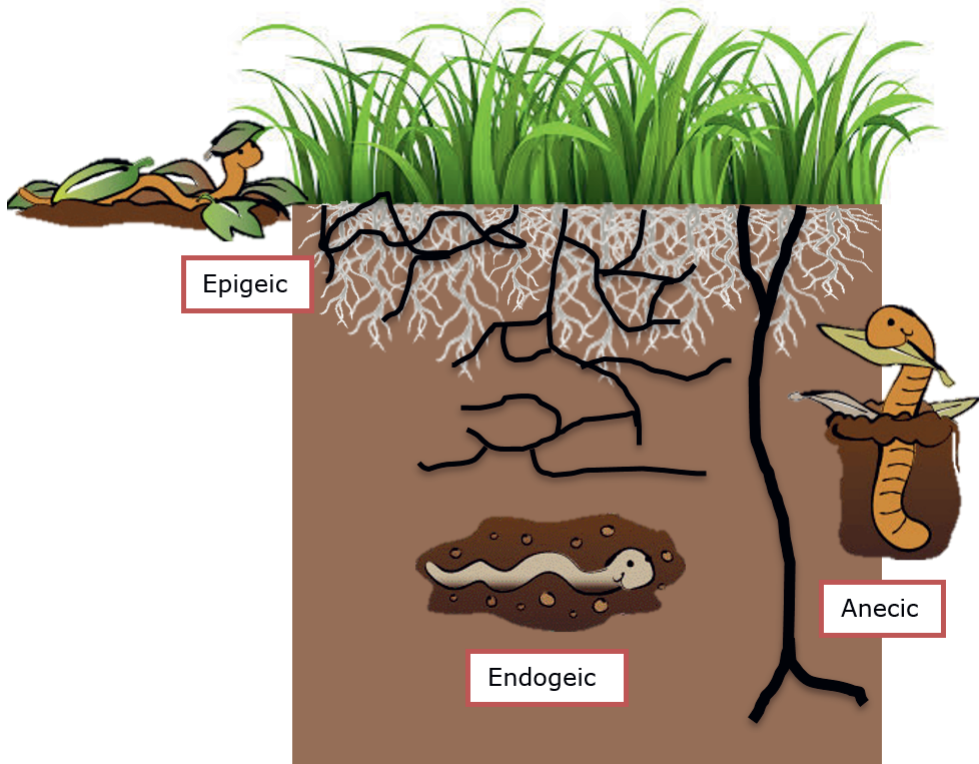


Figure 1.3 The three functional groups of earthworms described by Bouché (1977), based on the burrowing behaviour and food preference of earthworm species.

- **Epigeic** earthworms are litter dwellers and live in the litter layer and upper few cm of the soil, but rarely form burrows within the soil. They feed on relatively fresh surface litter and can be recognised by their pigmented bodies. Compost earthworms also belong to this category.
- **Endogeic** earthworms are soil dwellers and live deeper in the mineral soil. They make many non-permanent horizontal burrows. Endogeic earthworms are

geophagous and feed in the rhizosphere on dead roots, soil particles and the associated organic matter. Their bodies are pale and have little pigment.

- **Anecic** earthworms create a few vertical permanent burrows that can go as deep as the phreatic water level. While they ingest some soil, they mainly feed on relatively fresh surface litter which they drag deep down into their burrows to decompose further. This results in a large residue incorporation depth of the material they forage on. The anterior portion of anecic species is pigmented and the remainder of their body is pale.

Each functional group is represented by many different species. While these categories are useful to describe their behaviour in general terms, species can show plasticity in their behaviour (Neilson et al., 2000) and are increasingly recognised to show characteristics of multiple functional groups. Consequently, a recent study suggests that earthworm classification should not be this rigid (Bottinelli et al., 2020). In the field, a wide variety of earthworm species can be encountered, living together in **earthworm communities** (Didden, 2001; Lavelle and Spain, 2001; Van Vliet et al., 2007). In such communities earthworm species co-exist and interact, which makes the dynamics of earthworm populations and communities complex (Bohlen et al., 1995).

1.4.3 Earthworm occurrence

Earthworms represent the predominant soil fauna in most terrestrial systems (Lavelle and Spain, 2001). Worldwide more than 6000 species of earthworms occur on all continents with the exception of Antarctica (Anderson et al., 2017; Lavelle and Spain, 2001; Phillips et al., 2019). Earthworms are more abundant in some places than others and their densities can be as high as 1333 earthworms m⁻² in the west part of France (Cluzeau et al., 2012) to completely absent in some areas in North America that have not been colonised yet by earthworms after the last ice age (Hendrix and Bohlen, 2002). Although the data are a source of discussion, a large global analysis of **earthworm abundance** shows that earthworms are most abundant in temperate regions (Phillips et al., 2019). In the Netherlands at least 18 earthworm species can be found, belonging to all three functional groups (Van Eekeren et al., 2014). Earthworm abundance is strongly influenced by land use, soil texture, soil pH, vegetation, temperature, organic matter content and soil moisture content (Bohlen et al., 1995; Curry, 2004; Eriksen-Hamel and Whalen, 2006; Jänsch et al., 2013; Lavelle and Spain, 2001; Rutgers et al., 2009). Especially under grassland earthworm populations are high (Cluzeau et al., 2012; Didden, 2001; Lavelle and Spain, 2001; Rutgers et al., 2016; Van Eekeren et al., 2008; Yeates et al., 1998). Throughout literature, average earthworm densities for Dutch grasslands have been observed, varying from 252 (with 5 and 95 percentiles of 0 and 725; Rutgers et al. (2016)) to 384 (Didden, 2001), 421 (Van Vliet et al., 2007) or even 500 (with ranges from 300 to 900; Hoogerkamp et al. (1983)) earthworms m⁻².

1.5 Grassland

As earthworms are especially abundant under grassland, these systems are particularly **interesting to study the effect of earthworms on plant-available P**. Additionally, grasslands are relevant to study as they are extremely important to global food production since they provide feed for ruminants. They range from extensively managed grasslands to intensive livestock production systems. More than 25% of the world's land area is covered by grasslands, of which about two-thirds is used for agriculture (FAO, 2019). In the Netherlands, grasslands are the dominating form of land use, comprising more than 40% of agricultural land (FAO, 2019), and this fraction is even 54% according to the Dutch government (Rijksoverheid, 2021). The largest part of Dutch grassland is situated on non-calcareous sandy soils (48%), followed by substantial areas of grassland under non-calcareous clay (21%), calcareous clay (15%) and peat land (15%), and a minor part under calcareous sandy soils (1%) (Schoumans and Chardon, 2015). The distribution of grasslands is thereby very comparable to the total area of each soil type present in the Netherlands (Table 1.1). Dutch grasslands often have a neutral to high P-status (the median P status of grasslands is described as 'ample sufficient') (Reijneveld et al., 2010) and many of them have a P surplus (CBS, 2022). Hence, P uptake and grass biomass production will probably not be limited by P-deficient conditions in the near future. However, due to the restrictions on P application standards imposed by the Dutch manure policy, grass yields have the risk to decline in the future whereas the P content of grass may decrease below the requirements of lactating and high-yielding dairy cows (Valk and Šebek, 1999; Van Middelkoop et al., 2016).

Table 1.1 Overview of the five main soil types and total area of land in the Netherlands with maize, other arable crops, grass, and nature as a land use. Furthermore, the area of grassland for each soil type has been given. The numbers between brackets are the percentage of a soil type of the total area of this land use. Data is collected from Schoumans and Chardon (2015) and based on data from the national stratified soil survey (Finke et al., 2001).

Soil type	Total land area (ha)	Grassland (ha)
Non-calcareous sand	1 544 316 (53%)	748 136 (48%)
Calcareous clay	619 904 (21%)	227 116 (15%)
Non-calcareous clay	424 032 (14%)	325 693 (21%)
Peat	260 013 (9%)	230 383 (15%)
Calcareous sand	91 186 (3%)	22 821 (1%)
Total	2 939 451	1 554 149

1.6 Research objectives

In the search to discover sustainable ways to facilitate optimal P acquisition for grass growth, this research considers the contribution of earthworms to enhance the utilisation of soil P. Ultimately, adoption of P management strategies that harness earthworm-enhanced P-availability could result in a more sustainable P nutrition of grass and the adaptation of our agroecosystems to a future with less external P inputs from fertilisation. The main objective of this research is **to elucidate the effect of earthworms on plant-available P in soil and P uptake by grass, and to explore its potential to increase the sustainability of P nutrition of grass.**

In order to address its primary goal, specific research objectives of this thesis are:

1. To assess the effect of different earthworm species on plant-available P and relate this to a) physico-chemical soil properties and b) earthworm ecology.
2. To determine the controlling mechanisms of earthworm-enhanced P-availability and quantify the relative importance of all contributing mechanisms.
3. To assess the effect of earthworm diversity on plant-available P.
4. To evaluate if the effect of earthworms on plant-available P results in an increased grass P uptake and biomass production under realistic field conditions.

For addressing these research objectives, a thorough understanding of the various mechanisms involved in the earthworm-induced increase of plant-available P is needed, both from chemical and ecological perspectives and considering different spatial scales. Therefore, a variety of experimental approaches ranging from greenhouse pot experiments to a field experiment and from chemical analysis of earthworm casts to SCM is used to address these research objectives. These approaches are outlined in the different chapters of this thesis as further described in section 1.7.

1.7 Thesis outline

The research objectives are addressed in the different chapters of this thesis as shown in Fig. 1.4. In **Chapter 2** the first research objective is addressed. Eight earthworm species common to the Netherlands have been used in a greenhouse pot experiment with a non-calcareous sandy soil exhibiting a low P-status. With different extraction methods various soil P pools including water-extractable ortho-P have been measured in both earthworm casts and bulk soil, as well as a large range of important physico-chemical properties. Because the earthworms belong to different ecological categories, this experimental setup did not only allow for quantification of the variation among species in their effect on readily plant-available P, but also facilitates the relation to the variation in soil physico-chemical characteristics and earthworm ecological classification.

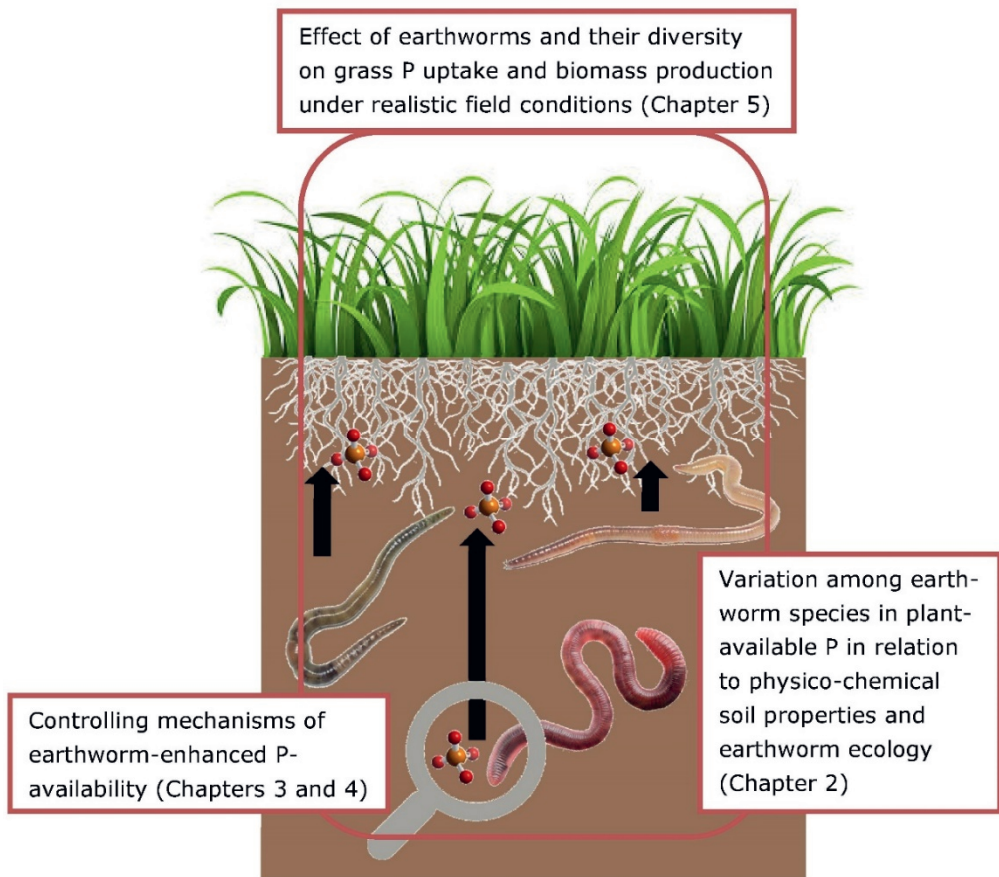


Figure 1.4 Schematic representation of the experimental chapters of this thesis (between brackets) and the research objectives (red boxes) they address.

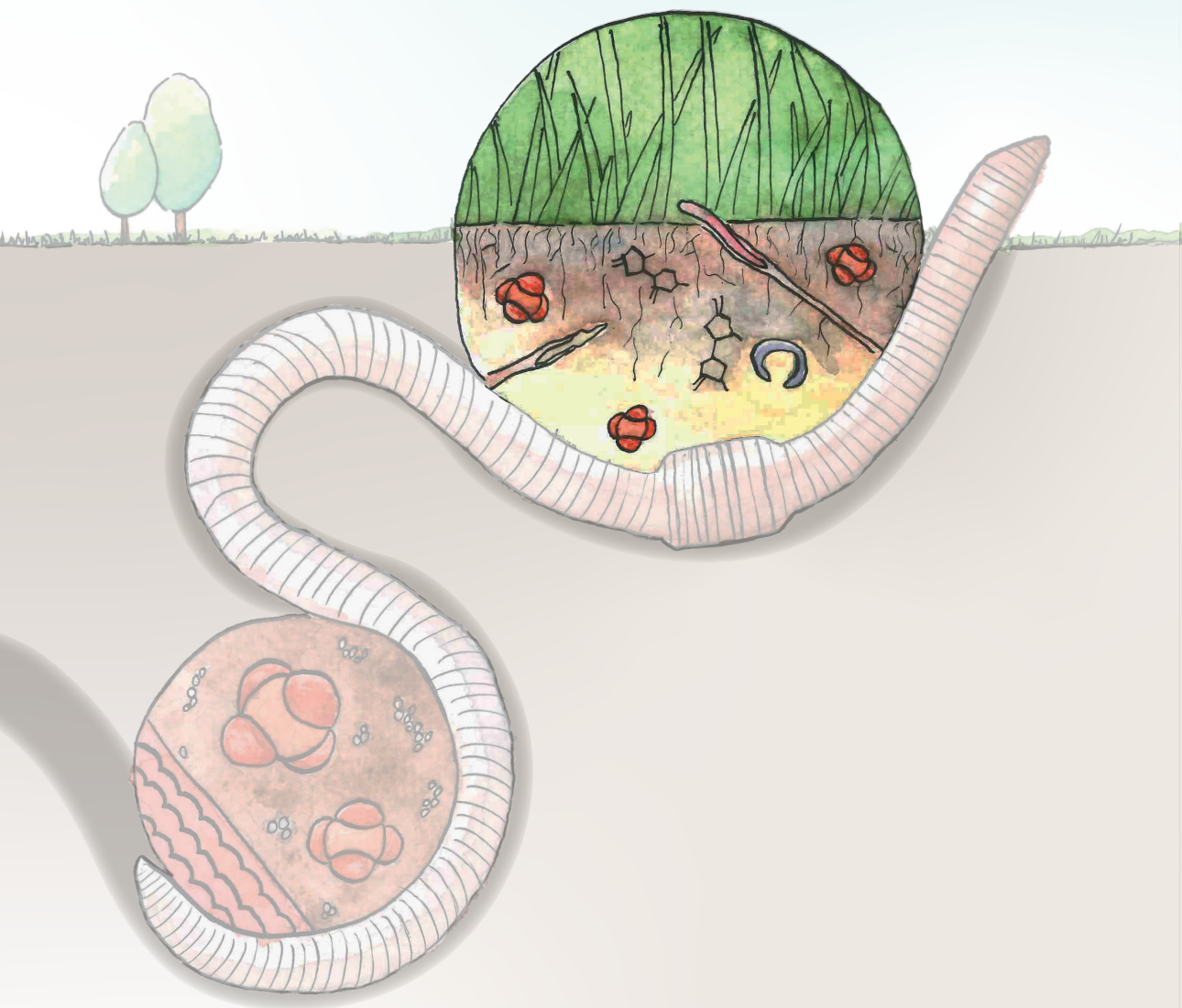
Chapter 3 focusses on the second research objective and discusses the different pathways underlying the earthworm-induced effect on water-extractable ortho-P using cast and bulk samples from another greenhouse pot experiment. Here the effects of three earthworm species across four soils differing in texture, pH, and metal-(hydr)oxide composition were studied. Surface complexation modelling was used to understand the mechanisms controlling earthworm-enhanced P-availability and to quantify their relative importance for the soil types and earthworm species studied.

Chapter 4 addresses one of the mechanisms controlling earthworm-enhanced P-availability, i.e., the pathway of mineralisation of organic P. We determined the contribution of five earthworm species in monocultures and mixtures to phosphatase activity in earthworm casts, burrows and bulk soil and assessed the effect of phosphatase activity on plant P uptake.

The third and fourth research objective are discussed in **Chapter 5**, which concerns a > two-year mesocosm field experiment using a non-calcareous sandy soil with a low P-

status. Five earthworm species are used in monoculture as well as in three-species and five-species mixtures to assess the effect of community composition on grass biomass production and P uptake.

In the last chapter, **Chapter 6**, the results from the previous chapters are brought together with additional analyses of data collected in the experimental chapters, in order to address the objectives of this research. The results are discussed in a broader context and the implications of the findings from this research as well as the conditions under which the effect of earthworms on plant-available P are most promising are debated. Additionally, I deliberate on some of the questions arising from my research and give recommendations for future research on how earthworms can be employed to increase the sustainability of P nutrition of grass.





Chapter 2

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.

2.1 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 (Gérard, 2016; Hinsinger, 2001). 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., 2004a). 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 (Ros et al., 2017; Vos et al., 2014) 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 (Drake and Horn, 2007; Tiunov and Scheu, 2000); (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 (Curry and Schmidt, 2007; Sharpley and Syers, 1976).

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 (Barois and Lavelle, 1986; Basker et al., 1994; Briones et al., 2008; Kuczak et al., 2006; Sharpley and Syers, 1976; Van Groenigen et al., 2019; Vos et al., 2014). 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 (Kuczak et al., 2006; Sharpley and Syers, 1976) to several hundreds of times (Ros et al., 2017; Vos et al., 2014).

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.

2.2 Materials and methods

2.2.1 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 CaCl₂ 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 (Schoomans 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 2.2.4. Based on these data, the P loading of reactive metal (hydr)oxides (α) was calculated according to Van der Zee and Van Riemsdijk (1988):

$$\alpha = P_{ox} / (Al_{ox} + Fe_{ox}) \quad (2.1)$$

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.

2.2.2 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 ($2.5\ \mu g\ B\ pot^{-1}$, $2.5\ \mu g\ Mn\ pot^{-1}$, $0.1\ \mu g\ Cu\ pot^{-1}$, $0.25\ \mu g\ Zn\ pot^{-1}$ and $0.05\ \mu g\ Mo\ pot^{-1}$). 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) *Aporrectodea 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.

2.2.3 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.

2.2.4 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., 2010a); (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., 2010a) and (iii) water-extractable ortho-P as a proxy of the amount of readily-available P in the soil solution (Koopmans et al., 2006; Torrent and Delgado, 2001).

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 rpm 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. (2010a): 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 rpm 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 $\delta^{13}\text{C}$ 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 $\text{Mg}(\text{ClO}_4)_2$. 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 $A_{254\text{-cor}}$:

$$A_{254\text{-cor}} = A_{254} - 0.085 \text{ Fe} + 0.0007 \quad (2.2)$$

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):

$$\text{Aromaticity} = 3.63 + 6.52 \times (100 \times A_{254\text{-cor}} / \text{DOC}) \quad (2.3)$$

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).

2.2.5 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.

2.3 Results

2.3.1 Physico-chemical soil properties and earthworm survival

The physico-chemical properties of the soil used in this study are shown in Table 2.1. 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).

Table 2.1. 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 ₂ O 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

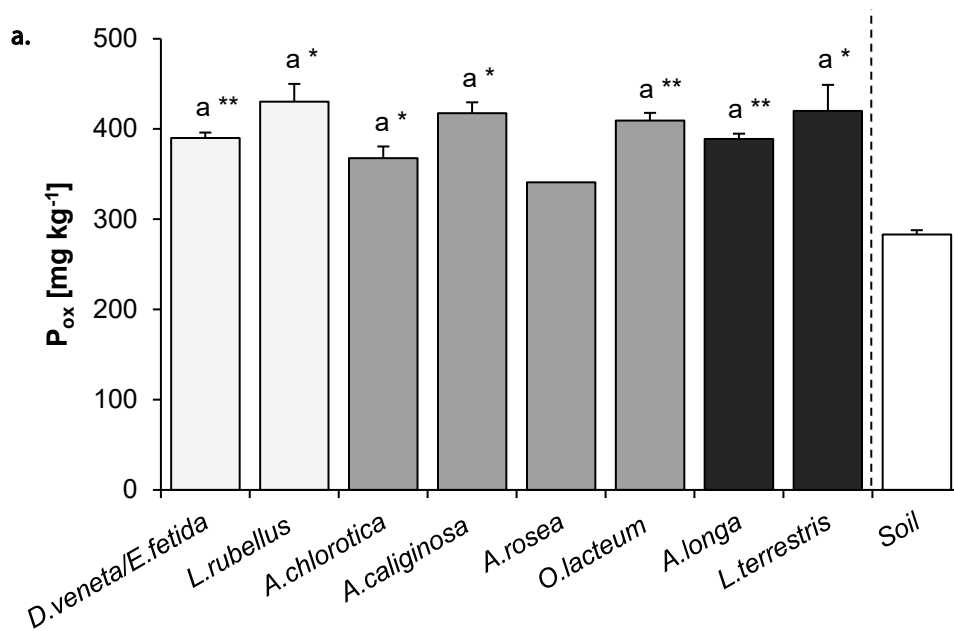
Earthworm survival differed per earthworm species, but was overall 83% after the final earthworm collection (results not shown). Even though casts of *A. rosea* were collected three times, all replicates had to be pooled for chemical analysis. Therefore, replicates were no longer present and this species 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.

2.3.2 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. 2.1a). 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. 2.1b; $p < 0.001$), 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* ($6.21 \pm 0.53 \text{ mg l}^{-1}$ or to $62.1 \pm 5.3 \text{ mg kg}^{-1}$), which were two orders of magnitude higher than those of the control bulk soil ($0.06 \pm 0.005 \text{ mg l}^{-1}$ or $0.6 \pm 0.05 \text{ mg kg}^{-1}$). 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* ($0.95 \pm 0.05 \text{ mg l}^{-1}$ or $9.5 \pm 0.5 \text{ mg kg}^{-1}$), but these ortho-P concentrations were



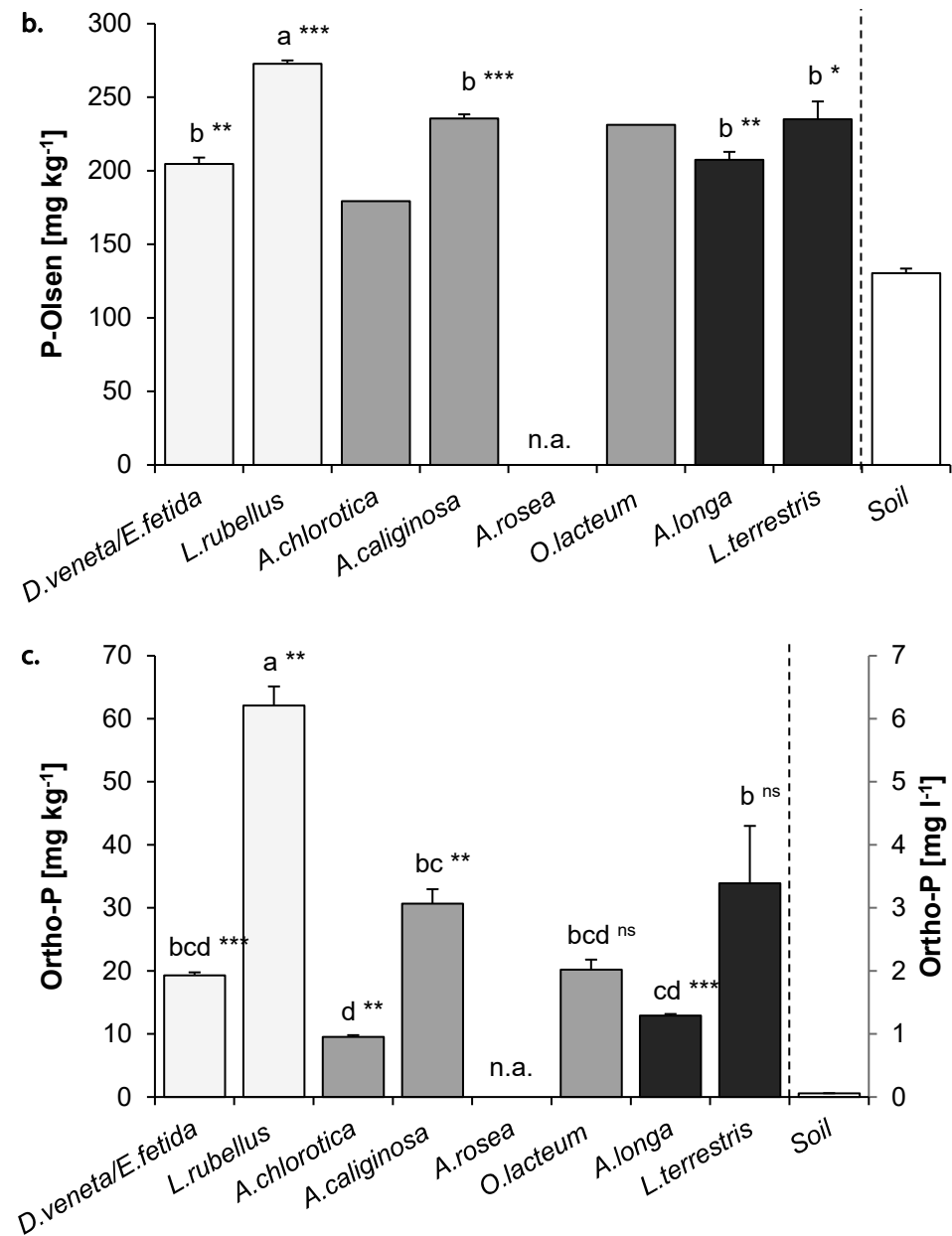


Figure 2.1. Physico-chemical parameters in bulk soil and earthworm cast: P_{ox} (mg kg⁻¹; a), P-Olsen (mg kg⁻¹; b) and water-extractable ortho-P (mg kg⁻¹ on the primary y-axis and in mg l⁻¹ on the secondary y-axis; c). Letters indicate significant differences among earthworm species and asterisks show 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).

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. 2.2a) 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. 2.2b) 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. 2.1c and 2.2b). According to Fig 2.3, 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. 2.2c). 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. 2.2d).

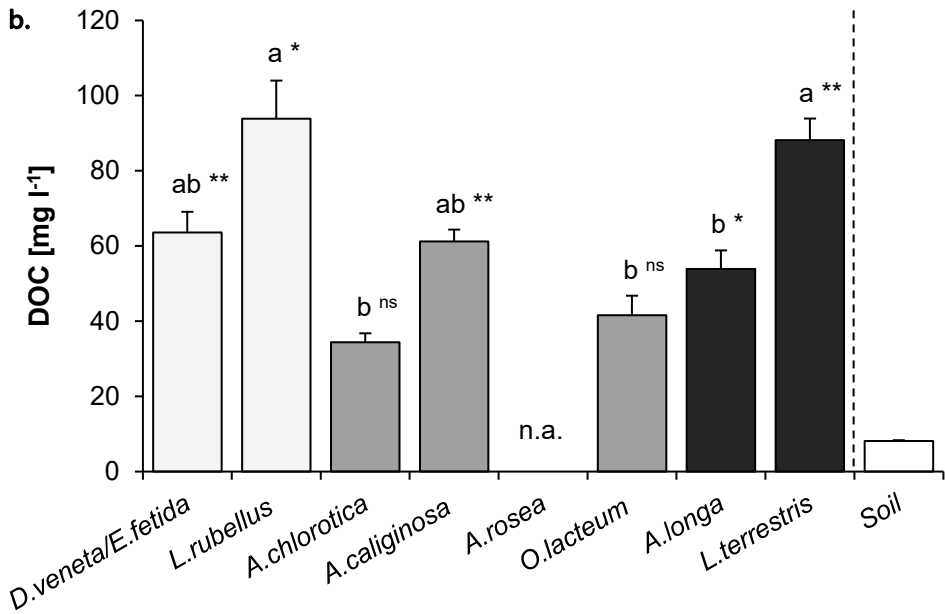
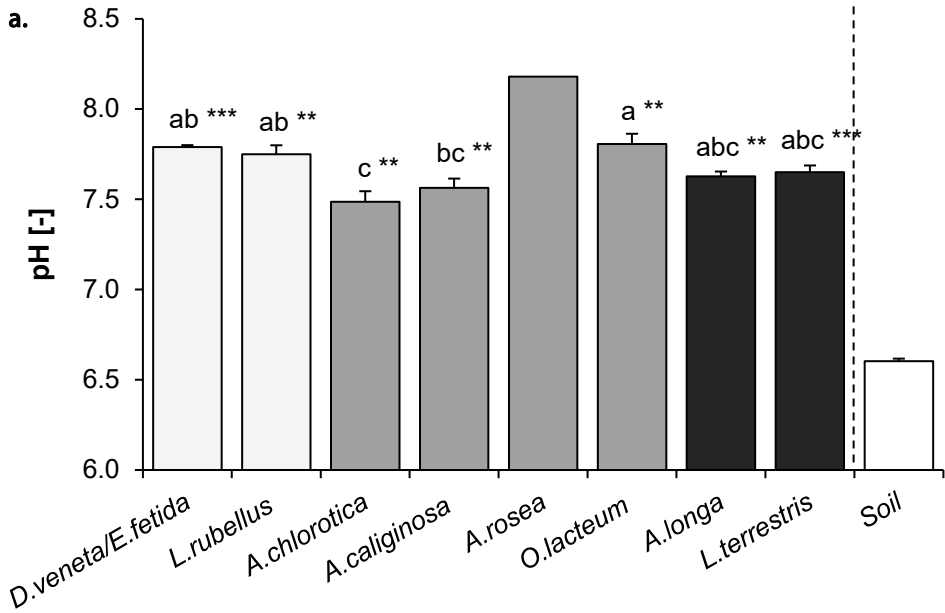
For moisture content, ionic strength, NH_4^+ and IC, significant variation among earthworm species was present as well. With the exception of *O. lacteum*, earthworm casts contained more moisture than the control bulk soil (Fig. S1a). The ionic strength and the NH_4^+ and IC concentrations in the water 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).

2.4. Discussion

2.4.1 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. 2.1a). This suggests that an elevated P_{ox} content is a common trait among the earthworm species that were studied here.



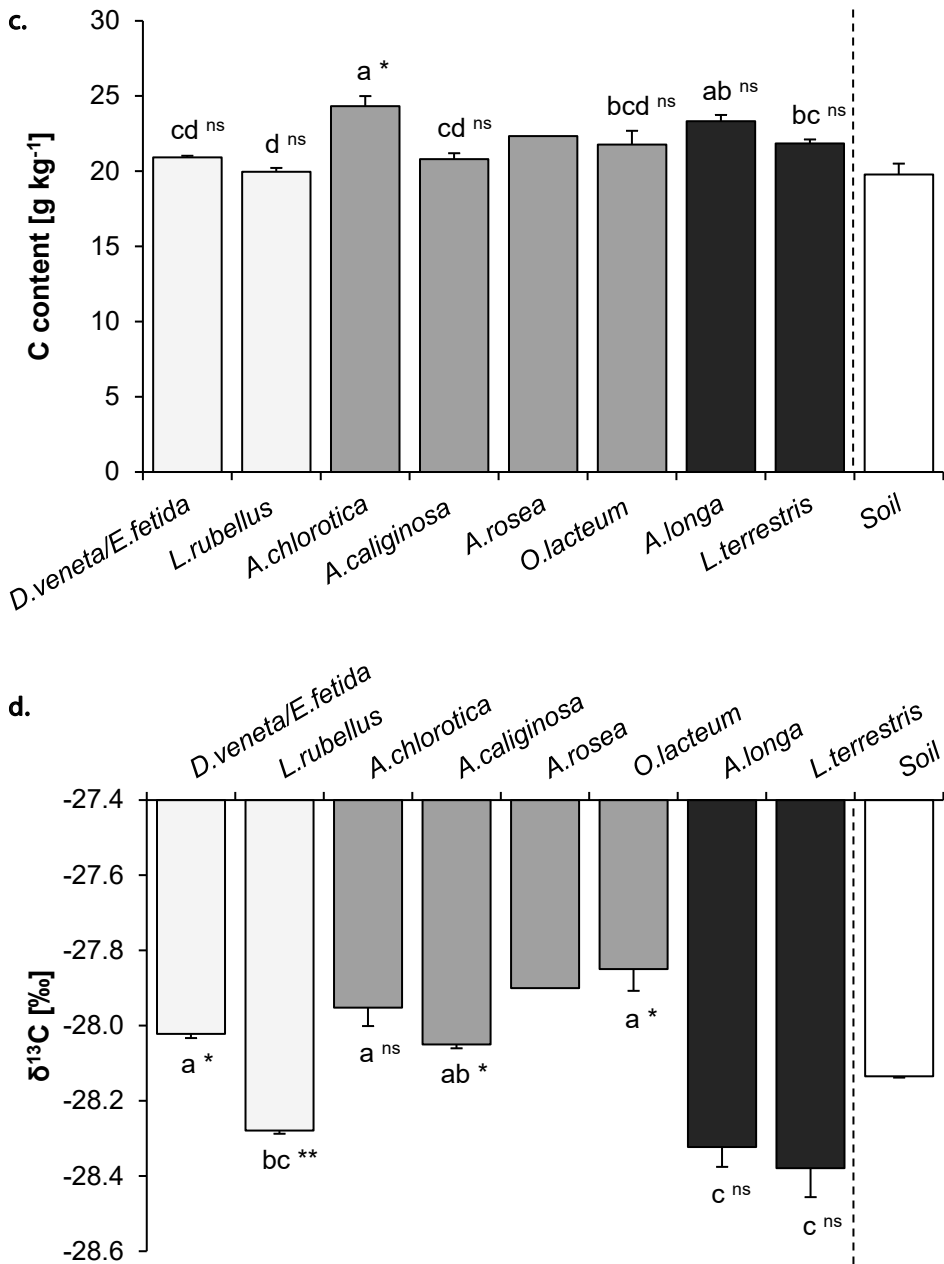


Figure 2.2. Physico-chemical parameters in bulk soil and earthworm cast: pH (a), DOC (mg l⁻¹; b), C content (mg kg⁻¹; c) and δ¹³C (‰; d). Letters indicate significant differences among earthworm species and asterisks show the level of significance between earthworm casts and bulk soil. Error bars are denoted as SE and colours indicate earthworm functional groups (light grey: epigeic; grey: endogeic; dark grey: anecic).

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. 2.1b), while the concentrations of water-extractable ortho-P are even more variable among the studied earthworms (Fig. 2.1c). 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 (Ros et al., 2017; Vos et al., 2014). 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}).

2.4.2 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 2.1.

Elevated pH

The pH may directly affect the concentration of ortho-P in the water extracts (pathway (i) in section 2.1). Our results show a significantly higher pH in earthworm casts compared to the control bulk soil (Fig. 2.2a), which is in agreement with literature (Barois and Lavelle, 1986; Basker et al., 1994; Kuczak et al., 2006; Ros et al., 2017; Sharpley and Syers, 1976; Van Groenigen et al., 2019; Vos et al., 2014). 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: $\text{Soil} + \text{H}_2\text{PO}_4^- (\text{aq}) \rightleftharpoons \text{Soil-H}_2\text{PO}_4$. 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: $\text{H}_2\text{PO}_4^- (\text{aq}) \rightleftharpoons \text{HPO}_4^{2-} + \text{H}^+ (\text{aq})$ ($pK_c = 6.9$ 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. 2.1c). 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. 2.2b) is in agreement with literature (Bolan and Baskaran, 1996; Ros et al., 2017; Vos et al., 2014). 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 2.1, 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. 2.3), 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.

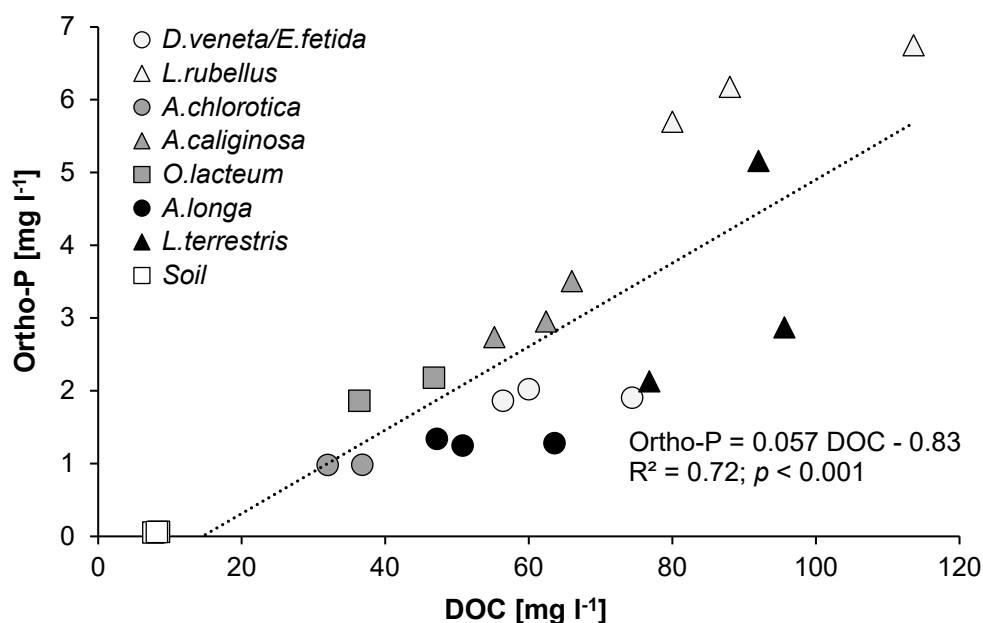


Figure 2.3. Correlation between the concentrations of DOC (mg l^{-1}) and ortho-P (mg l^{-1}) in the water extracts of the cast and bulk soil samples.

Stimulated mineralisation of organic P

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 2.1) 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 (Drake and Horn, 2007; Tiunov and Scheu, 2000); (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 2.1), 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. 2.2c).

Mineralisation can be illustrated by the following representative reaction equation:



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 (2.4), 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 (2.4)). 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 2.1; with a C mass fraction of 0.58, $42 \text{ g OM kg}^{-1} = 24.4 \text{ g C kg}^{-1} = 2.0 \text{ mol C kg}^{-1}$; $P_{\text{org}} = 99 \text{ mg P kg}^{-1} = 3.2 \text{ mmol P kg}^{-1}$; so the molar C/P ratio = 635), decomposition of approximately 50 g OM kg^{-1} soil is required (average $\Delta P_{\text{ox}} = 117 \text{ mg P kg}^{-1}$ (Fig. 2.1a) = $3.8 \text{ mmol P kg}^{-1}$, which according to the calculated molar C/P ratio of the OM yields $2.4 \text{ mol C kg}^{-1}$). This is impossible as our soil contains only 42 g OM kg^{-1} (Table 2.1). 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.

Selective feeding

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 2.1). 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., 2010a) 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 $\alpha = P_{\text{ox}} / (\text{Fe}_{\text{ox}} + \text{Al}_{\text{ox}})$ 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 ($<4 \mu\text{m}$) 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. 2.2d) 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 $\delta^{13}\text{C}$, 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 the model. The model that best described our data was:

$$Ortho-P = 0.048 * DOC + 0.013 * P_{ox} - 0.35 * C\ content - 0.10 * IC + 2.77 \quad (2.5)$$

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.

2.4.3 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 2.4.2, Fig. 2.2d 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.

2.5. 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.

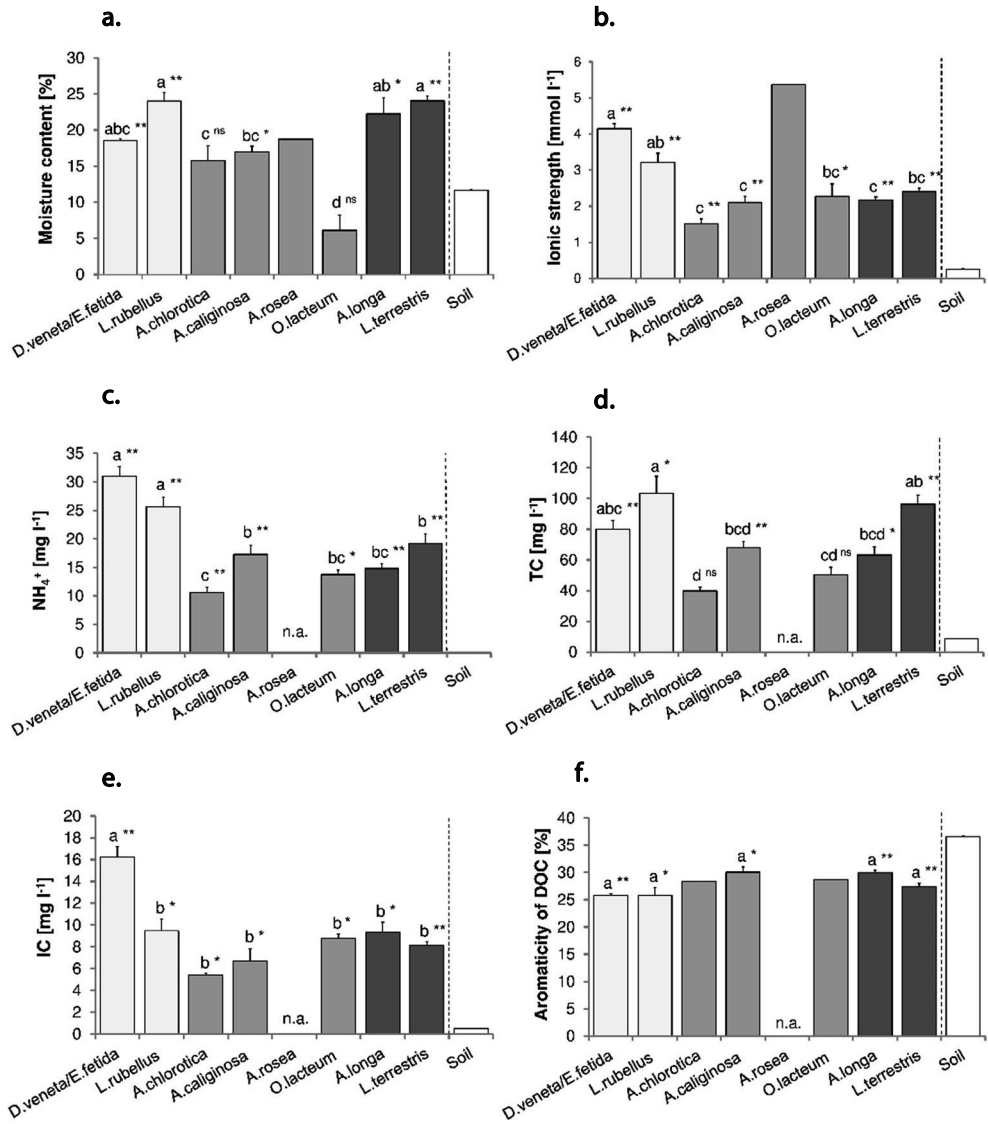
Acknowledgements

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



(Figure S1 continues on next page)

(Figure S1 continued)

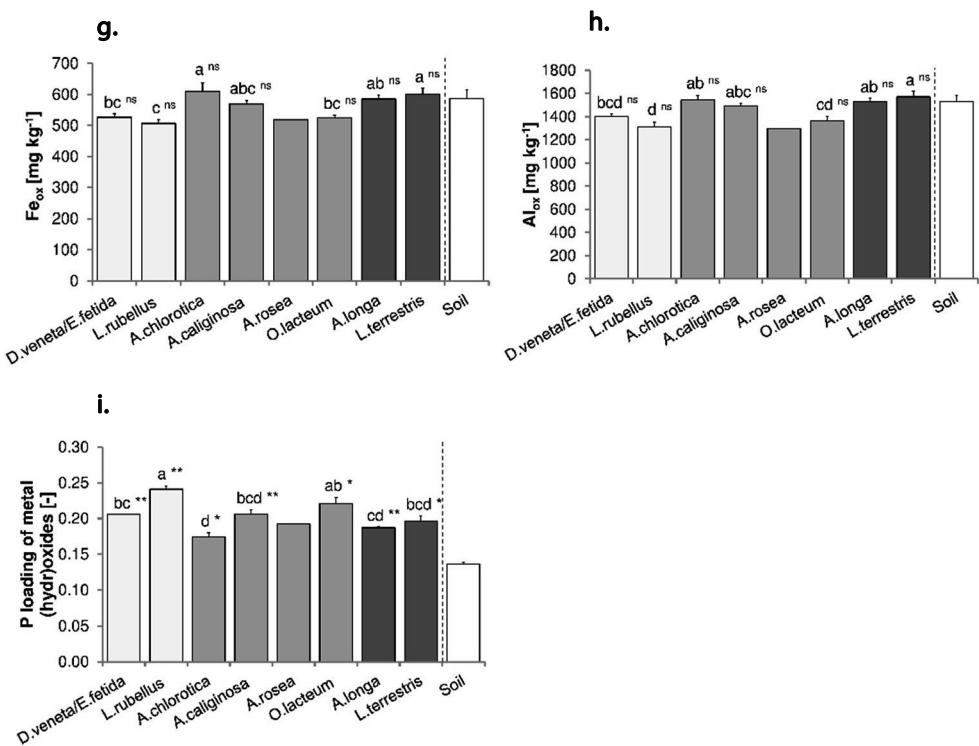
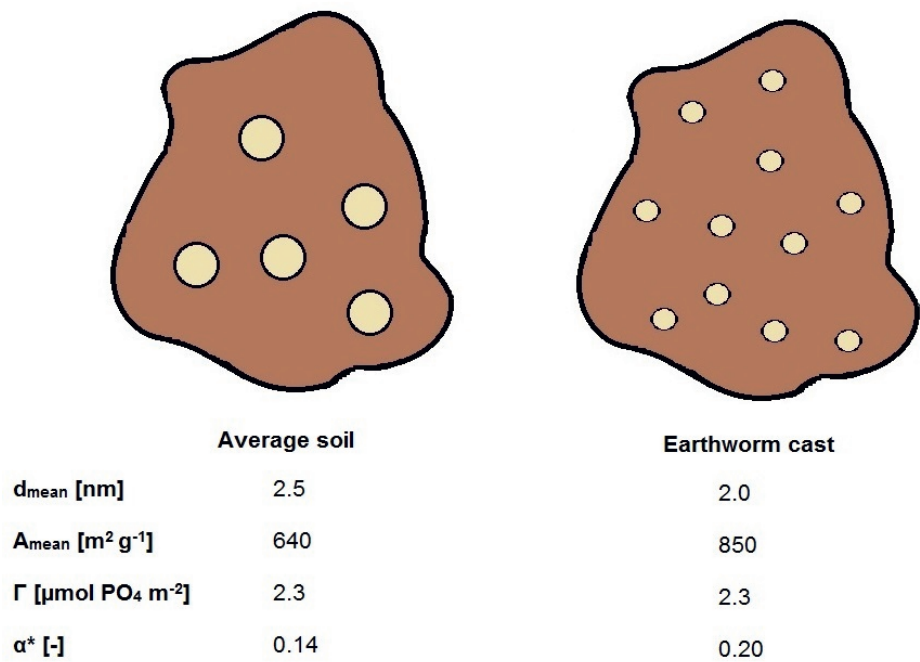


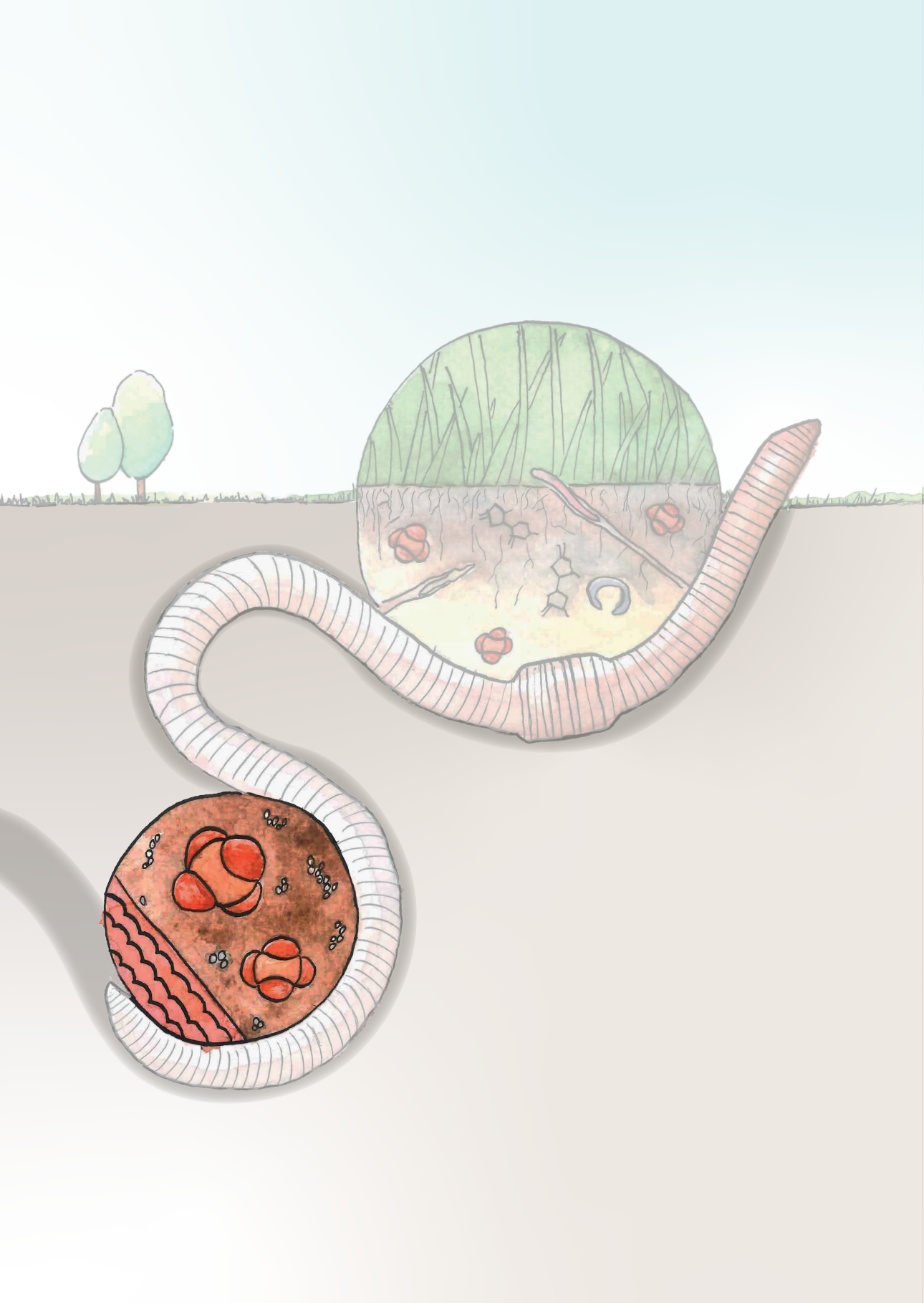
Figure S1. Physico-chemical parameters in bulk soil and earthworm cast: moisture content (%; a), ionic strength (mmol l⁻¹; b), NH₄⁺ (mg l⁻¹; c), TC (mg l⁻¹; d), IC (mg l⁻¹; e) aromaticity of DOC (%; f), content of Fe-(hydr)oxides (mg kg⁻¹; g), content of Al-(hydr)oxides (mg kg⁻¹; h) and the P loading 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).



$$* \alpha = P_{\text{ox}} / (\text{Fe}_{\text{ox}} + \text{Al}_{\text{ox}}) \approx (\Gamma * A_{\text{mean}}) / (\text{Fe}_{\text{ox}} + \text{Al}_{\text{ox}})$$

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

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 m² reactive surface of metal (hydr)oxide (Γ). In the calculations used for this figure, the chosen value of $\Gamma = 2.3 \mu\text{mol m}^{-2}$ is within the range of Γ values observed for a large series of agricultural top soils (Hiemstra et al., 2010a). As our data show the same oxide content (Fe_{ox} and Al_{ox} in $\mu\text{mol g}^{-1}$) in both systems, our calculations demonstrate that even a subtle difference in the mean particle size of the metal (hydr)oxides in two matrices ($d_{\text{mean}}=2.5 \text{ nm}$ for the average soil and $d_{\text{mean}}=2.0 \text{ nm}$ for earthworm cast) can already explain the significant difference in the amount of P reversibly bound per unit oxide mass (α 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.





Chapter 3

A new view on earthworm-enhanced solubility of soil phosphorus

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Abstract

Sustainability of agricultural systems is at stake, as phosphorus (P) is a non-renewable resource while its global reserves are limited. Stimulating earthworm activity can be a technology to increase the level of readily plant-available phosphate (PO_4). However, conclusive evidence on the mechanisms underlying an earthworm-enhanced PO_4 solubility is yet missing. We set up a greenhouse pot experiment in which we investigated the large increase in water-extractable PO_4 in casts of three earthworm species (*Lumbricus rubellus*, *Aporrectodea caliginosa*, *Lumbricus terrestris*) in soils with either predominantly Fe- or Al-(hydr)oxides. Oxalate-extractable PO_4 was increased in earthworm casts compared to bulk soil which can be attributed to the mineralisation of natural organic matter (NOM). Surface complexation modelling was used to elucidate the mechanisms that control earthworm-enhanced PO_4 solubility. Besides NOM mineralisation, two major mechanisms contributing to earthworm-enhanced PO_4 solubility are (i) a decrease in the reactive surface area (RSA) of the metal-(hydr)oxide fraction; and (ii) a decrease in the competition between NOM and PO_4 for binding sites of the metal-(hydr)oxides. As the newly discovered decrease of the RSA was only found for Fe-(hydr)oxide-dominated soils, earthworms have the largest potential to improve the sustainability of P use in those soils.

3.1. Introduction

Phosphorus (P) is an important element in the environment. It is often the limiting factor for plant growth (Hinsinger, 2001), and therefore P fertilisers are widely used in agriculture. However, phosphate rock, the prime source of mineral P fertiliser, is non-renewable, and its long-term availability is increasingly at stake (Cordell et al., 2009; Sharpley et al., 2018). On the other hand, P fertilisation beyond plant needs imposes large risks to the environment (Carpenter et al., 1998; Shepherd et al., 2016). To increase the sustainability of agricultural systems by decreasing the use of P fertilisers, many ways of improving the utilisation of soil P are currently explored (Rowe et al., 2016; Stutter et al., 2012). One of these techniques considers the effect of stimulating earthworm activity, which can temporally and locally increase the level of readily plant-available phosphate (PO_4) in soil (Le Bayon and Milleret, 2009), and thereby improve the P uptake of grass and enhance its biomass production (Mackay et al., 1982; Ros et al., 2017; Vos et al., 2014).

The effect of earthworms on the solubility of soil P is large. In earthworm casts, the readily-available PO_4 concentration can be several hundred times higher than in the surrounding bulk soil (Van Groenigen et al., 2019). The extent to which PO_4 solubility is enhanced varies strongly among earthworm species but is not distinctly related to their ecological classification (Vos et al., 2019). Various mechanisms for the increase of soil P by earthworms have been proposed as discussed in detail in previous studies (Le Bayon and Milleret, 2009; Ros et al., 2017; Vos et al., 2019; Vos et al., 2014). Summarised, the proposed mechanisms are: (a) desorption of PO_4 from mineral surfaces due to an increase of pH in earthworm casts; (b) release of PO_4 due to competitive binding of natural organic matter (NOM) from the earthworm gut to reactive sites of metal-(hydr)oxides; (c) release of additional PO_4 due to stimulated mineralisation of NOM through enhanced microbial activity in the earthworm gut; and (d) selective feeding by earthworms on soil particles with a higher P content. However, there is little conclusive evidence yet on the relative importance of each of these pathways.

To understand and quantify the solubility of PO_4 as a function of soil properties, surface complexation modelling (SCM) can be used. Following this approach, Ros et al. (2017) proposed increased NOM binding to Fe- and Al-(hydr)oxide particles as a major mechanism explaining the increase in water-extractable PO_4 in earthworm casts. However, those authors noted that the NOM surface loadings required to explain the observed PO_4 concentrations in casts were very high compared to the typical NOM loading of Dutch agricultural soils (Hiemstra et al., 2010b; Hiemstra et al., 2013). Another reason to question these results is the use of goethite as a proxy for the natural metal-(hydr)oxide fraction in the model approach, while ferrihydrite (Fh) particles may be responsible for most of the reactivity towards PO_4 (Mendez et al., 2020; Mendez et al., 2022).

Furthermore, due to the lack of precise chemical characterisation of casts, additional pathways of earthworm-enhanced PO_4 solubility may be yet unseen. Such an

unconsidered pathway could be a change in the reactive surface area (RSA) of soil metal-(hydr)oxide particles during passage through the earthworm gut, as a decrease of the RSA results in less binding capacity for PO_4 at the metal-(hydr)oxide surface and a subsequent release of PO_4 . Such a change in the RSA can be assessed with the probe-ion method (Hiemstra et al., 2010a; Koopmans et al., 2020), and could be caused by the chemical conditions inside the earthworm gut as a consequence of intense microbial processes. In the earthworm gut, NOM is rapidly decomposed, and oxygen and other electron acceptors are consumed in this process. These microbially-induced processes stimulate anoxic conditions (Drake and Horn, 2007), which may affect the RSA of the metal-(hydr)oxide fraction, particularly if this fraction is dominated by redox-sensitive Fe (Zhou et al., 2019).

The aim of the present study was firstly to reveal possibly omitted pathways by which earthworms affect PO_4 solubility, and secondly, to quantify the relative importance of all contributing mechanisms. To elucidate the role of the type of metal-(hydr)oxides, we conducted a greenhouse pot experiment with two soils where Fe-(hydr)oxides are more abundant than Al-(hydr)oxides, and with two soils with predominantly Al-(hydr)oxides. For the bulk soils and casts, the RSA was derived with the probe-ion method, and the Fe-(hydr)oxide speciation was determined both structurally and chemically. For the four soils, various types of earthworm species and earthworm-free controls with and without mineral P fertilisation were tested to induce variation in the intensity of NOM decomposition and soil chemical properties. This allowed us to unravel and quantify the various processes contributing to earthworm-enhanced PO_4 solubilisation in soils.

3.2. Materials and methods

3.2.1 Greenhouse experiment

A greenhouse pot experiment was used to generate earthworm casts and bulk soil samples. The experiment was set up as a full factorial randomized block design with soil type (four soils) and earthworm species (five treatments) as independent factors. Each treatment was replicated four times. The soil treatment consisted of four topsoils (0-25 cm) from the Netherlands with different physico-chemical properties (Table 3.1; analytical procedures are described in SI S1.1). The earthworm treatments included *Lumbricus rubellus* Hoffmeister, 1843 (Lr; feeds on surface litter), *Aporrectodea caliginosa* (Savigny, 1826) (Ac; feeds on roots, soil, and soil-associated NOM), *Lumbricus terrestris* Linnaeus, 1758 (Lt; ingests some soil, but mainly feeds on surface litter) (Bouché, 1977; Drake and Horn, 2007), a control without earthworms (P0), and a control without earthworms but with additional mineral P fertilisation (P+). All pots were sown with perennial ryegrass (*L. perenne*) and kept at 60% of the water holding capacity of each soil. Further experimental details are described in SI S1.2.

Table 3.1 Physico-chemical characteristics of the four soils used in this study.

	S1	S2	S3	S4
Coordinates [lat, long]	52°31'32.8"N 5°32'22.5"E	52°12'57.8"N 6°13'26.4"E	51°59'32.7"N 5°34'58.4"E	51°59'28.5"N 5°35'04.4"E
Land use	Intensively managed crops	Extensively managed pasture	Extensively managed pasture	Intensively managed pasture
Sand [%]	32.0	67.3	89.0	88.9
Silt [%]	33.0	14.5	6.1	4.2
Clay [%]	20.3	13.1	1.6	2.2
CaCO ₃ [%]	7.4	-	-	-
NOM [g kg ⁻¹]	46	47	41	40
pH-CaCl ₂ [-]	7.5	4.9	6.0	5.8
P-AL [mmol kg ⁻¹]	4.7	0.1	3.4	8.4
PO ₄ -ox [mmol kg ⁻¹]	11.1	3.1	4.9	13.0
P _{ox} [mmol kg ⁻¹]	11.9	5.7	8.8	18.7
Fe _{ox} [mmol kg ⁻¹] ^a	68.1 (82%)	125.8 (87%)	11.3 (16%)	12.5 (18%)
Al _{ox} [mmol kg ⁻¹] ^a	15.3 (18%)	18.6 (13%)	60.6 (84%)	58.7 (82%)
α [mol mol ⁻¹] ^b	0.14	0.04	0.12	0.26
Fe-DCB [mmol kg ⁻¹]	140.6	797.3	13.3	15.6
Fe _{ox} /Fe-DCB [%] ^c	48	16	85	80
Non-crystalline	100	16	82	75
Fe-(hydr)oxides [%] ^d				
Crystalline	0	84	18	25
Fe-(hydr)oxides [%] ^d				

^a The percentage between parentheses gives the relative amount of Fe and Al present in the oxalate pool, showing that this pool is dominated by Fe-(hydr)oxides in soils S1 and S2, and by Al-(hydr)oxides in soils S3 and S4.

^b The P loading of reactive metal-(hydr)oxides was calculated as $\alpha = P_{ox} / (Fe_{ox} + Al_{ox})$ with all oxalate-extractable elements in mol kg⁻¹.

^c This molar ratio represents the fraction of non-crystalline Fe-(hydr)oxides according to chemical extractions.

^d Calculated by scaling the Fe of the non-crystalline (Fh) and crystalline (goethite, lepidocrocite, and hematite) Fe-(hydr)oxides on the total pool of Fe-(hydr)oxides derived by XAS.

3.2.2 Chemical analyses

Earthworm casts and bulk soil of the two control treatments without earthworms (P0 and P+) were collected as described in SI S1.3. The moisture content of both matrices was determined by drying subsamples at 40°C for 24 h. The dried material was extracted with 0.2 M acid ammonium oxalate to determine the amounts of P, PO₄ (P_{ox}, PO₄-ox), and non-crystalline Fe- and Al-(hydr)oxides (Fe_{ox}, Al_{ox}) (Schwertmann, 1964). Concentrations of P, Fe, and Al were measured with inductively coupled plasma–atomic emission spectroscopy (ICP-AES). A subsample of the filtrate was diluted ten times with 0.01 M HCl to eliminate the interference of oxalate during PO₄ measurement (Hass et al., 2011) by segmented flow analysis (SFA) using the molybdenum blue method (Murphy and Riley, 1962). As the amount of cast was insufficient, the total (non-crystalline and crystalline) amount of Fe-(hydr)oxides was solely determined for the P0 bulk soils using a dithionite-citrate-bicarbonate extraction (DCB) (Holmgren, 1967; Mehra and Jackson, 1960). A structural characterisation of Fe in the P0 bulk soils and some cast samples was obtained by Fe K-edge X-ray absorption spectroscopy (XAS) (using reference spectra for Fh, goethite, lepidocrocite, hematite, and Fe in clay minerals; SI S3).

A modified P-Olsen method (Olsen et al., 1954) was used in the probe-ion approach (Hiemstra et al., 2010a; Koopmans et al., 2020) to calculate the RSA of casts and bulk soils in combination with SCM. For the extraction of 0.2 g dried material with 0.5 M NaHCO₃ (pH=8.5) at a solution-to-solid ratio (SSR) of 50 L kg⁻¹, 0.2 g washed powdered activated carbon was added in excess to facilitate NOM removal. Suspensions were shaken for 257 h (~10 days) at 20 strokes minute⁻¹ in an end-over-end shaker at 20°C. After centrifuging for 10 min at 3000 rotations minute⁻¹, the suspensions were filtered over a 0.45 µm-filter, acidified with 0.3 M HCl to pH=2.0, and put in an ultrasonic bath to remove the released CO₂. Subsequently, PO₄ was measured by SFA.

Since soil drying and rewetting increases P and dissolved organic carbon (DOC) release in water and weak salt extracts (Koopmans et al., 2006; Koopmans and Groenenberg, 2011), moist material was used for the extraction of casts and bulk soils with water as a proxy for the soil solution. Water extracts were prepared by adding ultra-pure water to moist material at a SSR of 10 L kg⁻¹ after correcting for the initial moisture content. The suspensions were shaken horizontally for 24 h with 75 strokes minute⁻¹ at 20°C, centrifuged for 10 min at 3750 rotations minute⁻¹, and the supernatants were filtered through a 0.45 µm-filter. The pH and electrical conductivity (EC) of the filtrates were measured, after which EC was converted to ionic strength (*I*) (Griffin and Jurinak, 1973). Aliquots of the filtrate were taken to measure (i) PO₄, NH₄, NO₃, total dissolved N (TDN), total dissolved C (TDC), and dissolved inorganic C (DIC) by SFA; (ii) total dissolved P (TDP) with inductively coupled plasma–mass spectrometry; (iii) Ca, Mg, Na, K, and S by ICP-AES. Dissolved organic N (DON) was calculated from the difference between TDN and mineral N forms (NH₄ and NO₃), DOC from the difference between TDC and DIC, and dissolved organic P (DOP) from the difference between TDP and PO₄.

3.2.3 Surface complexation modelling

3.2.3.1 Reactive surface area

To describe the adsorption of cations and anions on metal-(hydr)oxide surfaces, we used SCM based on a combination of the charge distribution model (Hiemstra and Van Riemsdijk, 1996) and the multisite ion complexation model (Hiemstra et al., 1996) (together further referred to as CD model). The probe-ion method (Hiemstra et al., 2010a; Koopmans et al., 2020) was used to calculate the RSA ($\text{m}^2 \text{kg}^{-1}$ cast or soil) of casts and bulk soils, by interpreting the measured equilibrium PO_4 concentration in the NaHCO_3 extracts in the absence of NOM with the CD model. During equilibration in NaHCO_3 , the total amount of reversible adsorbed PO_4 (R_{ev} in mol kg^{-1}) will be distributed over the solid and solution phase:

$$R_{\text{ev}} = \text{RSA} \times \Gamma + \text{SSR} \times C \quad (3.1)$$

where R_{ev} is represented by $\text{PO}_4\text{-ox}$, Γ (mol m^{-2}) the PO_4 loading of the reference oxide, SSR the solution-to-solid ratio employed during NaHCO_3 extraction (50 L kg^{-1}), and C the measured equilibrium PO_4 concentration in the NaHCO_3 extracts (M). The probe-ion method results in a RSA representing all soil surfaces that contribute to PO_4 binding. While those surfaces can consist of both metal-(hydr)oxides and oxidic clay minerals (Gérard, 2016; Mendez et al., 2020), only metal-(hydr)oxides were included in our SCM as clay minerals did not significantly contribute to the RSA of a wide range of Dutch topsoils (Mendez et al., 2020). The reference oxide for each matrix was either goethite (as proxy for crystalline Fe-(hydr)oxides) and/or Fh (as proxy for non-crystalline Fe-(hydr)oxides), as for both oxides a consistent set of CD model parameters is available (SI S2) (Hiemstra et al., 2010a; Hiemstra and Zhao, 2016; Mendez and Hiemstra, 2019; Mendez et al., 2020). The choice for the reference oxide for each soil was based on the solid Fe speciation given by XAS analyses (SI S3).

Subsequently, the RSA resulting from Eq. 3.1 can be scaled on the pool of metal-(hydr)oxides into the specific surface area (SSA in $\text{m}^2 \text{g}^{-1}$) and the corresponding mean spherical oxide particle size (d in nm). This calculation, which requires particle size-dependent values of the molar mass and mass density of the Fe- and Al-(hydr)oxides, was based on the set of equations and parameter values given by Hiemstra (2018) and Mendez et al. (2020) (SI S4).

3.2.3.2 Natural organic matter loading

As NOM can compete with PO_4 for reactive surface sites, the NOM loading of metal-(hydr)oxides may affect the PO_4 concentration in solution (Hiemstra et al., 2010b).

Therefore, this NOM loading was calculated for casts and bulk soils based on the obtained RSA (section 3.2.3.1), $\text{PO}_4\text{-ox}$, and the conditions in the water extracts.

Firstly, the free ion concentrations of NH_4 , NO_3 , Na, K, Ca, Mg, and $(\text{H})\text{CO}_3$ were calculated from their measured concentrations in the water extracts, pH, and I using ECOSAT which includes the NICA-Donnan model. Ion complexation with inorganic ligands and dissolved organic matter (DOM) was included in our model calculations and competition between protons and cations for binding to DOM was considered (Hiemstra et al., 2013; Weng et al., 2012). For the modelling, half of the DOM concentration was interpreted as generic fulvic acid (FA) (Ros et al., 2010) and the DOM concentrations were calculated from the measured DOC concentrations assuming a C content of 50%. Generic NICA-Donnan model parameters were used for proton and cation binding to FA (Milne et al., 2001; Milne et al., 2003).

Secondly, the RSA and the reference oxides used for its calculation, in combination with the calculated free ion concentrations and the pH and I of the water extracts, as well as the measured $\text{PO}_4\text{-ox}$ of the casts and bulk soil samples were used as input for the CD model to derive the NOM loading of each sample. In our model approach, three Fe-NOM surface species were defined (Hiemstra et al., 2013) (SI S2). The NOM loading ($\mu\text{mol m}^{-2}$; the total of all three included Fe-NOM surface species) was derived as a fitting parameter while the PO_4 concentration was fixed at the measured value using ECOSAT in combination with the program FIT (version 2.581) (Kinniburgh and Tang, 2004).

Statistical analyses were performed in R, version 4.0.2, and are described in SI S5.

3.3. Results and discussion

3.3.1 Soil properties and earthworm survival

The NOM content was 4-5% for all four soils, but the soils varied largely in their pH (4.9-7.5) and $\text{PO}_4\text{-ox}$ content (3.1-13.0 mmol kg^{-1} ; Table 3.1). The latter was 54-93% of P_{ox} , which is comparable to other Dutch topsoils (Mendez et al., 2020), and showed that the risk of eutrophication can be overestimated when P_{ox} instead of $\text{PO}_4\text{-ox}$ is used for the calculation of the degree of P saturation of soils (Koopmans et al., 2004a; Van der Zee and Van Riemsdijk, 1988). In soils S1 and S2, the metal-(hydr)oxide fraction consisted predominantly of Fe-(hydr)oxides, whereas this fraction was Al-(hydr)oxide-dominated in soils S3 and S4 (Table 3.1). The latter two soils had been part of a long-term (>30 years) P fertilisation trial on grassland and differed in the amount of P fertiliser they had received, resulting in differences in their P-status.

Earthworm survival during the greenhouse experiment was on average 77% but varied between earthworm species (Ac showed a survival of 63% while this was 87% and 83% for Lr and Lt). Since Ac produced smaller amounts of cast than the other species, all

four replicates had to be pooled to obtain sufficient material for chemical analysis, but even then not all analyses could be performed.

3.3.2 Controlling mechanisms of earthworm-enhanced PO_4 solubility

3.3.2.1 Mineralisation and pH

For earthworm casts, PO_4 concentrations in the different soil extracts (oxalate, NaHCO_3 , water) were elevated relative to the P0 bulk soil (Fig. 3.1 and Table S6.2). This could (partly) be caused by selective feeding of the earthworms, as NOM particles enriched with small embedded oxide particles will have a relatively larger P content than the average NOM particle (Ros et al., 2017; Vos et al., 2019). Furthermore, additional PO_4 entering the system through NOM mineralisation by earthworms could result in the observed increased amount of PO_4 -ox in casts. Enhanced mineralisation by earthworms is indicated by the elevated water-extractable DOC concentrations of the casts (Table S6.1). This agrees with literature (Bolan and Baskaran, 1996; Ros et al., 2017; Van Groenigen et al., 2019; Vos et al., 2019) and can be caused by the excretion of DOC-rich substances by the earthworms (e.g. mucus) (Lopez-Hernandez et al., 1993) or stimulated mineralisation inside the earthworm gut (Brown et al., 2000; Satchell and Martin, 1984; Wolters, 2000), resulting in the production of soluble organic molecules as a by-product of mineralisation (Kalbitz et al., 2000). Enhanced mineralisation is evidenced by the increased DON, S, and DOP concentrations in the water extracts of the casts (Table S6.1). The increase of these NOM constituents shows a good correlation with the increase of DOC (SI S7). Additionally, there is a general increase in cation and anion concentrations and /of the water extracts of the casts (Table S6.1), which is likely related to enhanced mineralisation inside the earthworm gut. Concentrations of elements that are not directly released by NOM mineralisation can still be increased, as mineralisation can lead to elevated NH_4 concentrations (Decaëns et al., 1999), which can subsequently be exchanged for other cations like Na or K at the soil cation exchange complex (Ros et al., 2017).

Focusing on the differences in the casts of the various earthworm species, the concentrations of DOC and the other mentioned components of the water extracts are generally higher for Lr than Lt. For Ac, our dataset is incomplete, but based on the DOC concentrations observed for this species in previous studies (Vos et al., 2019; Vos et al., 2014), the expected contribution of Ac to mineralisation is lower than for Lt. The suggested order of intensity of mineralisation is, therefore, $\text{Lr} > \text{Lt} > \text{Ac}$. This agrees with measurements on N mineralisation in the casts of these species (Postma-Blaauw et al., 2006), and matches the expected mineralisation potential based on the NOM quality in the food these earthworms ingest (section 3.2.1).

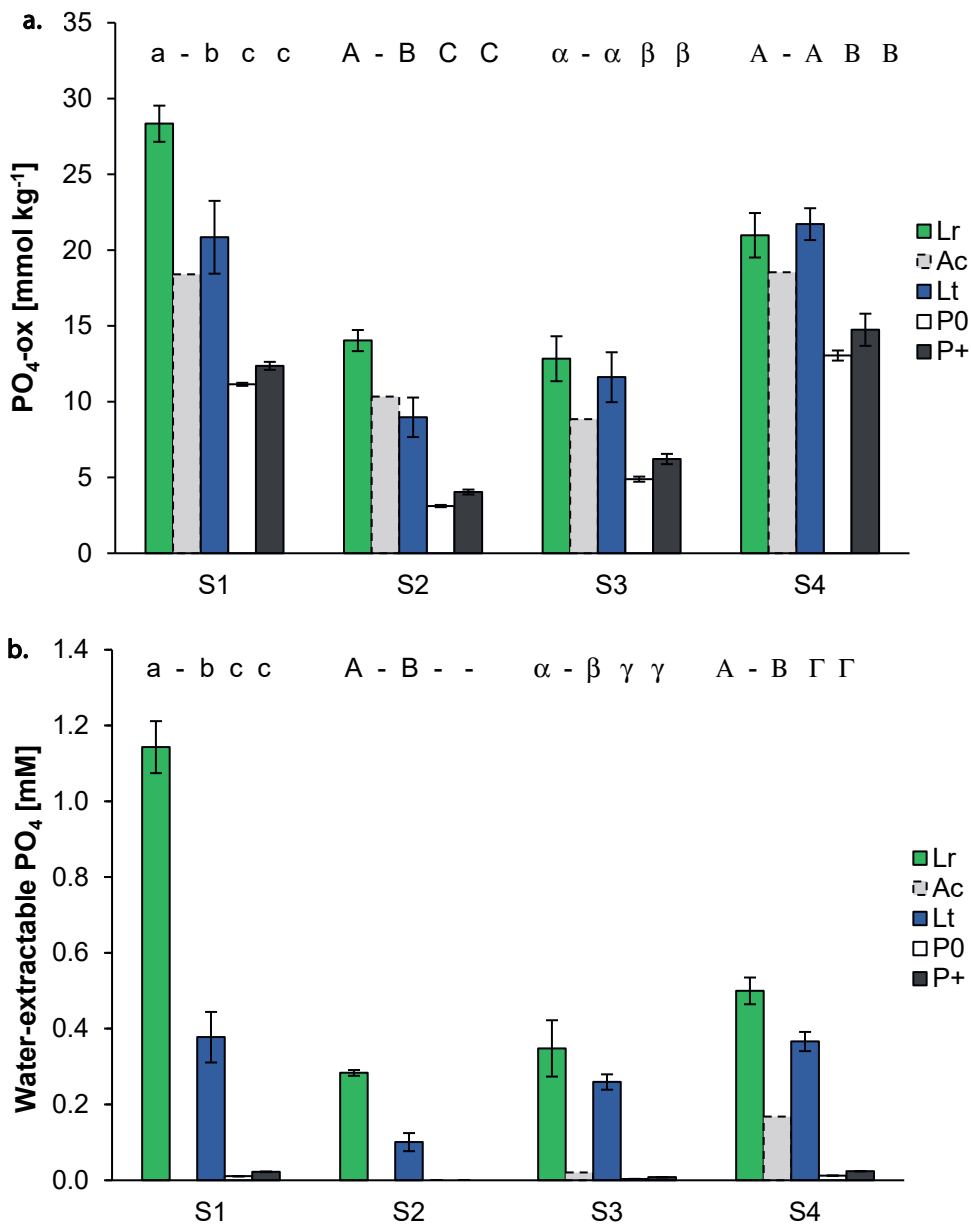


Figure 3.1 Oxalate-extractable PO₄ (a; PO₄-ox) and water-extractable PO₄ (b) of the four soils and earthworm casts. The water-extractable PO₄ concentrations of soil S2 (P0 and P+) were below the detection limit of the SFA. Due to limited cast production by Ac, water-extractable PO₄ could not be determined for soils S1 and S2, and only without replicates for soils S3 and S4. Hence, Ac was excluded from statistical analysis and, when available, its value is presented with a dotted bar. Error bars denote standard deviations. The significance of differences was determined for each soil separately.

The production of CO_2 by mineralisation inside the earthworm gut results in the enhancement of $p\text{CO}_2$, and the associated release of protons. However, often these protons are buffered by the production of alkalinity (HCO_3^-) during NOM mineralisation (Ros et al., 2017). This production of alkalinity can be the reason for the observed pH increase in earthworm casts for three of our soils (S2, S3, S4; Table S6.1), although other causes, like the earthworms' calciferous glands, are also possible (Briones et al., 2008). This pH increase in casts is widely recognised in literature (Basker et al., 1994; Kuczak et al., 2006; Sharpley and Syers, 1976; Van Groenigen et al., 2019; Vos et al., 2019) and may result in larger PO_4 desorption from mineral surfaces. However, in soil S1, we observed a decrease in the pH of casts. For this calcareous soil with a high pH an additional mechanism for the consumption of the produced alkalinity is present, as HCO_3^- release may lead to precipitation of Ca and neutralisation of the produced alkalinity according to $\text{HCO}_3^- + \text{Ca} \rightleftharpoons \text{CaCO}_3(\text{s}) + \text{H}^+$. Because of the pH decrease in soil S1, the final pH in the casts from all soils was rather similar ($\text{pH} \approx 7.5$). Hence, earthworms seem to create a more or less constant pH in their casts, regardless of the pH of the surrounding bulk soil.

3.3.2.2 Reactive surface area

The increase of $\text{PO}_4\text{-ox}$ because of the additional PO_4 added by mineralisation will affect the water-soluble PO_4 concentration, but a quantitative interpretation requires modelling for which the RSAs of casts and bulk soils are pivotal. The reference oxides required to determine the RSAs were given by XAS, which showed that the solid Fe speciation of the casts was identical to that of the corresponding bulk soils. The Fe-(hydr)oxides of soil S1 consisted solely of Fh, while in the other soils, also crystalline Fe-(hydr)oxides (i.e. goethite, lepidocrocite, and hematite) were present (Table S3.1). For all soils but soil S1, the fraction of non-crystalline Fe-(hydr)oxides according to XAS was in close agreement with the amount found by chemical extraction using oxalate and DCB (Table 3.1). For soil S1, however, the ratio $\text{Fe}_{\text{ox}}/\text{Fe-DCB}$ is much lower than seen by XAS. This may be due to the limited sensitivity of XAS for identifying different types of Fe-(hydr)oxides in samples in which most Fe is associated with clays, as is the case for soil S1 (only 22% of total Fe is present in the form of (hydr)oxides; Table S3.1). Another explanation might be the incomplete dissolution of non-crystalline Fe-(hydr)oxides after the two-hour oxalate extraction of soil S1 (Schwertmann et al., 1982). In our SCM, the relative occurrence of non-crystalline and crystalline Fe-(hydr)oxides measured with XAS will be used and brought to the absolute scale using the total amount of Fe-(hydr)oxides measured with DCB. Since for extraction with DCB no sufficient cast material was available, the total amount of Fe-(hydr)oxides in the casts was taken to be the same as in the corresponding bulk soils. The minor differences ($2 \pm 5\%$) between Fe_{ox} of the casts and corresponding P0 soils support this assumption (Table S6.2).

In soils S3 and S4, the Fe-(hydr)oxides are predominantly non-crystalline, while the total metal-(hydr)oxide fraction consists largely of non-crystalline Al-(hydr)oxides (Table

3.1). Therefore, the small fraction of crystalline Fe-(hydr)oxides compared to the total pool of metal-(hydr)oxides was ignored in our further calculations. Presently, no specific proxy with a consistent set of parameters for the CD model is available for natural Al-(hydr)oxides, but according to recent work with Al-(hydr)oxide-dominated weathered tropical soils, Fh was an adequate proxy in the CD modelling (Mendez et al., 2022). Therefore, Fh was chosen as reference oxide for soils S3 and S4. Fh was used as well for soil S1, but for soil S2 a mixture of goethite and Fh was used in the ratio measured by XAS (84% and 16% respectively; Table 3.1).

The RSA of our soils can be assessed with the PO_4 probe-ion method (Hiemstra et al., 2010a; Koopmans et al., 2020) using either Fh or a mixture of Fh and goethite as a proxy. For soils S1, S3, and S4, the procedure is relatively straightforward as only one proxy (i.e. Fh) is used in the interpretation of the data from the NaHCO_3 extracts, as described by Eq. 3.1 (section 3.2.3.1). Subsequently, these RSAs can be scaled on the pool of metal-(hydr)oxides into the SSA to derive the corresponding mean spherical size of the oxide particles according to the set of equations provided in section S4.1. This scaling requires the amount of non-crystalline oxides in each sample: $\text{Fe-DCB} \times \text{non-crystalline fraction XAS (Fe-(hydr)oxides)}$ and Al_{ox} (Al-(hydr)oxides) (Table 3.1).

For soil S2, the calculation of the RSA differs from the approach followed for soils S1, S3, and S4 because of the large contribution of crystalline Fe-(hydr)oxides to the total pool of metal-(hydr)oxides. The calculations were only possible if the SSA of one of the reference oxides was fixed at a chosen value (section S4.2). Therefore, the SSA of the crystalline Fe-(hydr)oxide fraction (SSA_{crys}) was set to $\text{SSA}_{\text{crys}} = 40 \text{ m}^2 \text{ g}^{-1}$, which falls within the range of values reported for synthetic goethite samples ($\approx 20\text{--}110 \text{ m}^2 \text{ g}^{-1}$) (Hiemstra et al., 1989) and which results in realistic SSA values for the non-crystalline fraction ($\text{SSA}_{\text{ncrys}}$) that are consistent and comparable with the results for the other Fe-(hydr)oxide-dominated soil S1 (SI S8). Although uncertain, the precise value of our estimate of SSA_{crys} did not influence our main conclusions. Further scaling of the RSA of soil S2 on the pool of metal-(hydr)oxides into the total SSA of non-crystalline and crystalline metal-(hydr)oxides and the corresponding mean spherical oxide particle size, requires the amounts of non-crystalline and crystalline Fe-(hydr)oxides. Consistently with the approach followed for non-crystalline Fe-(hydr)oxides, the amount of crystalline Fe-(hydr)oxides was estimated by $\text{Fe-DCB} \times \text{crystalline fraction XAS}$.

The effect of short-term P fertilisation on the RSA and particle size follows from comparing the P0 and P+ treatments of each soil in Fig. 3.2. This shows that the addition of P in our relatively short pot experiment did not lead to significant differences. The effect of long-term P fertilisation on the RSA and particle size can be assessed from a comparison of soils S3 and S4. These soils were taken from a long-term P fertilisation trial and have similar soil properties, but soil S4 received P fertiliser for >30 years, whereas soil S3 did not. This long-term P fertilisation has led to a suppression of particle growth in soil S4, therefore yielding a much larger RSA and smaller particle size for the different matrices,

which agrees with previous observations (Borch et al., 2007; Makris et al., 2005; Schwertmann, 1991). In the Fe-(hydr)oxide-dominated soils S1 and S2, the RSA of the casts was lower than for the corresponding bulk soils, while the particle size of the casts was larger. We explain this based on the prevailing redox conditions of chemical reduction in the earthworm gut which are due to the intense process of mineralisation (Drake and Horn, 2007; Zhou et al., 2019), consuming oxygen and other electron acceptors. Such anoxic conditions can lead to the formation of small amounts of Fe^{2+} and Fe reduction has been shown to occur in the earthworm gut (Zhou et al., 2019). Subsequently, this Fe^{2+} can act as a catalyst for the aging process of Fe-(hydr)oxides as is observed in synthetic systems (Hansel et al., 2005; Jones et al., 2017; Pedersen et al., 2005). This results in larger Fe-(hydr)oxide particles and thereby decreases the RSA of the casts. In contrast to Fe, Al is not redox-sensitive and Al-(hydr)oxides will therefore not be affected. Indeed, the RSA and particle size of the casts and bulk soils for the Al-(hydr)oxide-dominated soils either showed no significant differences (soil S3) or only relatively small and opposite differences to those in the Fe-(hydr)oxide-dominated soils between Lt and P+ (soil S4; Fig. 3.2). However, as Fh was used as a proxy for soils S3 and S4, while the metal-(hydr)oxide fraction of these soils was dominated by Al-(hydr)oxides, the specific differences for soil S4 should be considered with care as they can be the result of less accurate model parameters.

The decrease of the RSA in the casts from Fe-(hydr)oxide-dominated soils will lead to a higher PO_4 solubility in the water extracts as less RSA is available for PO_4 adsorption. Hence, a decrease of the RSA in combination with an increase in $\text{PO}_4\text{-ox}$ due to additional PO_4 input by enhanced mineralisation both contribute to the strong increase in PO_4 solubility for soils S1 and S2. For the Al-(hydr)oxide-dominated soils S3 and S4, earthworm-enhanced PO_4 solubility is likely primarily due to enhanced mineralisation, as the RSA of casts did not decrease compared to bulk soils.

When the increase in particle size of the metal-(hydr)oxides is related to a low redox potential in the earthworm gut, differences in particle size between earthworm species may be expected as their intensity of mineralisation may differ. More intense mineralisation by Lr than by Lt (section 3.3.2.1) could result in more growth of Fe-(hydr)oxide particles, leading to a lower RSA and larger particle size for Lr than for Lt. This was indeed found in soils S1 and S2, although the difference was not significant for soil S2. For the Al-(hydr)oxide-dominated soils, no significant differences in RSA and particle size were observed between earthworm species.

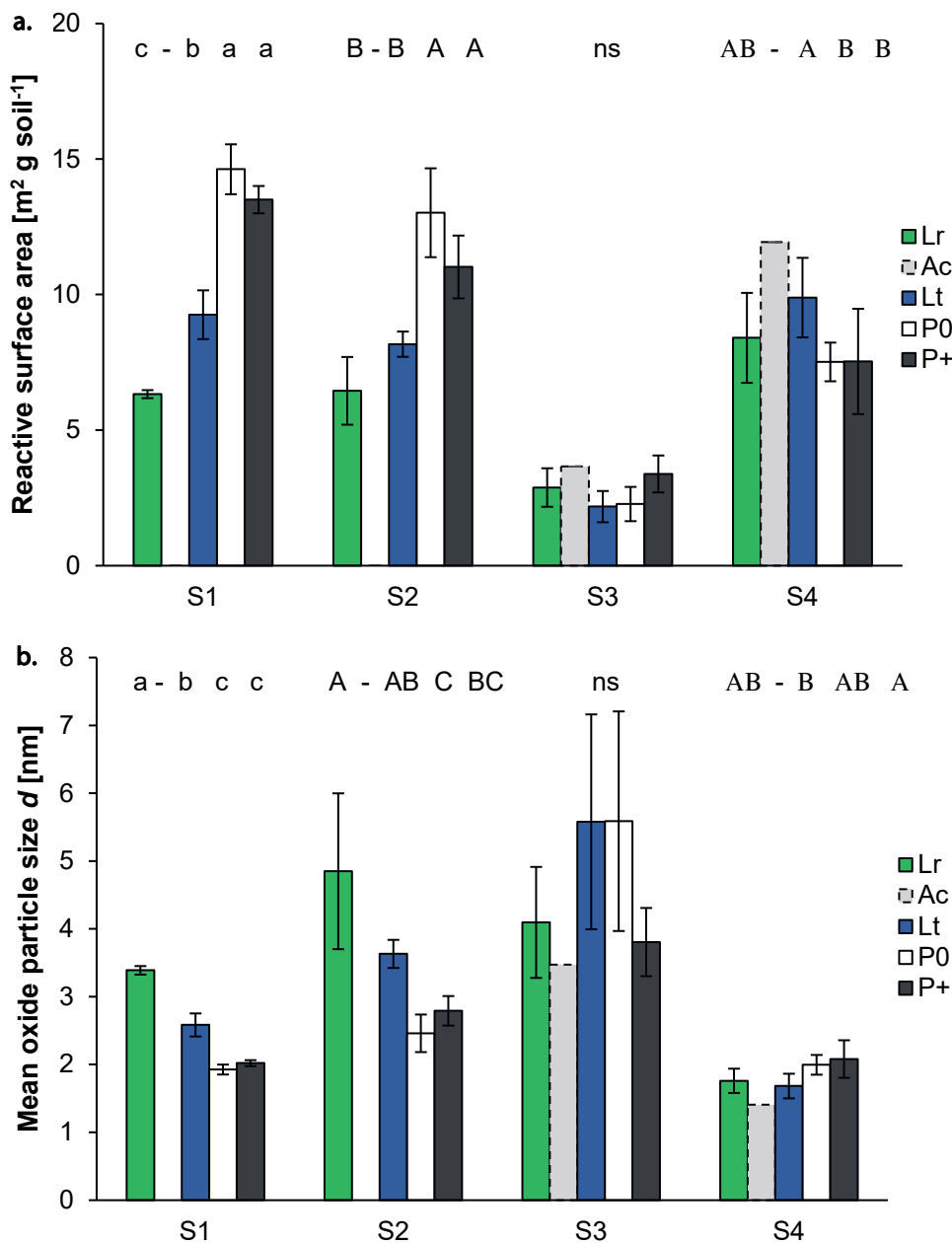


Figure 3.2 Reactive surface area (a; RSA) and mean oxide particle size (b; d) of the four soils and earthworm casts. Due to limited cast production by Ac, the RSA and d could not be determined for soils S1 and S2, and only without replicates for soils S3 and S4. Hence, Ac was excluded from statistical analysis and, when available, its value is presented with a dotted bar. Error bars denote standard deviations. The significance of differences was determined for each soil separately.

3.3.2.3 Natural organic matter loading

Another proposed mechanism to explain the increase in the water-solubility of PO_4 in earthworm casts is a change in competitive adsorption between NOM and PO_4 (Le Bayon and Milleret, 2009; Lopez-Hernandez et al., 1993; Ros et al., 2017). This contribution has been evaluated here with the CD model, by deriving the NOM loading (i.e. the total of the adsorbed Fe-NOM surface species in $\mu\text{mol m}^{-2}$) of the metal-(hydr)oxide surfaces. Using solely $\text{PO}_4\text{-ox}$ (as R_{ev}) and RSA as model input, measured PO_4 concentrations are often underpredicted by the model, which can be attributed to the absence of competitive binding between PO_4 and NOM in the model approach (Hiemstra et al., 2010b). This was the case for soils S1 and S2, and including NOM competition would increase the predicted PO_4 concentration. The data for $\text{PO}_4\text{-ox}$ and RSA can be unified with the PO_4 concentration measured in the water extracts by fitting the required NOM loading.

The results are depicted in Fig. 3.3, which shows an inverse relationship between the fitted NOM loading and adsorbed PO_4 . The bulk soils have a high NOM loading and a low PO_4 loading, but in the earthworm gut, PO_4 is released, which increases the PO_4 loading. In the Fe-(hydr)oxide-dominated soils S1 and S2, this PO_4 release is caused by mineralisation and a decrease of the RSA, and it varies per earthworm species matching the hypothesis of more intense mineralisation by Lr than Lt as Lr has a higher PO_4 loading than Lt. The consequence of the increased PO_4 loading is a decrease in the NOM loading and a decrease in the competitive effect of NOM adsorption on the PO_4 solubility of casts compared to the bulk soil. This finding is different from the suggestion made by Ros et al. (2017) who proposed an increase of the NOM loading of the metal-(hydr)oxides in casts. These authors did not measure the RSA of both bulk soil and casts and assumed there were no differences in the SSA of metal-(hydr)oxides between both matrices (Ros et al., 2017), which can explain this discrepancy. The NOM loadings required to explain the measured PO_4 concentrations in water extracts from casts in Ros et al. (2017) were very high ($>2 \mu\text{mol m}^{-2}$), considering that the NOM loading of Dutch agricultural soils is typically $1.6 \pm 0.3 \mu\text{mol m}^{-2}$ (Hiemstra et al., 2013). In our study, a more realistic NOM loading of $1.1 \pm 0.6 \mu\text{mol m}^{-2}$ for the casts of the Fe-(hydr)oxide-dominated soils was obtained.

For the Al-(hydr)oxide-dominated soils S3 and S4, we followed a different approach to fit the NOM loading, as for some cast samples the predicted PO_4 concentration in the absence of NOM competition in the CD modelling was already higher than the measured PO_4 concentration in the water extracts. This was likely due to the combination of a low to very low NOM loading of casts and, in part, an underestimation of the RSA by the probe-ion method. We did additional measurements to improve our RSA estimates, but those did not yield satisfying results (SI S9). In our alternative approach, we therefore increased the RSA in the CD modelling for one sample of soil S3 for which water-extractable PO_4 was most overpredicted until the fitted NOM loading became zero. Subsequently, this RSA was applied to all other samples of soil S3 as no differences in the RSA were observed between

treatments (section 3.3.2.2). Fixing the RSA allowed us to fit the NOM loading of the other samples of soil S3. The same approach was followed for soil S4. Similar to the Fe-(hydr)oxide-dominated soils, the NOM loading of the Al-(hydr)oxide-dominated soils was lower for the casts than for the corresponding bulk soils (Fig. 3.3). This strengthens the validity of our conclusions on the importance of the release of PO_4 from mineralisation and the decrease in the RSA of the Fe-(hydr)oxide-dominated soils being the major mechanisms for enhancing PO_4 solubility in the cast, while the competitive effect of NOM adsorption on the PO_4 concentration in the water extracts decreases in casts compared to the bulk soil.

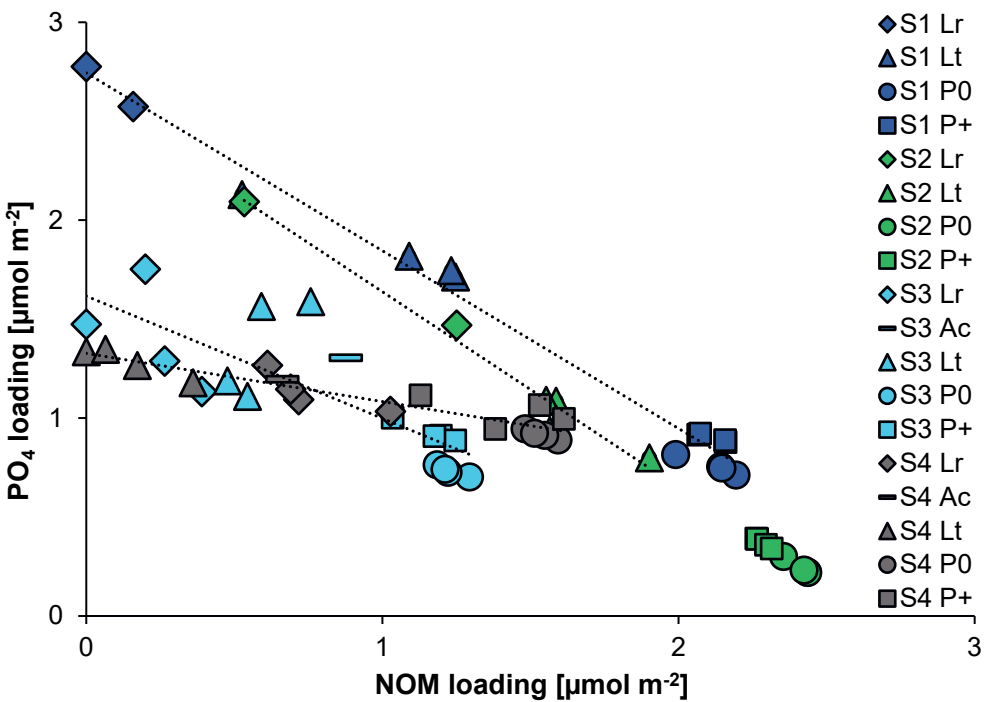


Figure 3.3 Relationship between the NOM and PO_4 loading of metal-(hydr)oxides for the four soils and earthworm casts. For the bulk soil samples (P0 and P+) of soil S2, no NOM loading could be modelled as the water-soluble PO_4 concentration was below the detection limit of the SFA. The NOM loading of these samples has been calculated from the regression line between the NOM and PO_4 loading of the casts from this soil but should be interpreted with care as it is based on extrapolation. Colours refer to soils and symbols to treatments. Dotted lines are regression lines for each soil. The calculations for the Al-(hydr)oxide-dominated soils S3 and S4 were done using adapted values for the RSA (see text).

3.3.3 Scenario analysis

The contribution of the different controlling mechanisms of earthworm-enhanced PO_4 solubility in earthworm casts is illustrated in Fig. 3.4. Overall, the strong increase of pH in soils S2, S3, and S4 (a; Table S6.1) has relatively little effect on the increase in PO_4 solubility. A major contribution is made by PO_4 addition to the system through stimulated mineralisation inside the earthworm gut, leading to an increase of $\text{PO}_4\text{-ox}$ (b; Fig. 3.1a). Both mechanisms were already acknowledged in the literature (Le Bayon and Milleret, 2009; Ros et al., 2017; Vos et al., 2019; Vos et al., 2014). A new finding in our work is the decrease in the RSA of casts (c). We observed this only for the Fe-(hydr)oxide-dominated soils, whereas this process is absent or minor in the Al-(hydr)oxide-dominated soils (Fig. 3.2a). Another new conclusion is related to the role of competitive binding between NOM and PO_4 , as in contrast to a previous suggestion (Ros et al., 2017), we observed a lower NOM loading in earthworm casts than in the bulk soil (d; Fig. 3.3). This decrease of interfacial NOM is a feedback process resulting from the increase in PO_4 loading induced by processes (a)-(c). Consequently, the competitive effect of NOM adsorption exerted on PO_4 solubility decreases in casts compared to their corresponding bulk soils.

All these controlling mechanisms of earthworm-enhanced PO_4 solubility are directly or indirectly related to the capacity of an earthworm to mineralise the organic material they ingest, which therefore governs the ability of the various earthworm species to enhance PO_4 solubility in soils. Although the magnitude of earthworm-enhanced PO_4 solubility, therefore, differs per earthworm species, the same controlling mechanisms are involved within a soil.

The identification and understanding of the various mechanisms involved in earthworm-enhanced PO_4 solubility in our study show how nature can teach us how to increase the sustainability of agricultural systems by decreasing P fertiliser inputs while still increasing the level of readily plant-available soil P to support optimal plant growth. As the decrease of the RSA was only present in soils where the metal-(hydr)oxide fraction was dominated by Fe-(hydr)oxides, earthworms have the largest potential to improve the sustainability of P use in those soils. Since the redox sensitivity of Fe-(hydr)oxide-dominated soils contributed to increased PO_4 solubility, this can potentially be relevant for other oxyanions with a high affinity for binding to soil metal-(hydr)oxides like arsenate (As(V)) (Stachowicz et al., 2008). In As(V)-contaminated soils, a decrease of the RSA caused by earthworms might lead to increased levels of soluble As(V), translating into higher toxicity. Such side-effects of earthworm-enhanced PO_4 solubility should be taken into account when considering the use of earthworms to make the P nutrition of our agricultural systems more sustainable.

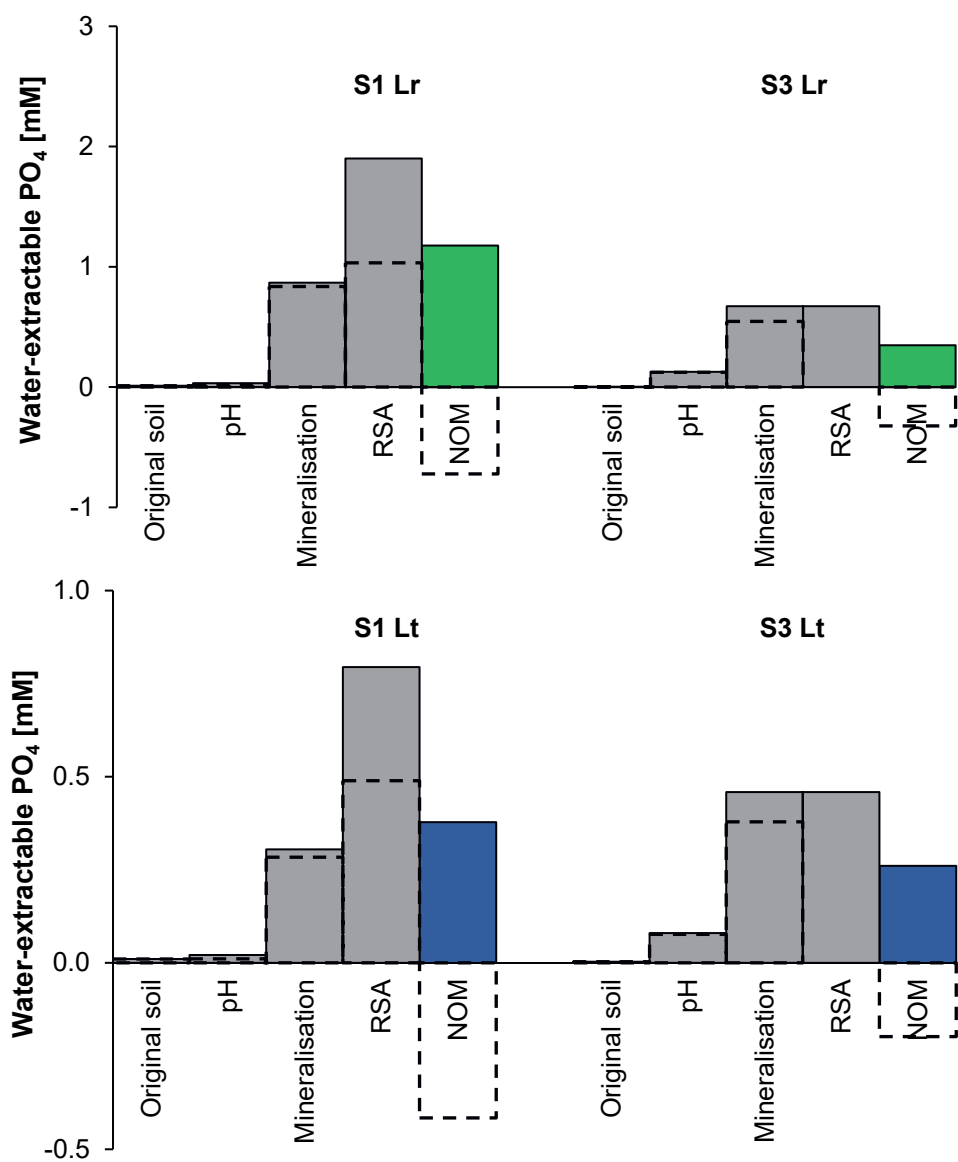


Figure 3.4 Additive effects of the controlling mechanisms of earthworm-enhanced PO₄ solubility on water-extractable PO₄ for soils (columns: soil S1 (left) as representative of a Fe-(hydr)oxide-dominated soil and soil S3 (right) as representative of an Al-(hydr)oxide-dominated soil) and earthworm species (rows: the two species for which sufficient cast material was collected for replicates of the corresponding measurements, Lr and Lt). The controlling mechanisms indicated on the x-axis are: change in pH (pH), increased NOM mineralisation releasing additional PO₄ (Mineralisation), a decrease in RSA increasing water-soluble PO₄ as there is less surface area available for PO₄ adsorption (RSA), and a

decrease in competition for adsorption sites between PO_4 and NOM (NOM) as the NOM loading becomes lower in earthworm casts. Starting point for each figure is the condition of the controlling mechanisms of earthworm-enhanced PO_4 solubility in the P0 soil (original soil). Subsequently, the CD model was used to predict the PO_4 concentration in the water extracts by changing consecutively the pH, $\text{PO}_4\text{-ox}$, and the RSA to the values measured in earthworm casts. Lastly, the fitted NOM content was included. This approach is shown by the different bars in each figure, in which every sequential grey scaled bar represents the additive effect of a controlling mechanism and the coloured bars match with the observed PO_4 concentration for the specific treatment (Fig. 3.1b). Dotted bars indicate the separate effect of a controlling mechanism, which is calculated as the additive effect of each successively mentioned mechanism minus the additive effect of the previously mentioned mechanism(s). Because of the possible effects of the interplay of the controlling mechanisms, the calculation of the separate effects might yield different values of the dotted bars.

Acknowledgements

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Supporting information

S1 Greenhouse experiment

S1.1 Analytical procedures for soil characterisation

The physico-chemical properties of the four soils were determined on dried (40°C) and sieved (2 mm) soil following the analytical procedures described below:

- Soil texture (% sand, silt, and clay): sieve and pipette method as described by Houba et al. (1997).
- CaCO_3 : Scheibler method as described by Allison (1960).
- NOM: estimated from loss-on-ignition in a muffle furnace (550°C) and corrected for water loss from the crystalline clay structure according to $\text{NOM} = \text{NOM measured by loss-on-ignition} - 0.07 \times \% \text{Clay}$ (Houba et al., 1997).
- pH- CaCl_2 : measured in a 0.01M CaCl_2 extract obtained at a solid-to-solution ratio of 1:10 (w:v) and shaken for 2 h on a horizontal shaker at 180 strokes min^{-1} (Houba et al., 2000).
- P-AL: measured by segmented flow analysis (SFA) after the extraction with acetic acid and ammonium lactate as described by Egnér et al. (1960). In the Netherlands, P-AL is routinely used as a soil extraction method in agricultural practice for the P fertiliser recommendation of grassland (Reijneveld et al., 2010).
- $\text{PO}_4\text{-ox}$, P_{ox} , Fe_{ox} and Al_{ox} : see main text section 3.2.2.
- α : the P loading of reactive metal-(hydr)oxides was calculated as $\alpha = \text{P}_{\text{ox}} / (\text{Fe}_{\text{ox}} + \text{Al}_{\text{ox}})$ with all oxalate-extractable elements expressed in mol kg^{-1} . This equation was originally developed for non-calcareous sandy soils (Van der Zee and Van Riemsdijk, 1986). For consistency, it is applied to the calcareous clay soil S1, but its value should be interpreted with care.
- Fe-DCB: a DCB solution of $170 \text{ g L}^{-1} \text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ and $17 \text{ g L}^{-1} \text{Na}_2\text{S}_2\text{O}_4$ was used to extract the total (non-crystalline and crystalline) amount of Fe-(hydr)oxides at a solution-to-solid ratio of 60 L kg^{-1} . The suspension was shaken for 16 h on a horizontal shaker with 180 strokes min^{-1} at 20°C. After centrifugation for 10 min at 3000 rotations min^{-1} Fe was measured with inductively coupled plasma-atomic emission spectroscopy (ICP-AES). This procedure was based on the method described by Holmgren (1967) and Mehra and Jackson (1960).
- Non-crystalline and crystalline Fe-(hydr)oxides: Calculated by scaling the Fe of the non-crystalline (Fh) and crystalline (goethite, lepidocrocite, and hematite) Fe-(hydr)oxides on the total pool of Fe-(hydr)oxides derived by XAS (SI S3).

51.2 Experimental setup pot experiment

A greenhouse pot experiment was set up as a full factorial randomized block design with soil type (four soils) and earthworm species (five treatments) as independent factors. Each treatment was replicated four times, resulting in a total of $4 \times 5 \times 4 = 80$ pots. The replicates were distributed over four blocks and both the location of the blocks and the locations of the pots within the blocks were randomized weekly.

Mitscherlich pots (diameter: 20 cm; height: 23 cm) were filled with 6.5 kg air-dried, sieved (5 mm), and homogenised soil. All soils were fertilised equally with N (125 kg ha^{-1} ($=4.0 \text{ mmol kg}^{-1} \text{ soil}$) equally divided over NH_4 and NO_3 and added as NH_4NO_3), K (100 kg ha^{-1} ($=1.1 \text{ mmol kg}^{-1} \text{ soil}$) as KCl), Ca (50 kg ha^{-1} ($=0.6 \text{ mmol kg}^{-1} \text{ soil}$) as CaCl_2), Mg (25 kg ha^{-1} ($=0.5 \text{ mmol kg}^{-1} \text{ soil}$) as MgSO_4 and MgCl_2), S (12 kg ha^{-1} ($=0.2 \text{ mmol kg}^{-1} \text{ soil}$) as MgSO_4), and trace elements (B, Mn, Cu, Zn, and Mo). In the case of the control with P fertiliser (P+), 100 kg P ha^{-1} ($=1.4 \text{ mmol kg}^{-1} \text{ soil}$) as NaH_2PO_4 was added as well. De-ionised water was added to all pots to obtain a water holding capacity (WHC) of 60%. An unfertilised germination layer of 0.5 kg air-dried soil was moistened with de-ionised water to the same WHC and placed on top of the fertilised soil. Subsequently, 4.0 g of perennial ryegrass (*Lolium perenne*) seeds were sown per pot. The grass was pre-grown to stimulate tillering and root formation, and 30 days after sowing additionally fertilised with N (50 kg ha^{-1} ($=1.6 \text{ mmol kg}^{-1} \text{ soil}$) equally divided over NH_4 and NO_3 and added as NH_4NO_3) and K (40 kg ha^{-1} ($=0.5 \text{ mmol kg}^{-1} \text{ soil}$) as KCl). During the pre-growing period, the grass was cut twice 5 cm above the soil surface (21 and 33 days after sowing). The grass cuttings were shredded and left on the soil surface (first time) to decompose and serve as earthworm feed, or harvested (second time). The earthworms were added 35 days after sowing.

The five earthworm treatments included *Lumbricus rubellus* Hoffmeister, 1843 (Lr; feeds on surface litter), *Aporrectodea caliginosa* (Savigny, 1826) (Ac; feeds on roots, soil and soil-associated natural organic matter (NOM)), *Lumbricus terrestris* Linnaeus, 1758 (Lt; ingests some soil, but mainly feeds on surface litter) (Bouché, 1977; Drake and Horn, 2007), a control without earthworms (P0) and a control without earthworms but additional mineral P fertilisation (P+). Earthworm density was (Lr) 29, (Ac) 60, (Lt) 66, (-) 0, and (+) 0 g earthworm pot^{-1} respectively, which corresponded with (Lr) 1751, (Ac) 2546, (Lt) 477, (-) 0 and (+) 0 individuals m^{-2} . These values are higher than earthworm densities observed in pastures in the Netherlands (Didden, 2001; Van Vliet et al., 2007), because normal field densities were expected to yield insufficient cast material for chemical analysis. The earthworm species Lr and Ac were collected from fields and park areas in and near Wageningen, the Netherlands, whereas the species Lt was purchased from Star Food Holland BV, Barneveld, the Netherlands. The earthworms were kept in dark mesocosms under controlled conditions (15°C) with tree leaves as feed. Earthworms were added to the pots after their intestines had been voided for 48 h according to the wet filter paper method (Dalby et al., 1996). Only adult earthworms or large juveniles were used in this experiment.

In the centre of each pot a watering tube was placed through which the grass was watered with de-ionised water every other day. The soil moisture content was adjusted to exactly 60% of WHC once a week by watering the pots gravimetrically. After the addition of the earthworms, a wireframe was placed on each pot to support an elevated tulle netting which prevented the earthworms from escaping but permitted light entry and grass growth.

S1.3 Sample collection

Grass was harvested 33 days after the addition of the earthworms (68 days after sowing). One day later, the earthworms were collected by hand sorting them from the pots. They were rinsed with water to remove any adhering soil particles. Earthworm casts for chemical analysis were excreted over the next 48 h when the earthworms were left in 100 mL polyethylene cups in a dark and temperature-controlled (15°C) room. The cups were shortly opened and gently shaken by hand every day to activate the earthworms. Since the amount of cast collected from Ac was limited, all four replicates had to be pooled to obtain sufficient cast material for chemical analysis, but even then not all chemical analyses could be performed. The moist soil of the treatments without earthworms (P0 and P+) was homogenised per pot, sieved over 2 mm, and stored in airtight cups until the cast material was collected and chemical analysis of all samples was conducted at the same time.

S2 Surface complexation reactions

Table S2.1 (next pages) Formation reactions of surface species with charge distribution coefficients (Δz) and affinities ($\log K$) for surface complexation reactions as used in the CD-MUSIC model for ferrihydrite. Surface densities were set at 3.0 nm⁻² ($\equiv\text{FeOH}^{-1/2}$ (a); monodentate), 2.51 nm⁻² ($\equiv\text{FeOH}^{-1/2}$ (bl); bidentate low affinity), 0.29 nm⁻² ($\equiv\text{FeOH}^{-1/2}$ (bh); bidentate high affinity) and 1.4 nm⁻² ($\equiv\text{Fe}_3\text{O}^{-1/2}$) and the capacitance values of the extended Stern layer at $C_1=1.15 \text{ F m}^{-2}$ and $C_2=0.90 \text{ F m}^{-2}$. All values are taken from literature (Hiemstra et al., 2013; Hiemstra and Zhao, 2016; Mendez and Hiemstra, 2019; Mendez and Hiemstra, 2020).

Surface species	$\equiv\text{FeOH}^{-1/2}(\text{a})$	$\equiv\text{FeOH}^{-1/2}(\text{bl})$	$\equiv\text{FeOH}^{-1/2}(\text{bh})$	$\equiv\text{Fe}_{30}^{-1/2}$	$\equiv\text{HNOM}^{-1}$	Δz0	Δz1	Δz2	H^{+}	Na^{+}	K^{+}	NH_4^{+}	Ca^{2+}	Mg^{2+}	NO_3^{-}	PO_4^{3-}	CO_3^{2-}	$\log K$
$\equiv\text{FeOH}(\text{a})$	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
$\equiv\text{FeOH}_2(\text{a})$	1	0	0	0	0	+1	0	0	1	0	0	0	0	0	0	0	0	8.1
$\equiv\text{FeOH}(\text{a})\cdots\text{Na}$	1	0	0	0	0	0	+1	0	0	1	0	0	0	0	0	0	0	-0.60
$\equiv\text{FeOH}(\text{a})\cdots\text{K}$	1	0	0	0	0	0	+1	0	0	0	1	0	0	0	0	0	0	-1.30
$\equiv\text{FeOH}(\text{a})\cdots\text{NH}_4$	1	0	0	0	0	0	+1	0	0	0	0	1	0	0	0	0	0	-1.30
$\equiv\text{FeOH}_2(\text{a})\cdots\text{NO}_3$	1	0	0	0	0	+1	-1	0	1	0	0	0	0	0	1	0	0	7.42
$\equiv\text{FeOH}(\text{bl})$	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
$\equiv\text{FeOH}_2(\text{bl})$	0	1	0	0	0	+1	0	0	1	0	0	0	0	0	0	0	0	8.1
$\equiv\text{FeOH}(\text{bl})\cdots\text{Na}$	0	1	0	0	0	0	+1	0	0	1	0	0	0	0	0	0	0	-0.60
$\equiv\text{FeOH}(\text{bl})\cdots\text{K}$	0	1	0	0	0	0	+1	0	0	0	1	0	0	0	0	0	0	-1.30
$\equiv\text{FeOH}(\text{bl})\cdots\text{NH}_4$	0	1	0	0	0	0	+1	0	0	0	0	1	0	0	0	0	0	-1.30
$\equiv\text{FeOH}_2(\text{bl})\cdots\text{NO}_3$	0	1	0	0	0	+1	-1	0	1	0	0	0	0	0	1	0	0	7.42
$\equiv\text{FeOH}(\text{bh})$	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
$\equiv\text{FeOH}_2(\text{bh})$	0	0	1	0	0	+1	0	0	1	0	0	0	0	0	0	0	0	8.1
$\equiv\text{FeOH}(\text{bh})\cdots\text{Na}$	0	0	1	0	0	0	+1	0	0	1	0	0	0	0	0	0	0	-0.60
$\equiv\text{FeOH}(\text{bh})\cdots\text{K}$	0	0	1	0	0	0	+1	0	0	0	1	0	0	0	0	0	0	-1.30
$\equiv\text{FeOH}(\text{bh})\cdots\text{NH}_4$	0	0	1	0	0	0	+1	0	0	0	0	1	0	0	0	0	0	-1.30
$\equiv\text{FeOH}_2(\text{bh})\cdots\text{NO}_3$	0	0	1	0	0	+1	-1	0	1	0	0	0	0	0	1	0	0	7.42
$\equiv(\text{FeO})_2\text{PO}_2(\text{bl})$	0	2	0	0	0	+0.46	-1.46	0	2	0	0	0	0	0	0	1	0	28.31
$\equiv\text{Fe}_3\text{O}$	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
$\equiv\text{Fe}_3\text{OH}$	0	0	0	1	0	+1	0	0	1	0	0	0	0	0	0	0	0	8.1
$\equiv\text{Fe}_3\text{O}\cdots\text{Na}$	0	0	0	1	0	0	+1	0	0	1	0	0	0	0	0	0	0	-0.60

(Table S2.1 continues on next page)

(Table S2.1 continued)

Surface species	$\equiv\text{FeOH-1/2 (a)}$	$\equiv\text{FeOH-1/2 (bl)}$	$\equiv\text{FeOH-1/2 (bh)}$	$\equiv\text{Fe3O-1/2}$	$\equiv\text{HNOM-1}$	Az0	Az1	Az2	H ⁺	Na ⁺	K ⁺	NH ₄ ⁺	Ca ²⁺	Mg ²⁺	NO ₃ ⁻	PO ₄ ³⁻	CO ₃ ²⁻	logK
$\equiv\text{Fe}_3\text{O}\cdots\text{K}$	0	0	0	1	0	0	+1	0	0	0	1	0	0	0	0	0	0	-1.30
$\equiv\text{Fe}_3\text{O}\cdots\text{NH}_4$	0	0	0	1	0	0	+1	0	0	0	0	1	0	0	0	0	0	-1.30
$\equiv\text{Fe}_3\text{OH}\cdots\text{NO}_3$	0	0	0	1	0	+1	-1	0	1	0	0	0	0	0	1	0	0	7.42
$\equiv(\text{FeO})_2\text{PO}_2(\text{bh})$	0	0	2	0	0	+0.46	-1.46	0	2	0	0	0	0	0	0	1	0	28.31
$\equiv(\text{FeO})_2\text{POOH}(\text{bl})$	0	2	0	0	0	+0.65	-0.65	0	3	0	0	0	0	0	0	1	0	33.52
$\equiv(\text{FeO})_2\text{POOH}(\text{bh})$	0	0	2	0	0	+0.65	-0.65	0	3	0	0	0	0	0	0	1	0	33.52
$\equiv\text{FeOPO}_2\text{OH}(\text{a})$	1	0	0	0	0	+0.28	-1.28	0	2	0	0	0	0	0	0	1	0	26.36
$\equiv\text{FeOPO}_2\text{OH}(\text{bl})$	0	1	0	0	0	+0.28	-1.28	0	2	0	0	0	0	0	0	1	0	26.36
$\equiv\text{FeOPO}_2\text{OH}(\text{bh})$	0	0	1	0	0	+0.28	-1.28	0	2	0	0	0	0	0	0	1	0	26.36
$\equiv\text{FeOPO}(\text{OH})_2(\text{a})$	1	0	0	0	0	+0.33	-0.33	0	3	0	0	0	0	0	0	1	0	29.84
$\equiv\text{FeOPO}(\text{OH})_2(\text{bl})$	0	1	0	0	0	+0.33	-0.33	0	3	0	0	0	0	0	0	1	0	29.84
$\equiv\text{FeOPO}(\text{OH})_2(\text{bh})$	0	0	1	0	0	+0.33	-0.33	0	3	0	0	0	0	0	0	1	0	29.84
$(\equiv\text{FeO})_2\text{PO}_2\text{Ca}(\text{bl})$	0	2	0	0	0	+0.62	-1.08	+1.46	2	0	0	0	1	0	0	1	0	30.09
$(\equiv\text{FeO})_2\text{PO}_2\text{Ca}(\text{bh})$	0	0	2	0	0	+0.62	-1.08	+1.46	2	0	0	0	1	0	0	1	0	30.09
$\equiv\text{FeOPO}_3\text{Ca}(\text{a})$	1	0	0	0	0	+0.24	-1.30	+1.06	1	0	0	0	1	0	0	1	0	22.27
$\equiv\text{FeOPO}_3\text{Ca}(\text{bl})$	0	1	0	0	0	+0.24	-1.30	+1.06	1	0	0	0	1	0	0	1	0	22.27
$\equiv\text{FeOPO}_3\text{Ca}(\text{bh})$	0	0	1	0	0	+0.24	-1.30	+1.06	1	0	0	0	1	0	0	1	0	22.27
$(\equiv\text{FeOH})_2\text{Ca}(\text{bl})$	0	2	0	0	0	+0.94	+1.06	0	0	0	0	0	1	0	0	0	0	2.64
$(\equiv\text{FeOH})_2\text{Ca}(\text{bh})$	0	0	2	0	0	+0.94	+1.06	0	0	0	0	0	1	0	0	0	0	5.13
$(\equiv\text{FeOH})_2\text{Mg}(\text{bl})$	0	2	0	0	0	+0.89	+1.11	0	0	0	0	0	0	1	0	0	0	1.87

(Table S2.1 continued)

Surface species	$\equiv\text{FeOH}^{-1/2}(\text{a})$	$\equiv\text{FeOH}^{-1/2}(\text{bl})$	$\equiv\text{FeOH}^{-1/2}(\text{bh})$	$\equiv\text{Fe}_{30}^{-1/2}$	$\equiv\text{HNOM}^{-1}$	Δz_0	Δz_1	Δz_2	H^+	Na^+	K^+	NH_4^+	Ca^{2+}	Mg^{2+}	NO_3^-	PO_4^{3-}	CO_3^{2-}	$\log K$
$(\equiv\text{FeOH})_2\text{Mg}(\text{bh})$	0	0	2	0	0	+0.89	+1.11	0	0	0	0	0	0	1	0	0	0	4.09
$\equiv\text{FeOPO}_3\text{Mg}(\text{a})$	1	0	0	0	0	+0.22	-1.55	+1.33	1	0	0	0	0	1	0	1	0	22.00
$\equiv\text{FeOPO}_3\text{Mg}(\text{bl})$	0	1	0	0	0	+0.22	-1.55	+1.33	1	0	0	0	0	1	0	1	0	22.00
$\equiv\text{FeOPO}_3\text{Mg}(\text{bh})$	0	0	1	0	0	+0.22	-1.55	+1.33	1	0	0	0	0	1	0	1	0	22.00
$(\equiv\text{FeO})_2\text{CO}(\text{bl})$	0	2	0	0	0	+0.66	-0.66	0	2	0	0	0	0	0	0	0	1	21.73
$(\equiv\text{FeO})_2\text{CO}(\text{bh})$	0	0	2	0	0	+0.66	-0.66	0	2	0	0	0	0	0	0	0	1	21.73
$(\equiv\text{FeO})_2\text{CO}(\text{bl})\cdots\text{Na}$	0	2	0	0	0	+0.65	+0.35	0	2	1	0	0	0	0	0	0	1	22.38
$(\equiv\text{FeO})_2\text{CO}(\text{bh})\cdots\text{Na}$	0	0	2	0	0	+0.65	+0.35	0	2	1	0	0	0	0	0	0	1	22.38
$\equiv\text{FeOCO}_2(\text{a})$	1	0	0	0	0	+0.34	-1.34	0	1	0	0	0	0	0	0	0	1	11.6
$\equiv\text{FeOCO}_2(\text{bl})$	0	1	0	0	0	+0.34	-1.34	0	1	0	0	0	0	0	0	0	1	11.6
$\equiv\text{FeOCO}_2(\text{bh})$	0	0	1	0	0	+0.34	-1.34	0	1	0	0	0	0	0	0	0	1	11.6
$\equiv\text{FeNOM}$	0	1	0	0	1	+1.5	-1	-0.5	0	0	0	0	0	0	0	0	0	0
$\equiv\text{FeOH}_2\text{NOM}$	0	1	0	0	1	+2	-1.5	-0.5	0	0	0	0	0	0	0	0	0	0.6
$\equiv\text{FeNOMH}$	0	1	0	0	1	+1.5	-0.5	0	1	0	0	0	0	0	0	0	0	3.3

S3 X-ray absorption spectroscopy (XAS)

The four bulk soils (P0 treatment) and earthworm casts from soil S1 (Lt and Lr) and from soil S2 (Lt) were analysed by Fe K-edge XAS, including X-ray absorption near-edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) spectroscopy. For XAS analysis, the dried samples were ground and mixed with a minor amount of cellulose using an agate mortar and pestle, and prepared as 7 mm or 13 mm diameter pellets. The XAS analyses were performed at the Accelerator Test Facility and Synchrotron Radiation Source at the Karlsruhe Institute of Technology (KIT). Soils S3 and S4 were analysed at the SUL-X beamline, the other samples (soils S1 and S2 and corresponding earthworm casts) at the XAS beamline. The X-ray photon energy was calibrated using a metallic Fe foil (first maximum of the first derivative of the absorption edge set to 7112 eV). The incident photon intensity was measured with an ionisation chamber, the Fe K α fluorescence signal at a 90° angle to the incident beam using a 1-element Vortex (XAS beamline) or 7-element silicon drift detector (SUL-X beamline). Reference spectra were available from earlier work (Regelink et al., 2014). For data extraction and data interpretation by linear combination fitting (LCF), the software code ATHENA was used (Ravel and Newville, 2005).

In Fig. S3.1, the XANES and EXAFS spectra of reference materials, the soils, and earthworm casts are shown. For soils S1 and S2, overlay plots of soil and corresponding cast spectra (soil S1 and Lt and Lr casts named “S1 – overlay” and soil S2 and Lt cast named “S2 – overlay” in Fig. S3.1) reveal no detectable differences. The speciation of solid Fe in the earthworm casts thus did not detectably vary from the speciation of Fe in the respective soils. To enhance the quality of the spectra for LCF analysis, the average spectra of the normalised soil and cast spectra were used for soils S1 and S2 (spectra named “Soil 1” and “Soil 2” in Fig. S3.1).

The soil spectra indicated variations in the speciation of solid Fe among the four soils. These variations were characterised by LCF analysis of the EXAFS spectra over the k-range 2–10 Å⁻¹, following a similar approach as in previous work (Regelink et al., 2014). Eight reference spectra were used for LCF analysis: 2-line ferrihydrite (Fh), goethite (Gt), hematite (Hm), and lepidocrocite (Lp) as proxies for Fe in Fe(III)-(hydr)oxides and smectite (SWy-2), illite (IMt-1), nontronite (NAu-2), and biotite (Bio) as proxies for Fe in clay minerals with varying Fe contents and Fe redox state. Using these eight reference spectra, all possible 2- to 4-component fits were calculated (with individual fractions constrained to 0–100% while their sum was unconstrained). For each soil, the best n-component fit was retained whose r-factor (normalised sum of squared residuals) was at least 20% lower than the r-factor of the best (n–1)-component fit. From the normalised fractions of Fh, Gt, Hm, and Lp, the fraction of Fe in Fe(III)-(hydr)oxides (sum of Fh, Gt, Hm, and Lp) was calculated. The remainder of Fe was present in clay minerals. From the fractions of the clay minerals and their Fe(II) contents (7% for SWy-2; 12% for IMt-1; 0% for NAu-2 and 87% for Bio), the fraction of Fe(II) was estimated. The LCF results of the four soils listed in Table S3.1 reveal

variation in the fraction of Fe in Fe-(hydr)oxides as well as in the nature of the Fe-(hydr)oxides.

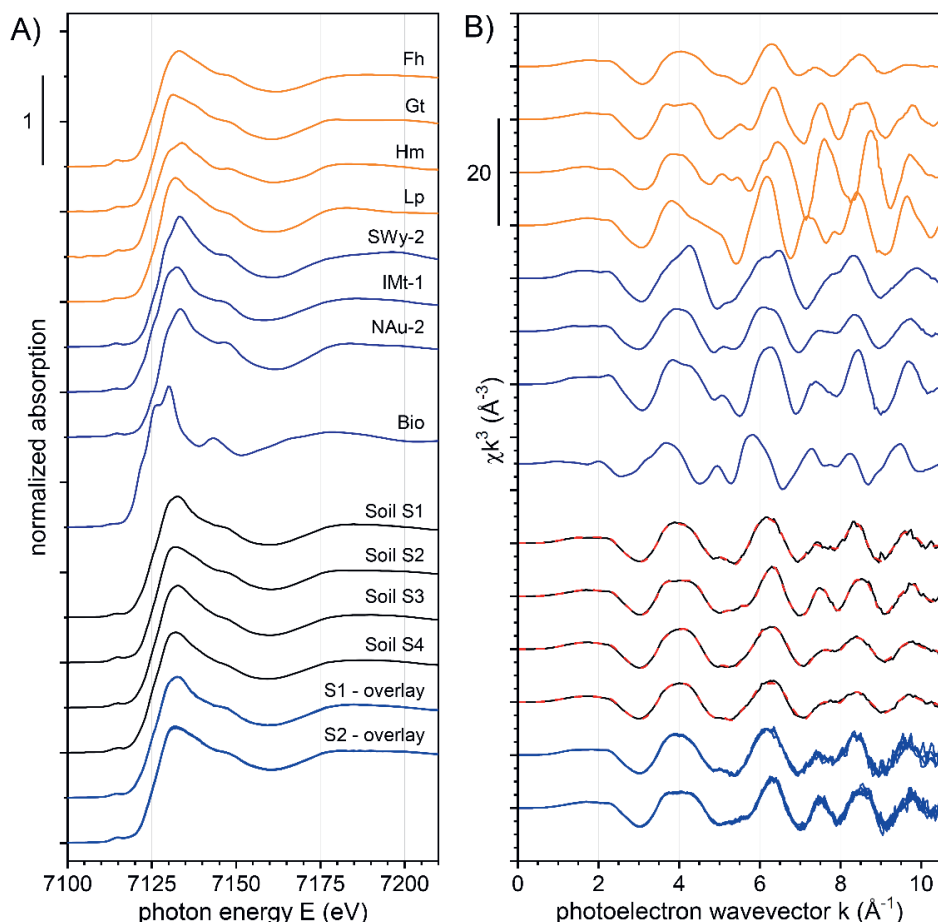


Figure S3.1 Fe K-edge XANES and EXAFS spectra of Fe-(hydr)oxide (Fh: ferrihydrite; Gt: goethite; Hm: hematite; Lp: lepidocrocite; orange lines) and clay mineral references (SWy-2: smectite; IMt-1: illite; NAu-2: nontronite; Bio: biotite; dark blue lines) and soil and casts. For soils S1 and S2, spectra collected for the soil and corresponding cast samples are shown in overlay plots (blue lines). Spectra of the soils S1, S2, S3, and S4 (black lines) are overlain by LCF-based reconstructions (red dashed lines). The spectra shown for soils S1 and S2 correspond to the average of the corresponding normalised soil and cast spectra. LCF results used for LCF reconstructions are listed in Table S3.1.

Table S3.1 EXAFS LCF analysis results. Normalised fitted fractions (as a percentage) together with the fitted sum and the r-factor are given.

	Normalised fitted fractions ^a							Fit sum	r-factor	Fraction		
	Fh	Gt	Hm	Lp	SWy	IMt	NAu			Bio	Fe(III)- oxides ^b	Fe(II) in clays ^c
					-2	-1	-2					
S1	22				24		31	22	1.05	0.014	22	21
S2	15	67		10	7				0.97	0.008	93	1
S3	42		9		34			15	1.12	0.007	51	15
S4	33		11		36			20	1.05	0.010	44	20

^a Fh=2-line ferrihydrite; Gt=goethite; Hm=hematite; Lp=lepidocrocite; SWy-2=smectite; IMt-1=illite; NAu-2=nontronite; Bio=biotite.

^b Sum of the Fe(III)-hydroxides including Fh, Gt, Hm, and Lp; the remaining Fe was present in clay minerals.

^c Estimated from the fractions of clay minerals and their Fe(II) contents (7% for SWy-2; 12% for IMt-1; 0% for NAu-2 and 87% for Bio).

S4 Calculation of metal-(hydr)oxide properties

The calculations of metal-(hydr)oxide properties presented in this section are based on the equations given by Hiemstra (2018) and Mendez et al. (2020). In this section, every reference to “soil” can be interpreted as either earthworm cast or bulk soil.

S4.1 Soil consisting of only non-crystalline oxides (S1, S3 and S4)

For soil S1, the metal-(hydr)oxide fraction consists entirely of non-crystalline oxides (Table 3.1 in the main text), because the Fe-(hydr)oxides consist solely of Fh (Table S3.1). For soils S3 and S4, the crystalline oxide fraction ≈ 0 , as those soils are dominated by non-crystalline Al-(hydr)oxides, and the small Fe-(hydr)oxide fraction in these soils consists mainly of non-crystalline oxides (Table 3.1 in the main text). For these soils, the small fraction of crystalline Fe-(hydr)oxides compared to the total pool of metal-(hydr)oxides was ignored in our further calculations.

S4.1.1 Probe-ion method

To calculate the RSA_{soil} ($m^2 kg_{soil}^{-1}$), Eq. 3.1 in the main text can be rewritten to:

$$RSA_{soil} = RSA_{ncrys} = (R_{ev} - SSR \times C) / \Gamma_{ncrys} \quad (S4.1)$$

where R_{ev} is the total amount of reversibly bound PO_4 ($mol kg^{-1}$) represented by PO_4 -ox, Γ_{ncrys} ($mol m^{-2}$) the PO_4 loading of the non-crystalline metal-(hydr)oxides as calculated with

the CD-model using Fh as a proxy, SSR the solution-to-solid ratio employed during NaHCO_3 extraction (50 L kg^{-1}), and C the measured equilibrium PO_4 concentration in the NaHCO_3 extract (M).

This RSA_{soil} can be scaled to the amount of non-crystalline oxides involved into the SSA_{soil} ($\text{m}^2 \text{ g}_{\text{ncrys}}^{-1}$):

$$\text{SSA}_{\text{soil}} = \text{SSA}_{\text{ncrys}} = \text{RSA}_{\text{soil}} / m_{\text{ncrys}} \quad (\text{S4.2})$$

where m_{ncrys} ($\text{g}_{\text{ncrys}} \text{ kg}_{\text{soil}}^{-1}$) is the mass content of non-crystalline oxides (Fh and Al-(hydr)oxides), with:

$$m_{\text{ncrys}} = \text{non-crystalline fraction XAS} \times \text{Fe}_{\text{DCB}} \times M_{\text{nano-Fh}} + \text{Al}_{\text{ox}} \times M_{\text{nano-Al(OH)}_3} \quad (\text{S4.3})$$

with Fe_{DCB} and Al_{ox} in $\text{mol kg}_{\text{soil}}^{-1}$, and $M_{\text{nano-Fh}}$ and $M_{\text{nano-Al(OH)}_3}$ in g mol^{-1} .

In order to calculate m_{ncrys} , the molar masses ($M_{\text{nano-Fh}}$ and $M_{\text{nano-Al(OH)}_3}$) of the non-crystalline oxides are required for which, in turn, their mass densities ($\rho_{\text{nano-Fh}}$ and $\rho_{\text{nano-Al(OH)}_3}$) are needed. Both the molar masses and mass densities of the non-crystalline oxides are size-dependent and can be calculated according to the theoretical approach described below.

S4.1.2 Theory

The starting point to calculate $M_{\text{nano-Fh}}$ and $M_{\text{nano-Al(OH)}_3}$ is to determine the mass density ($\rho_{\text{nano-Fh}}$ and $\rho_{\text{nano-Al(OH)}_3}$ in g m^{-3}) of both non-crystalline oxides. Using the same chosen value for the spherical particle diameter (d in m) of both non-crystalline oxides as a starting point, $\rho_{\text{nano-Fh}}$ and $\rho_{\text{nano-Al(OH)}_3}$ can be calculated:

$$\rho_{\text{nano-Fh}} = (M_{\text{core-Fh}} / (n_{\text{o-Fh}} \times V_{\text{o-Fh}})) - (M_{\text{core-Fh}} / n_{\text{o-Fh}} - M_{\text{H}_2\text{O}}) \times 6 / d \times N_{\text{H}_2\text{O}} \quad (\text{S4.4a})$$

$$\rho_{\text{nano-Al(OH)}_3} = (M_{\text{core-Al(OH)}_3} / (n_{\text{o-Al(OH)}_3} \times V_{\text{o-Al(OH)}_3})) - (M_{\text{core-Al(OH)}_3} / n_{\text{o-Al(OH)}_3} - M_{\text{H}_2\text{O}}) \times 6 / d \times N_{\text{H}_2\text{O}} \quad (\text{S4.4b})$$

in which M_{core} is the molar mass (g mol^{-1}) of the bulk mineral, n_{o} is the number of oxygen atoms in the mineral core expressed in mol oxygen per mol metal ions in the mineral core ($\text{mol}_{\text{o}} \text{ mol}^{-1}$), V_{o} the lattice volume ($\text{m}^3 \text{ mol}_{\text{o}}^{-1}$), $M_{\text{H}_2\text{O}}$ the molar mass of water (g mol^{-1}), and $N_{\text{H}_2\text{O}}$ is the density of chemisorbed water (mol m^{-2}). The values of the parameters of Fh and Al(OH)_3 needed for these equations can be found in Table S4.1. Subsequently, we can calculate SSA_{Fh} and $\text{SSA}_{\text{Al(OH)}_3}$ ($\text{m}^2 \text{ g}_{\text{Fh}}^{-1}$ and $\text{m}^2 \text{ g}_{\text{Al(OH)}_3}^{-1}$) by using the same chosen d values for both non-crystalline oxides as in the above given Eqs. S4.4a and S4.4b following:

$$\text{SSA}_{\text{Fh}} = 6 / (\rho_{\text{nano-Fh}} \times d) \quad (\text{S4.5a})$$

$$SSA_{Al(OH)_3} = 6 / (\rho_{nano-Al(OH)_3} \times d) \quad (S4.5b)$$

This allows us to calculate $M_{nano-Fh}$ and $M_{nano-Al(OH)_3}$:

$$M_{nano-Fh} = M_{core-Fh} / (1 - SSA_{Fh} \times N_{H_2O-Fh} \times M_{H_2O}) \quad (S4.6a)$$

$$M_{nano-Al(OH)_3} = M_{core-Al(OH)_3} / (1 - SSA_{Al(OH)_3} \times N_{H_2O-Al(OH)_3} \times M_{H_2O}) \quad (S4.6b)$$

Next, we can calculate the mass fractions of both non-crystalline oxides relative to the total mass of non-crystalline oxides in the soil:

$$f_{Fh} = (Fe-DCB \times \text{non-crystalline fraction XAS} \times M_{nano-Fh}) / (Fe-DCB \times \text{non-crystalline fraction XAS} \times M_{nano-Fh} + Al_{ox} \times M_{nano-Al(OH)_3}) \quad (S4.7a)$$

$$f_{Al(OH)_3} = (Al_{ox} \times M_{nano-Al(OH)_3}) / (Fe-DCB \times \text{non-crystalline fraction XAS} \times M_{nano-Fh} + Al_{ox} \times M_{nano-Al(OH)_3}) \quad (S4.7b)$$

with Fe-DCB and Al_{ox} in $\text{mol kg}_{soil}^{-1}$. The non-crystalline fraction XAS for the non-crystalline Fe-(hydr)oxides of soils S1, S3, and S4 can be found in Table 3.1 in the main text.

The mass-weighted average SSA_d ($\text{m}^2 \text{g}_{ncrys}^{-1}$) for the non-crystalline oxides (Fh and Al-(hydr)oxides) based on our chosen value of d can be calculated according to:

$$SSA_d = f_{Fh} \times SSA_{Fh} + f_{Al(OH)_3} \times SSA_{Al(OH)_3} \quad (S4.8)$$

Table S4.1 Parameters for Fh and Al-(hydr)oxides ($Al(OH)_3$) as used in Eqs. S4.4 and S4.6. All values are taken from Hiemstra (2018) and Mendez et al. (2020).

	Fh	$Al(OH)_3$
M_{core} [g mol^{-1}]	81.65	78.00
n_o [$\text{mol}_o \text{mol}^{-1}$]	1.6	3.0
V_o [$\text{m}^3 \text{mol}_o^{-1}$]	1.07×10^{-5}	1.07×10^{-5}
M_{H_2O} [g mol^{-1}]	18	18
N_{H_2O} [mol m^{-2}]	12.6×10^{-6}	6.3×10^{-6}

S4.1.3 Combining the probe-ion method and theory

The probe-ion method results in a value for SSA_{soil} (Eq S4.2) for the $M_{nano-Fh}$ and $M_{nano-Al(OH)_3}$ used in Eq S4.3. Simultaneously, the theoretical approach results in a SSA_d for the chosen value of d ; in the calculation of SSA_d (Eq S4.8), the same values of $M_{nano-Fh}$ and $M_{nano-Al(OH)_3}$ are used as in the calculation of SSA_{soil} (Eq S4.2). To determine the final value of d as

reported in our study, an iterative calculation can be done until a value of d is found for which $SSA_d = SSA_{soil}$.

S4.2 Soil consisting of non-crystalline and crystalline oxides (S2)

S4.2.1 Probe-ion method

In a soil with both non-crystalline and crystalline oxides like soil S2 the RSA_{soil} ($m^2 kg_{soil}^{-1}$) is calculated based on both oxide types:

$$RSA_{soil} = RSA_{ncrys} + RSA_{crys} \quad (S4.9)$$

Therefore, Eq. 3.1 in the main text can be rewritten as:

$$R_{ev} = RSA_{ncrys} \times \Gamma_{ncrys} + RSA_{crys} \times \Gamma_{crys} + SSR \times C \quad (S4.10)$$

Eq S4.10 can, in turn, be rewritten to:

$$RSA_{ncrys} = (R_{ev} - SSR \times C - RSA_{crys} \times \Gamma_{crys}) / \Gamma_{ncrys} \quad (S4.11)$$

where R_{ev} is the total amount of reversibly bound PO_4 ($mol kg^{-1}$) represented by PO_4 -ox, Γ_{ncrys} and Γ_{crys} ($mol m^{-2}$) are the PO_4 loadings of the non-crystalline and crystalline metal-(hydr)oxides as calculated with the CD-model using Fh and goethite as a proxy respectively, SSR is the solution-to-solid ratio employed during $NaHCO_3$ extraction ($50 L kg^{-1}$), and C the measured equilibrium PO_4 concentration in the $NaHCO_3$ extract (M). In order to solve this equation, we need to calculate RSA_{crys} , which can be done according to:

$$RSA_{crys} = m_{crys} \times SSA_{crys} \quad (S4.12)$$

For this, we need an estimate for SSA_{crys} and m_{crys} . SSA_{crys} is a chosen value for which a realistic SSA_{ncrys} is calculated according to the procedure described below. For this study, SSA_{crys} is set at a value of $40 m^2 g^{-1}$ (Hiemstra et al., 1989). For m_{crys} , the following equation can be used:

$$m_{crys} = Fe-DCB \times \text{crystalline fraction XAS} \times M_{nano-goet} \quad (S4.13)$$

with Fe-DCB in $mol kg_{soil}^{-1}$. The crystalline fraction XAS for the crystalline Fe-(hydr)oxides of soil S2 can be found in Table 3.1 in the main text. For $M_{nano-goet}$ we used a value of $89 g mol^{-1}$ (Hiemstra and Van Riemsdijk, 2009).

Subsequently, the RSA_{soil} can be scaled to the amount of non-crystalline and crystalline oxides involved in the SSA_{soil} ($m^2 g_{ncrys+crys}^{-1}$):

$$SSA_{\text{soil}} = RSA_{\text{soil}} / (m_{\text{ncrys}} + m_{\text{crys}}) \quad (\text{S4.14})$$

with m_{ncrys} calculated according to Eq S4.3 and m_{crys} according to Eq S4.13.

In order to calculate m_{ncrys} , the molar masses ($M_{\text{nano-Fh}}$ and $M_{\text{nano-Al(OH)3}}$) as well as the mass densities ($\rho_{\text{nano-Fh}}$ and $\rho_{\text{nano-Al(OH)3}}$) of the non-crystalline oxides are required. As mentioned before, these values are size-dependent and can be calculated according to the theoretical approach described below. The difference between S4.1.2 and S4.2.2 is the inclusion of crystalline oxides (with goethite as proxy) in the total pool of metal-(hydr)oxides. For these crystalline oxides, we used the fixed SSA_{crys} and $M_{\text{nano-goet}}$ as given above.

S4.2.2 Theory

The starting point to calculate $M_{\text{nano-Fh}}$ and $M_{\text{nano-Al(OH)3}}$ is to determine the mass density ($\rho_{\text{nano-Fh}}$ and $\rho_{\text{nano-Al(OH)3}}$ in g m^{-3}) of the non-crystalline oxides according to Eqs. S4.4a and S4.4b using the same chosen value for $d(m)$ for both non-crystalline oxides. Subsequently, SSA_{Fh} and $SSA_{\text{Al(OH)3}}$ ($\text{m}^2 \text{g}_{\text{Fh}}^{-1}$ and $\text{m}^2 \text{g}_{\text{Al(OH)3}}^{-1}$) are calculated with Eqs. S4.5a and S4.5b whereas SSA_{crys} is a chosen value. The calculation of SSA_{Fh} and $SSA_{\text{Al(OH)3}}$ allows, in turn, the calculation of $M_{\text{nano-Fh}}$ and $M_{\text{nano-Al(OH)3}}$ according to Eqs. S4.6a and S4.6b.

In addition, we can determine the mass fractions of the non-crystalline and crystalline oxides with respect to the total mass of oxides in the soil:

$$f_{\text{Fh}} = (\text{Fe-DCB} \times \text{non-crystalline fraction XAS} \times M_{\text{nano-Fh}}) / (\text{Fe-DCB} \times \text{non-crystalline fraction XAS} \times M_{\text{nano-Fh}} + \text{Al}_{\text{ox}} \times M_{\text{nano-Al(OH)3}} + \text{Fe-DCB} \times \text{crystalline fraction XAS} \times M_{\text{nano-goet}}) \quad (\text{S4.15a})$$

$$f_{\text{Al(OH)3}} = (\text{Al}_{\text{ox}} \times M_{\text{nano-Al(OH)3}}) / (\text{Fe-DCB} \times \text{non-crystalline fraction XAS} \times M_{\text{nano-Fh}} + \text{Al}_{\text{ox}} \times M_{\text{nano-Al(OH)3}} + \text{Fe-DCB} \times \text{crystalline fraction XAS} \times M_{\text{nano-goet}}) \quad (\text{S4.15b})$$

$$f_{\text{crys}} = (\text{Fe-DCB} \times \text{crystalline fraction XAS} \times M_{\text{nano-goet}}) / (\text{Fe-DCB} \times \text{non-crystalline fraction XAS} \times M_{\text{nano-Fh}} + \text{Al}_{\text{ox}} \times M_{\text{nano-Al(OH)3}} + \text{Fe-DCB} \times \text{crystalline fraction XAS} \times M_{\text{nano-goet}}) \quad (\text{S4.15c})$$

with Fe-DCB and Al_{ox} in $\text{mol kg}_{\text{soil}}^{-1}$. The non-crystalline fraction XAS and crystalline fraction XAS for the non-crystalline and crystalline Fe-(hydr)oxides of soil S2 can be found in Table 3.1 in the main text.

The mass-weighted average SSA_d ($\text{m}^2 \text{g}_{\text{ncrys+crys}}^{-1}$) for the non-crystalline and crystalline oxides (Fh, Al-(hydr)oxides and crystalline oxides) for the chosen value of d can be calculated according to:

$$SSA_d = f_{\text{Fh}} \times SSA_{\text{Fh}} + f_{\text{Al}} \times SSA_{\text{Al(OH)3}} + f_{\text{crys}} \times SSA_{\text{crys}} \quad (\text{S4.16})$$

S4.1.3 Combining the probe-ion method and theory

The probe-ion method results in a value for SSA_{soil} (Eq S4.14) for the $M_{nano-Fh}$ and $M_{nano-Al(OH)_3}$ used in Eq S4.3 and the given $M_{nano-goet}$. Simultaneously, the theoretical approach results in a SSA_d value for the chosen value of d , which contains the same values of $M_{nano-Fh}$, $M_{nano-Al(OH)_3}$ and $M_{nano-goet}$. To determine the final value of d , an iterative calculation can be done until a d is found for which $SSA_d = SSA_{soil}$.

S5 Statistical analyses

Statistical analyses were performed in R, version 4.0.2 (R core team, 2020). Linear mixed-effect models from the package nlme (Pinheiro et al., 2020) were used to test the effect of treatment (Lr, Ac, Lt, P0, and P+) with block as a random factor. The models were tested for normality (Shapiro-Wilk test) and homoscedasity (Levene test) of the residuals (package base). A type III SS ANalysis Of VAriance (ANOVA) or, when the prerequisite conditions of normality and homoscedasity were not met, the non-parametric Kruskal-Wallis test, was carried out for each soil separately with $\alpha < 0.05$ (package base). When significant differences were observed, Tukey's honestly significant difference posthoc test was used (packages emmeans (Lenth et al., 2020) and multcomp (Hothorn et al., 2018)).

S6 Chemical characterisation of soil and earthworm casts

Table S6.1 (next pages) Chemical composition of the water extracts of bulk soil and earthworm casts. The underscored values are given as the free ion concentrations that are used as input for the CD model and which are recalculated with the NICA-Donnan model from the measured concentrations to account for complexation with dissolved organic matter (section 2.3.2 in the main text). Presented values are averages and, between brackets, standard deviations.

	pH [-]	/ [mM]	NH ₄ [mM]	NO ₃ [mM]	Na [mM]	K [mM]	Ca [mM]	Mg [mM]
Lr	7.68 (0.06)	11.0 (1.6)	5.90 (0.87)	0.12 (0.10)	0.14 (0.05)	1.33 (0.24)	0.16 (0.04)	0.24 (0.15)
Ac	-	-	-	-	-	-	-	-
S1	Lt	7.61 (0.07)	7.3 (1.1)	3.58 (0.75)	0.29 (0.20)	0.12 (0.03)	0.83 (0.08)	0.28 (0.06)
	P0	8.21 (0.03)	2.9 (0.2)	0.00 (0.00)	0.64 (0.04)	0.36 (0.01)	0.17 (0.01)	0.10 (0.01)
	P+	8.21 (0.02)	2.9 (0.1)	0.00 (0.00)	0.60 (0.02)	0.46 (0.01)	0.18 (0.03)	0.10 (0.01)
	Lr	7.62 (0.01)	9.2 (0.2)	4.43 (0.17)	0.07 (0.08)	0.16 (0.06)	0.52 (0.01)	0.015 (0.01)
	Ac	-	-	-	-	-	-	-
S2	Lt	7.45 (0.20)	4.3 (1.6)	2.07 (0.80)	0.01 (0.01)	0.03 (0.04)	0.18 (0.10)	0.008 (0.006)
	P0	5.43 (0.01)	1.1 (0.1)	0.01 (0.00)	0.35 (0.04)	0.00 (0.00)	0.02 (0.01)	0.076 (0.006)
	P+	5.47 (0.04)	0.90 (0.15)	0.01 (0.00)	0.27 (0.05)	0.04 (0.01)	0.02 (0.00)	0.064 (0.006)
	Lr	7.94 (0.37)	7.2 (2.3)	2.53 (0.78)	0.00 (0.00)	0.26 (0.25)	0.74 (0.42)	0.001 (0.001)
	Ac	7.26 (-)	3.6 (-)	1.65 (-)	0.36 (-)	0.08 (-)	0.11 (-)	0.082 (-)
S3	Lt	7.45 (0.29)	5.5 (2.0)	2.09 (0.84)	0.00 (0.00)	0.20 (0.21)	0.51 (0.27)	0.002 (0.003)
	P0	6.65 (0.06)	0.73 (0.18)	0.00 (0.00)	0.14 (0.07)	0.02 (0.01)	0.07 (0.02)	0.075 (0.013)
	P+	6.65 (0.03)	0.80 (0.11)	0.00 (0.00)	0.15 (0.05)	0.09 (0.01)	0.08 (0.02)	0.06 (0.01)
	Lr	7.72 (0.11)	7.2 (1.6)	2.71 (0.70)	0.07 (0.12)	0.13 (0.13)	0.80 (0.35)	0.001 (0.00)
	Ac	7.32 (-)	5.8 (-)	3.25 (-)	0.16 (-)	0.00 (-)	0.00 (-)	0.00 (-)
S4	Lt	7.19 (0.27)	6.1 (2.5)	2.58 (1.25)	0.02 (0.04)	0.01 (0.02)	0.41 (0.25)	0.001 (0.001)
	P0	6.17 (0.04)	1.2 (0.1)	0.00 (0.00)	0.33 (0.04)	0.02 (0.00)	0.13 (0.01)	0.08 (0.01)
	P+	6.22 (0.05)	1.1 (0.2)	0.00 (0.00)	0.28 (0.06)	0.09 (0.01)	0.11 (0.02)	0.064 (0.01)

(Table S6.1 continues on next page)

(Table S6.1 continued)

	(H)CO ₃ [mM]	TDP [mM]	PO ₄ [mM] ^a	DOC [mM]	DON [mM]	DOP [mM]	S [mM]
S1	Lr	0.011 (0.001)	1.14 (0.07)	10.8 (0.1)	0.88 (0.02)	0.042 (0.006)	0.40 (-)
	Ac	-	-	-	-	-	-
	Lt	0.007 (0.002)	0.38 (0.07)	5.28 (1.21)	0.45 (0.14)	0.133 (0.018)	0.28 (0.05)
	P0	0.005 (0.000)	0.011 (0.000)	0.89 (0.05)	0.064 (0.006)	0.003 (0.000)	0.12 (0.01)
S2	P+	0.005 (0.000)	0.027 (0.001)	0.95 (0.04)	0.059 (0.006)	0.004 (0.001)	0.12 (0.00)
	Lr	0.008 (0.000)	0.41 (0.03)	15.4 (2.3)	1.20 (0.14)	0.125 (0.026)	0.45 (0.02)
	Ac	-	-	-	-	-	-
	Lt	0.002 (0.002)	0.19 (0.05)	10.7 (2.7)	0.66 (0.39)	0.089 (0.029)	0.29 (0.09)
S3	P0	0.000 (0.000)	0.003 (0.001)	0.52 (0.06)	0.049 (0.005)	0.003 (0.001)	0.017 (0.001)
	P+	0.000 (0.000)	0.006 (0.000)	0.67 (0.05)	0.061 (0.005)	0.005 (0.000)	0.02 (0.00)
	Lr	0.015 (0.015)	0.42 (0.09)	11.3 (2.9)	0.71 (0.25)	0.075 (0.019)	0.33 (0.10)
	Ac	0.000 (-)	0.034 (-)	-	0.31 (-)	0.013 (-)	-
S4	Lt	0.002 (0.000)	0.31 (0.03)	7.58 (2.58)	0.41 (0.29)	0.055 (0.013)	0.28 (0.08)
	P0	0.000 (0.000)	0.005 (0.000)	0.59 (0.01)	0.043 (0.012)	0.001 (0.000)	0.021 (0.001)
	P+	0.000 (0.000)	0.011 (0.000)	0.62 (0.01)	0.037 (0.002)	0.003 (0.000)	0.021 (0.001)
	Lr	0.007 (0.003)	0.62 (0.03)	9.97 (1.26)	0.76 (0.19)	0.116 (0.004)	0.33 (0.08)
S5	Ac	0.000 (-)	0.21 (-)	-	1.49 (-)	0.045 (-)	-
	Lt	1.14 (0.37)	0.46 (0.04)	8.73 (2.93)	0.55 (0.36)	0.093 (0.016)	0.30 (0.09)
	P0	0.000 (0.000)	0.016 (0.001)	0.58 (0.01)	0.052 (0.007)	0.004 (0.000)	0.029 (0.004)
	P+	0.001 (0.001)	0.032 (0.001)	0.62 (0.03)	0.047 (0.005)	0.008 (0.000)	0.029 (0.001)

^aThese values are displayed graphically in Fig. 3.1b in the main text.

Table S6.2 Characterisation of bulk soil and earthworm casts determined on dried (40°C) material. Presented values are averages and, between brackets, standard deviations.

	P-Olsen [mmol kg ⁻¹]	P _{ox} [mmol kg ⁻¹]	Fe _{ox} [mmol kg ⁻¹]	Al _{ox} [mmol kg ⁻¹]	α [mol mol ⁻¹]	Moisture content [-]
Lr	21.5 (1.2)	30.4 (1.6)	63.2 (0.2)	11.4 (0.0)	0.41 (0.02)	0.30 (0.04)
Ac	-	20.1 (1.0)	62.3 (-)	11.0 (-)	0.27 (-)	-
S1	13.1 (1.8)	22.4 (2.7)	67.8 (2.2)	12.0 (0.6)	0.28 (0.03)	0.31 (0.03)
P0	4.7 (0.1)	12.4 (0.2)	67.5 (1.1)	13.2 (0.2)	0.15 (0.00)	0.17 (0.00)
P+	5.6 (0.1)	13.6 (0.3)	67.9 (1.4)	13.3 (0.3)	0.17 (0.00)	0.17 (0.00)
Lr	8.5 (1.2)	17.1 (0.7)	120.0 (1.0)	15.7 (0.0)	0.13 (0.00)	0.27 (0.00)
Ac	-	12.9 (-)	111.0 (-)	15.1 (-)	0.10 (-)	-
S2	4.1 (0.8)	11.8 (1.4)	127.0 (6.0)	16.3 (0.6)	0.08 (0.01)	0.28 (0.03)
P0	0.7 (0.1)	5.7 (0.2)	120.0 (4.0)	17.1 (0.5)	0.04 (0.00)	0.15 (0.00)
P+	1.1 (0.0)	6.8 (0.2)	120.0 (5.0)	17.3 (0.6)	0.05 (0.00)	0.15 (0.00)
Lr	10.6 (1.2)	15.5 (1.7)	9.0 (0.6)	47.0 (3.0)	0.28 (0.02)	0.25 (0.02)
Ac	6.7 (-)	11.4 (-)	10.0 (-)	52.0 (-)	0.19 (-)	0.12 (-)
S3	9.8 (1.2)	14.1 (1.8)	9.4 (0.4)	49.0 (2.0)	0.24 (0.03)	0.31 (0.05)
P0	3.9 (0.2)	7.4 (0.2)	9.8 (0.2)	51.0 (2.0)	0.12 (0.00)	0.10 (0.01)
P+	4.7 (0.0)	8.8 (0.5)	10.0 (0.7)	52.0 (3.0)	0.14 (0.00)	0.11 (0.01)
Lr	13.9 (0.6)	24.7 (1.6)	11.0 (0.9)	49.0 (4.0)	0.41 (0.01)	0.26 (0.09)
Ac	11.1 (-)	22.5 (-)	11.9 (-)	52.0 (-)	0.35 (-)	0.33 (-)
S4	13.8 (0.8)	26.1 (1.2)	12.4 (0.3)	56.0 (2.0)	0.38 (0.02)	0.27 (0.06)
P0	8.2 (0.1)	16.6 (0.3)	11.9 (0.3)	54.0 (1.0)	0.25 (0.00)	0.13 (0.01)
P+	9.4 (0.3)	18.6 (1.5)	12.3 (1.0)	55.0 (4.0)	0.27 (0.00)	0.12 (0.00)

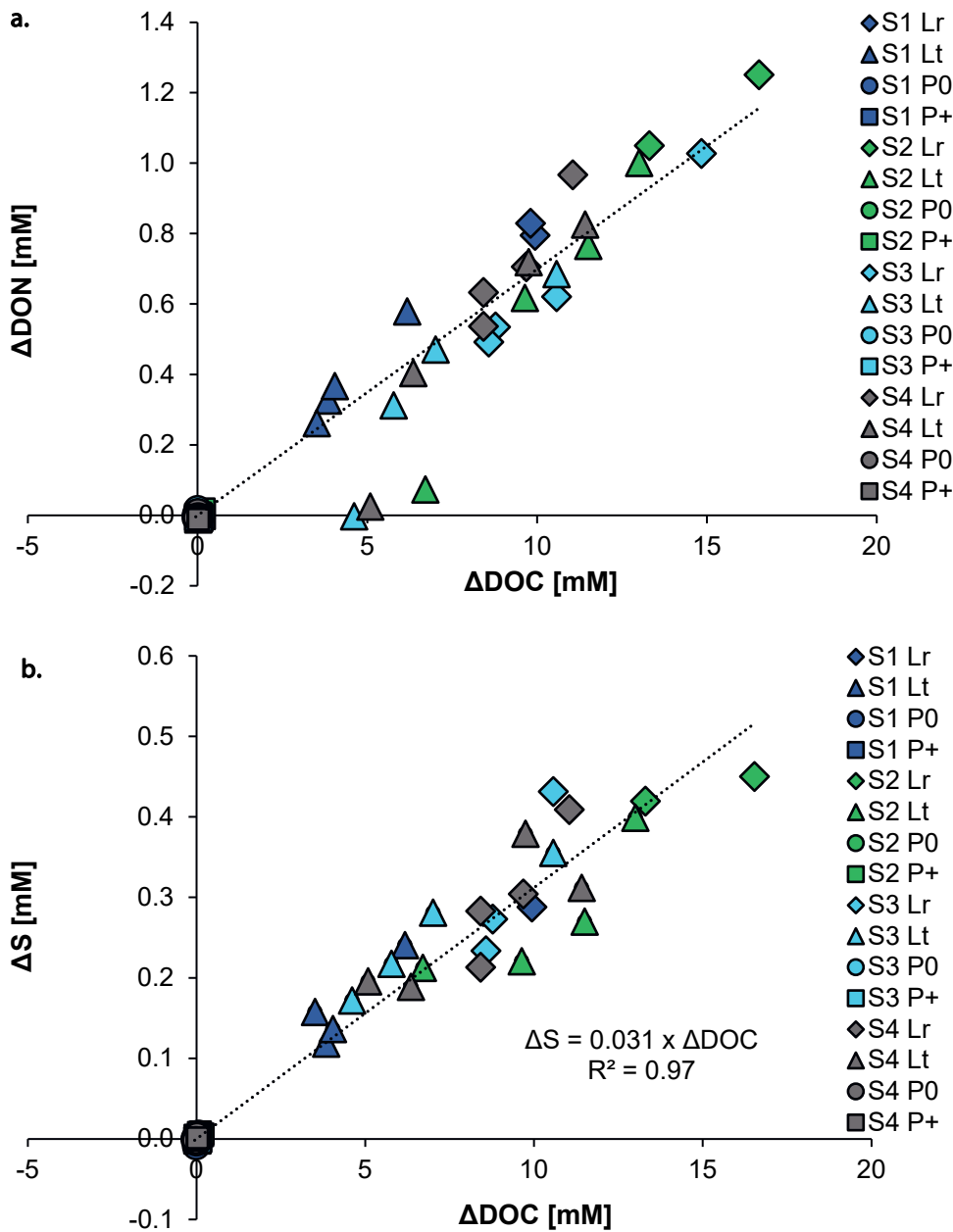
S7 Mineralisation

The changes between the P0 treatment and the other treatments (Δ) in important constituents of water-soluble NOM like the organic fraction of N (dissolved organic N; DON), S, and P (dissolved organic P; DOP) correlate very well with the change in the concentration of DOC (Fig. S9.1). Strong correlations with Δ DOC are observed for Δ DON ($R^2=0.96$) and Δ S ($R^2=0.97$). Hence, the increase in these organic fractions seems to be closely related to mineralisation. Since the correlation between Δ DOC and Δ S was strong, the main part of the measured S may have been of organic origin.

The average ratio of N:C calculated from DON and DOC ($0.070 \text{ mol mol}^{-1}=0.082 \text{ g g}^{-1}$; Fig. S9.1a) is in between the N:C ratio of nutrient-rich (0.12 g g^{-1}) and nutrient-poor (0.039 g g^{-1}) soil NOM as found in a meta-analysis of about 2000 globally distributed soil samples (Tipping et al., 2016). Since the N:C ratio agrees with the broad range found for soil NOM, the earthworms in our pot experiment could indeed have been feeding on soil-associated NOM. This agrees with the expected feeding behaviour of *Ac* (Bouché, 1977; Drake and Horn, 2007). However, the earthworms used to calculate this average N:C ratio mainly feed on surface litter (Lr and Lt) (Bouché, 1977; Drake and Horn, 2007). Therefore, the N:C ratio of *L. perenne* would be expected, which is 0.060 g g^{-1} for an assumed nutrient content of this grass species of 580 g C kg^{-1} and 35 g N kg^{-1} (Reuter and Robinson, 1997). The slight deviation between our observation and this theoretical number could be caused by a higher N content of the well-N fertilised grass in our greenhouse pot experiment or a relatively more decreased C content than N content in the decomposed NOM in casts.

For the change in the concentration of DOP between P0 and the other treatments, the correlation between Δ DOC and Δ DOP is somewhat weaker ($R^2=0.81$) than for Δ DON and Δ S. Also, the P:C ratio calculated from DOP and DOC ($0.009 \text{ mol mol}^{-1}=0.0232 \text{ g g}^{-1}$) is higher than observed in the above mentioned meta-analysis ($0.001\text{--}0.016 \text{ g g}^{-1}$ for nutrient-poor and nutrient-rich soil NOM respectively) (Tipping et al., 2016) or expected for *L. perenne* (0.006 g g^{-1} assuming a P content of 3.5 g P kg^{-1}) (Reuter and Robinson, 1997). Furthermore, the P:C ratio we observed is higher than the P:C ratio of a set of non-calcareous sandy soils in the Netherlands (0.0054 g g^{-1} ; $R^2=0.83$) (Koopmans et al., 2006). In Fig. S9.1c, the samples of the Fe-(hydr)oxide-dominated soil S1 (a calcareous clay soil) are furthest from the regression line. If we therefore only consider the data of the three sandy soils (S2, S3, and S4) like the soils used by Koopmans et al. (2006), the model fit of our data improves largely ($R^2=0.95$). However, the slope of the regression line remains high, which indicates the large DOP content of the NOM in our samples. This ratio is mainly caused by the high values of DOP in the water extracts of the casts and less by those of the bulk soils. Apparently, other mechanisms besides mineralisation may be responsible for the increase in the DOP concentration in casts, like desorption of organic P compounds from the mineral oxide surfaces.

For all panels of Fig. S9.1, the casts of Lr show the highest concentrations of the organic fractions, followed by those of Lt (Ac could not be measured). This confirms our hypothesis on the intensity of mineralisation of Lr>Lt, as discussed in the main text (section 3.2.1).



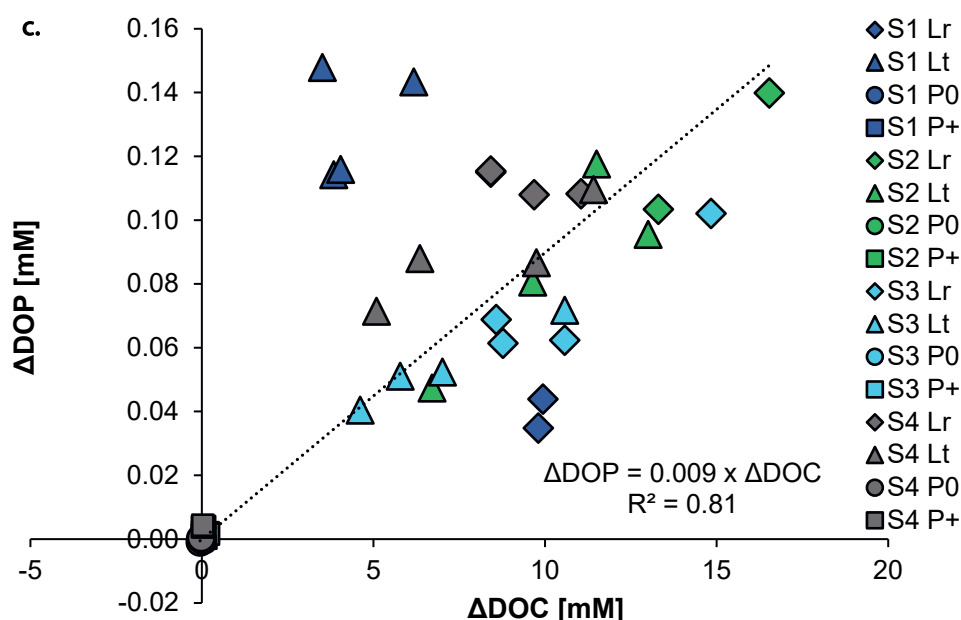


Figure S7.1 Correlation between the change in DOC between P0 and the other treatments (Δ), and the same change in other dissolved main constituents of NOM: a) DON, b) S and c) DOP. Colours refer to soils and symbols to treatments. Dotted lines are regression lines and the resulting equations and model fit are shown in the different panels.

S8 Specific surface area

The total SSA of the metal-(hydr)oxides present in earthworm casts and bulk soil (SSA_{soil} ; Fig. S8.1a) of soils S1, S3, and S4 was calculated using only the non-crystalline Fh as a reference oxide. Therefore, the SSA_{soil} of these soils equals the SSA of the non-crystalline fraction (SSA_{ncrys} ; Fig. S8.1b). For soil S2, goethite was additionally used as a proxy for the large crystalline fraction of Fe-(hydr)oxides present in this soil (Table 3.1 in the main text). Calculating the RSA of soil S2 was only possible if the SSA of one of the reference oxides was fixed to a chosen value. Therefore the SSA of the crystalline Fe-(hydr)oxide fraction (SSA_{crys}) was set to $SSA_{\text{crys}} = 40 \text{ m}^2 \text{ g}^{-1}$, which falls within the range of values reported for synthetic goethite samples ($\approx 20\text{--}110 \text{ m}^2 \text{ g}^{-1}$) (Hiemstra et al., 1989). This resulted in realistic values for SSA_{ncrys} that are realistic and comparable to SSA_{ncrys} values for the other Fe-(hydr)oxide-dominated soil S1 (Fig. S8.1b). Additionally, the corresponding mean particle size d for soil S2 (Fig. 3.2b in the main text) following from the set of equations in S4 S1.2 was realistic considering the range of values for d observed by Mendez et al. (2020) (1.5–5.1 nm). Using a lower SSA_{crys} led to unrealistic values of d , reaching values of $d < 1.5 \text{ nm}$ when $SSA_{\text{crys}} = 0 \text{ m}^2 \text{ g}^{-1}$ (i.e. no contribution of goethite). The upper boundary of SSA_{crys} was $70 \text{ m}^2 \text{ g}^{-1}$, as increasing it further resulted in negative values of SSA_{ncrys} . However, the

precise value of our estimate of SSA_{crys} did not influence our main conclusions concerning the contribution of the RSA of earthworm casts in increasing the solubility of PO_4 .

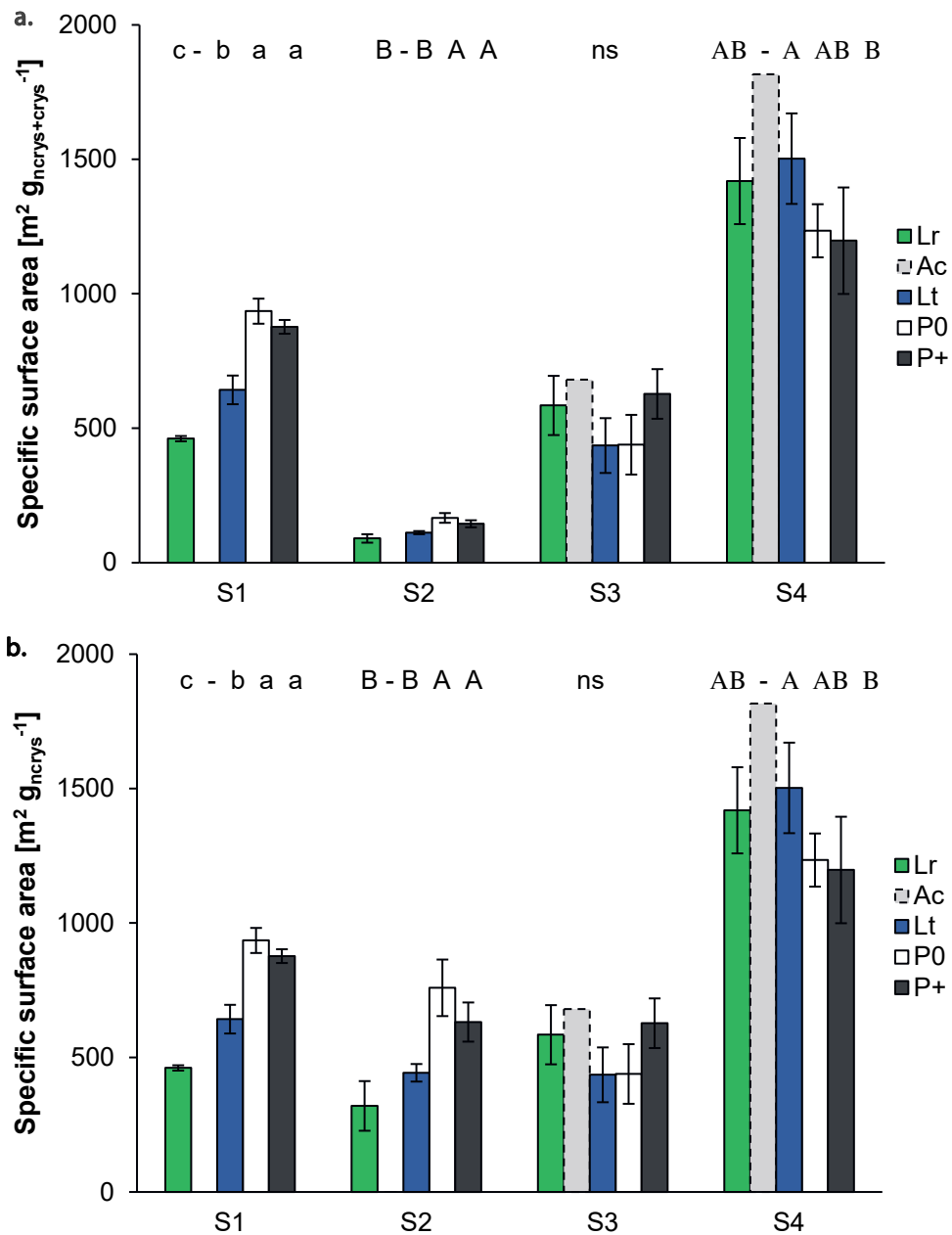


Figure S8.1 Specific surface area of the total pool of non-crystalline and crystalline metal-(hydr)oxides ($SSA_{soil}=SSA_{ncrys}+SSA_{crys}$) (a) and the non-crystalline metal-(hydr)oxides (SSA_{ncrys}) (b) of the four soils and earthworm casts. Due to limited cast production by Ac,

SSA_{soil} and SSA_{ncrys} could not be determined for soils S1 and S2, and only without replicates for soils S3 and S4. Hence, A_c was excluded from statistical analysis and, when available, its value is presented with a dotted bar. Error bars denote standard deviations. The significance of differences was determined for each soil separately. Please note: since crystalline Fe-(hydr)oxides were not included in the calculation of the SSA_{soil} for soils S1, S2, and S3, the results presented in panels (a) and (b) are the same. Since crystalline Fe-(hydr)oxides were accounted for in the calculation of SSA_{soil} for soil S2, the results presented for this soil in the two panels are different. Furthermore, SSA_{ncrys} was calculated by using the non-crystalline oxide fraction, which was mainly composed of Al-(hydr)oxides in the Al-(hydr)oxide-dominated soils S3 and S4. As the mass density of Al-(hydr)oxides is much lower than the mass density of Fe-(hydr)oxides, the SSA_{ncrys} of soils S3 and S4 is higher than the SSA_{ncrys} of the Fe-(hydr)oxide-dominated soils S1 and S2 if all oxides particles would have approximately the same mean particle size d . As the value of d of soil S3 was larger than of soil S4 (Fig. 3.2b in the main text) because of suppressed particle growth by long-term P fertilisation in soil S4, this larger SSA_{ncrys} is not visible for soil S3, but only for soil S4.

S9 Additional measurements for soil S3

For the calculation of the NOM loading, values of the RSA are required as model input. However, the uncertainty in the estimates of the RSA for soil S3 is clearly demonstrated when the measured PO_4 concentrations in the water extracts prepared from the samples of this soil are plotted against the PO_4 loading of the metal-(hydr)oxides. The PO_4 loading was calculated as $(R_{\text{ev}} - SSR \times C)/RSA$, with R_{ev} being the measured PO_4 -ox content of the samples from soil S3 and C representing the measured PO_4 concentration in the water extracts having a SSR of 10 L kg^{-1} . Figure S9.1 shows how the experimental results for the samples of soil S3 relate to the theoretical maximum curves of the bulk soil and casts. These theoretical maximum curves are calculated with the CD model in ECOSAT, using the average conditions in soil and casts (pH, I , and the free ion concentrations of NH_4 , NO_3 , Na, K, Ca, Mg, and $(H)CO_3$) without considering competition with NOM. In these model calculations, F_h was used as a reference oxide. As including competition between PO_4 and NOM for adsorption onto binding sites of metal-(hydr)oxides would lead to a lowering of these theoretical maximum curves, all measured data points should align with the calculated adsorption isotherm when there is no NOM competition or they should be located below the calculated adsorption isotherm when there is NOM competition. While this was the case for soils S1 and S2 in which the fraction of metal-(hydr)oxides is dominated by Fe-(hydr)oxides (data not shown), this was not the case for the Al-(hydr)oxide-dominated soil S3 (and to a lesser extent for soil S4). Hence, the RSA of these Al-(hydr)oxide-dominated soils may in reality have been higher than calculated with the

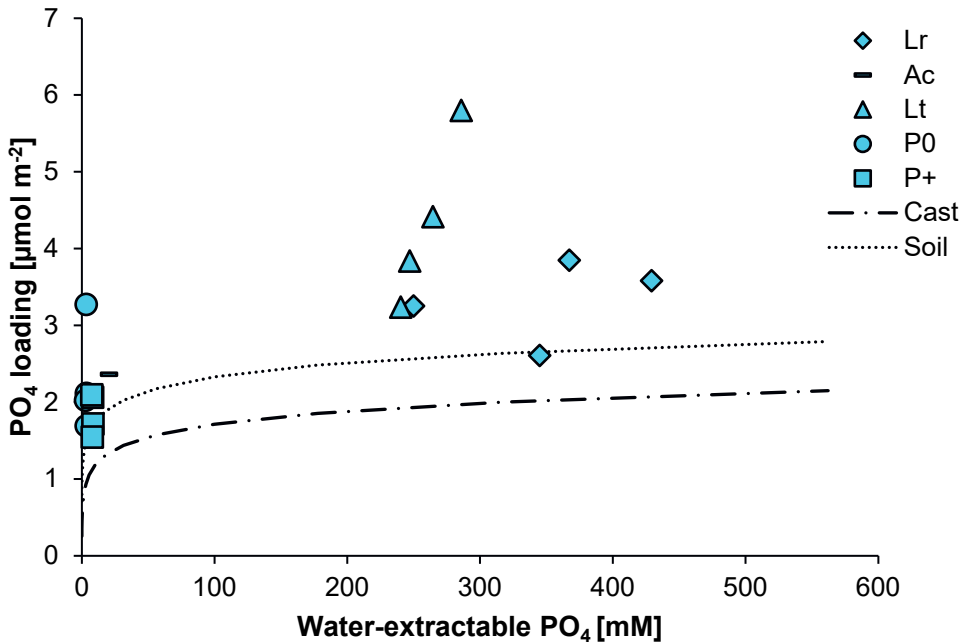


Figure S9.1 Concentrations of PO₄ in the water extracts plotted against the PO₄ loading of the metal-(hydr)oxides for the measured data points of soil S3 and the theoretical maximum curves for soil (dotted line) and earthworm casts (dash-dotted line).

probe-ion method from the measured PO₄-ox content and equilibrium PO₄ concentration in the NaHCO₃ extract.

The absolute values for the RSA of soils S3 and S4 are, therefore, incorrect, although the relative difference between the samples may still be correct. A plausible reason for the observed discrepancy might be the use of Fh as a proxy for the metal-(hydr)oxides in these soils while the soils predominantly contain Al-(hydr)oxides. Ideally, a more specific proxy for this type of oxide should be used, but such a proxy with a consistent set of parameters for the CD model does currently not exist for this type of material. In an attempt to improve our assessment of the RSA and to elucidate inconsistencies for soil S3, an additional experiment was done. Since no cast material was left for this additional experiment, we only used the samples of the bulk soil for soil S3. We selected two soil samples from the four replicates of the P0 treatment and two soil samples from the four replicates of the P+ treatment of this soil. The RSA was assessed following the approach of Hiemstra et al. (2010a) in which the solution-to-solid ratio (SSR) of each sample was varied in the NaHCO₃ equilibration measurements of the probe-ion method. So rather than using a single SSR for the NaHCO₃ equilibration measurement as described in section 2.3.1 of the main text, we used multiple SSRs in this additional experiment. The

experimental approach with multiple SSRs allowed the construction of a desorption isotherm. The SSRs employed during NaHCO_3 extraction were 2, 5, 10, 20, and 50 L kg^{-1} .

The PO_4 concentrations in the P-Olsen extract for the $\text{SSR}=50 \text{ L kg}^{-1}$ in this additional experiment were very close to the originally measured concentrations in which the same SSR was employed (on average 3% difference), indicating a high degree of precision between both experiments. By using an increasing SSR, the measured concentration of PO_4 in the NaHCO_3 extracts will decrease as a sample becomes more diluted. Because of PO_4 buffering processes, this dilution is not linear, as PO_4 is released from the soil surfaces when the samples become more strongly diluted (Fig. S9.2) (Mendez et al., 2022). The resulting relationships can be used to model the RSA. However, in the resulting curves, the PO_4 concentration at the lowest SSR value (2 L kg^{-1}) was lower than at further dilution. Partly, this can be attributed to the lower pH value at this lowest SSR, leading to more PO_4 adsorption. In the modelling, we have accounted for this, resulting in the dotted lines in the panels of Fig. S9.2 as the best fit.

Describing the relation between the equilibrium PO_4 concentration in the NaHCO_3 extracts and the SSR yielded an average value for the RSA of soil S3 of $9.3 \text{ m}^2 \text{ g soil}^{-1}$, which is much higher than the RSA for this soil as determined with the probe-ion method in which one single SSR was used for the NaHCO_3 extraction (section 2.3.1; on average $2.8 \text{ m}^2 \text{ g soil}^{-1}$ for the P0 and P+ treatments; Fig. 3.2a in the main text). However, the RSA value as determined in this additional experiment with multiple SSRs showed considerable variation between the four samples used. Furthermore, the fitted amount of reversibly bound PO_4 (R_{ev}) was much higher than the measured values of $\text{PO}_4\text{-ox}$ (data not shown). If in the modelling the experimental value of $\text{PO}_4\text{-ox}$ was used as input for R_{ev} , the average value for the RSA of soil S3 decreased from 9.3 to $4.1 \text{ m}^2 \text{ g soil}^{-1}$, but the quality of the model fit was lower and the large variation in the RSA of the different samples remained. Moreover, the RSA was insufficiently high to sufficiently lower the P loading of the data points in Fig. S9.1 to align or fall below the theoretical maximum curve for soil S3. Therefore, the results from this additional experiment did not yield a conclusive assessment of the RSA of soil S3 and are not considered in the data evaluation in the main text.

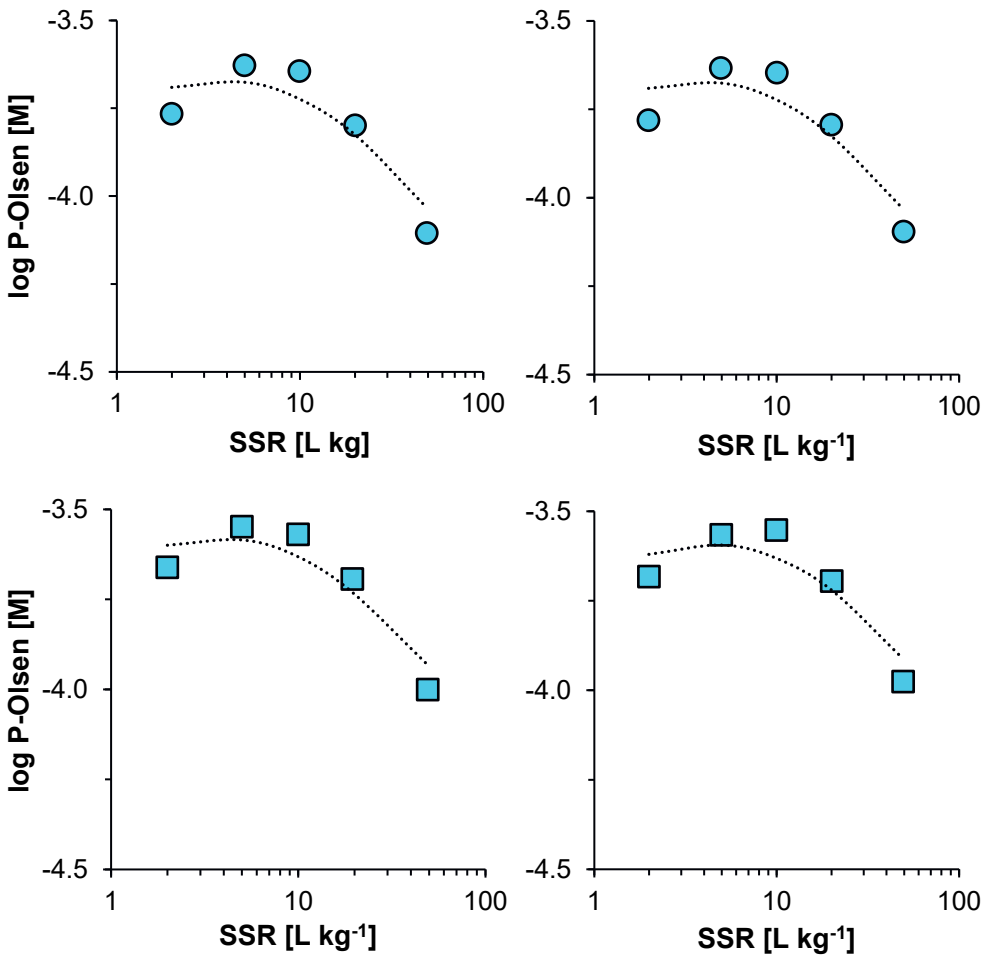
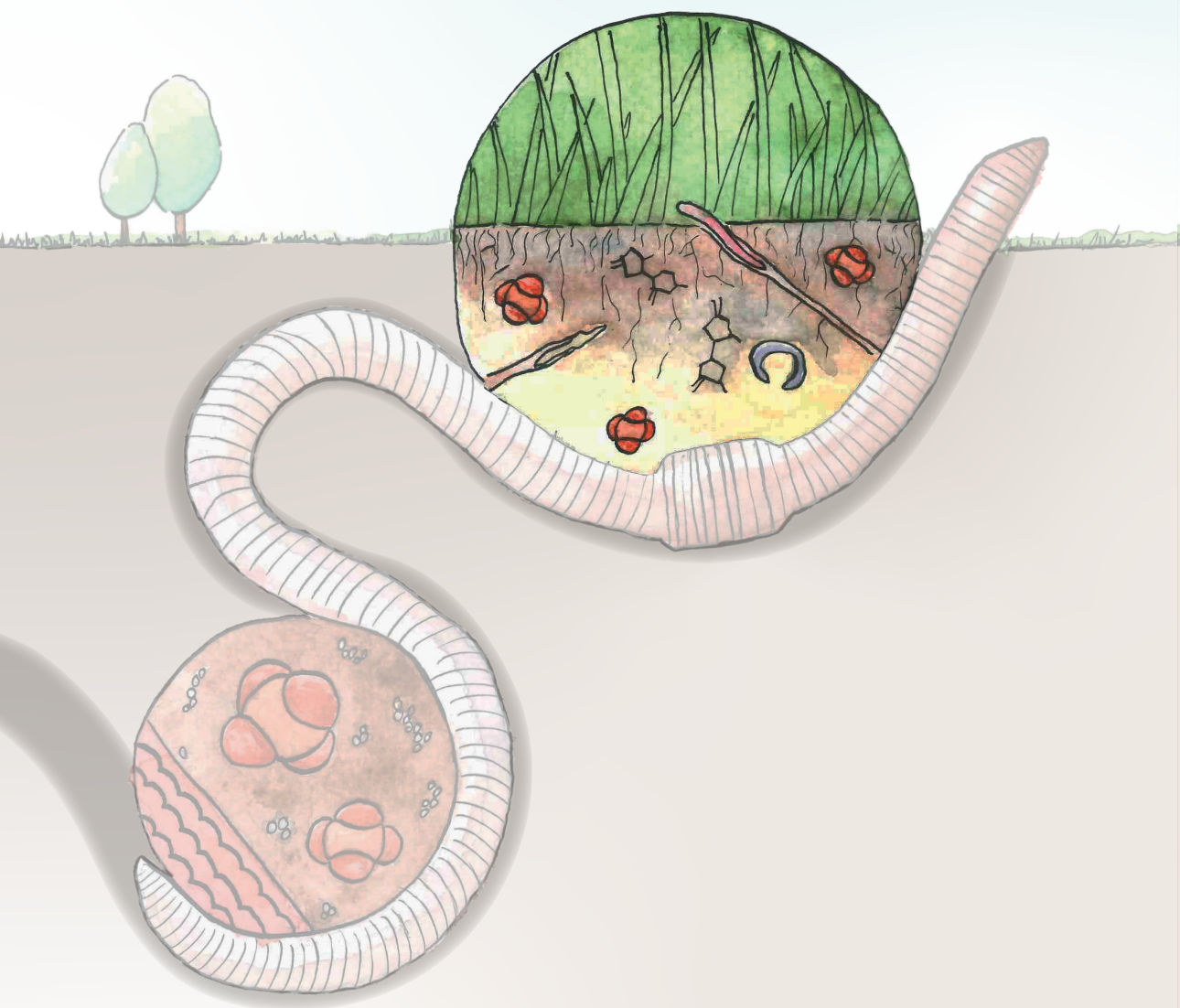


Figure S9.2 Measured data points (top row: two replicates of the P0 treatment; bottom row: two replicates of the P+ treatment) and modelled relationship (dotted line) between the logarithm of the PO_4 concentration in the NaHCO_3 extract (P-Olsen) and the SSR for the additional measurements of soil S3.





Chapter 4

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

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Jan Willem van Groenigen

Abstract

It has been established that earthworms can increase the solubility of phosphorus (P) in soil, and that this is to a large extent controlled by mineralisation of organic P. Phosphatase enzymes catalyse this mineralisation process, but a consistent comparison of their activity between earthworm species and for different soil spheres is still missing. To better understand the activity of phosphatase enzymes (here assessed as phosphomonoesterase, PME) to earthworm-enhanced P-availability, we set up a greenhouse pot experiment with five different earthworm species. The PME activity was assessed in earthworm casts, burrows, and bulk soil for all earthworm species and their mixture, at the pH-H₂O of both soil and casts. The PME activity measured at both pH values was highly correlated ($R^2=0.97$; $p<0.001$), 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*. Large variation in PME activity in the drilosphere was observed among earthworm species, but this variation was not related to earthworm functional groups. Furthermore, our data suggests that PME activity in earthworm casts is linked to P uptake by grass through mineralisation of organic P.

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

Phosphorus (P) is an essential nutrient for plant growth and is taken up by plant 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 soil mineral phase (Hesterberg, 2010; Morel et al., 2000). Earthworms are able to increase ortho-P solubility in the soil solution (Le Bayon and Milleret, 2009). Many of the controlling mechanisms of earthworm-enhanced P-availability are triggered by the mineralisation of organic P: a) it results in a direct contribution to the total pool of adsorbed P; b) it leads to a pH increase thereby decreasing ortho-P adsorption; and c) it 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 consist of monoester P compounds (Koopmans et al., 2007; Magid et al., 1996). The mineralisation of organic P is catalysed by phosphatase enzymes, which occur in different forms in soil and soil organisms (Kiss et al., 1975). These enzymes stimulate the hydrolysis of organic P and are produced by plant roots and microorganisms when relatively little of P in soils is available for uptake (Olander and Vitousek, 2000; Tadano et al., 1993; Toor et al., 2003). Usually, the activity of phosphomonoesterases (PME) is much higher than that of phosphodiesterases (Condon et al., 2005). In this study we therefore focus on the activity of PME.

Besides the production of phosphatases by plant roots and microorganisms, these enzymes can additionally be excreted by earthworms and the microorganisms in their gut (Satchell and Martin, 1984). The activity of phosphatases in the drilosphere, the part of the soil influenced by earthworms (Brown et al., 2000), has been studied over the past decades, and was often found to be strongly increased 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 was observed (Satchell and Martin, 1984). Although various earthworm species and different soil spheres have been studied in literature, these studies often used varying methods to determine the phosphatase activity or collect (surface) casts. A consistent comparison of the phosphatase activity among earthworm species for different soil spheres is still missing. Furthermore, measurements of phosphatase activity are often conducted at a buffered and optimal pH for enzyme activity (i.e., pH=6.5; Tabatabai and Bremner (1969)). However, phosphatase pH optima are highly variable and linked to the pH of the soil (Wade et al., 2021). This necessitates analysis at different pH values which are close to those of the corresponding soil sphere.

In order to assess PME activity in earthworm casts relative to bulk soil, we set up a greenhouse pot (diameter: 20 cm; height: 23 cm) experiment using the non-calcareous sandy soil with a low P-status described by Vos et al. (2019) (7.05 kg air-dry weight). The

soil organic matter content of this soil was 42 g kg^{-1} whereas the total P content amounted to 343 mg kg^{-1} of which 29% was organic (i.e., 99 mg kg^{-1}). The pots were sown with *Lolium perenne* and earthworm species commonly observed in the Netherlands were added as single-species treatments: *Lumbricus rubellus* Hoffmeister, 1843 (Lr); *Aporrectodea caliginosa* (Savigny, 1826) (Aca), *Allolobophora chlorotica* (Savigny, 1826) (pink morphotype; Ach); *Lumbricus terrestris* Linnaeus, 1758 (Lt) and *Aporrectodea longa* (Ude, 1895) (Al). A treatment with a mixture of all five species in which the total earthworm weight was distributed equally over all species (Mix), as well as control treatments without earthworms, either unamended (0EW0P) or amended with mineral P (0EW+P), were included. Furthermore, a 0EW+Cast 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 to distinguish between the effects of earthworms on cast fertility and earthworm activity. Earthworm densities in all treatments were approximately $286 \text{ g fresh (empty gut) earthworm m}^{-2}$, which is within the range observed in grasslands in the Netherlands (Van Vliet et al., 2007). All treatments were replicated four times.

The grass was cut every three weeks at 5 cm above the soil surface and the experiment was harvested 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 were determined according to Novozamsky et al. (1983). At the end of the experiment, 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). Other samples were collected by scraping the earthworm burrow walls (burrows), and shaking the rooted soil part (bulk soil). The moisture content of all soil sphere samples was determined by drying subsamples for 24 h at 40°C . Subsequently, PME activity was measured according to Tabatabai and Bremner (1969) on fresh samples of all casts of which sufficient material was present and two replicates of each treatment of the other two soil spheres. In short, a *p*-nitrophenyl phosphate solution was provided as a substrate and the PME hydrolytic activity is determined by colorimetric measurement of the conversion product of this substrate (*p*-nitrophenol). The method was successfully downscaled to measure small amounts of cast material. The 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 cast (Vos et al., 2019). 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).

Our results demonstrate large differences in PME activity among earthworm species (Fig. 4.1a). The PME activity was significantly higher ($p < 0.001$) in casts than in burrows, which was, in turn, significantly higher ($p < 0.001$) than in bulk soil (unpaired t-tests). This agrees well with the general observation of elevated PME activity in the

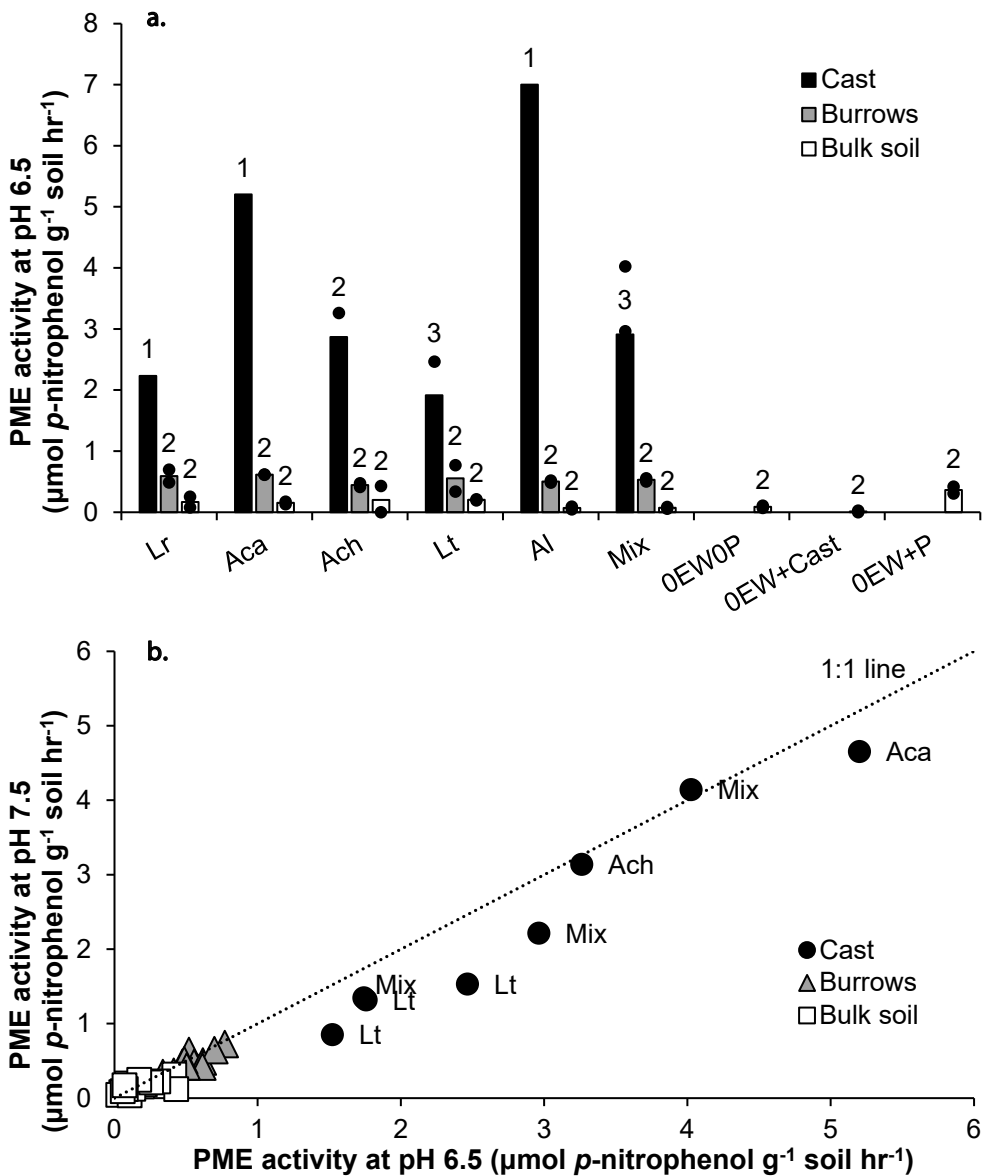


Figure 4.1 The PME activity for the various treatments and soil spheres as measured at pH 6.5 (a), and the correlation between PME activity as measured at pH 6.5 and 7.5 (b). In panel (a), data collected at pH 6.5 are presented, as for the treatments with little sample material PME activity could not be measured at both pH levels. Dots indicate the PME activity of each measured replicate, which amounted to two for both burrows and bulk soil. Because of the limitation of sample material, the number of replicates for PME activity measurements differed per treatment in casts (for all measured samples the number of replicates (n) is indicated above the graph).

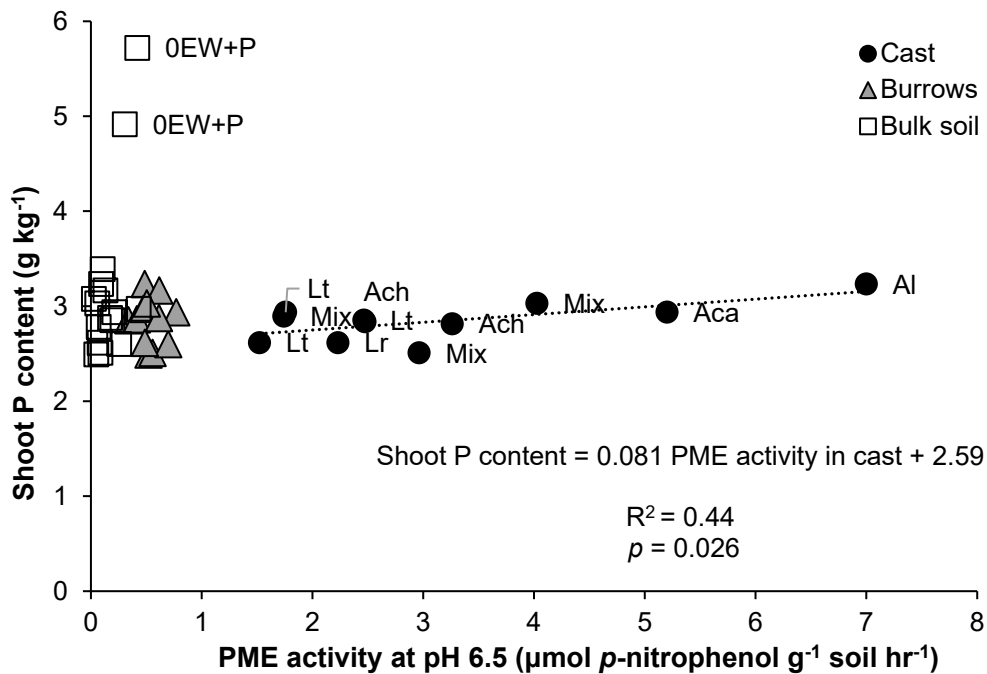
drilosphere compared to the bulk soil (Hoang et al., 2016; Satchell and Martin, 1984; Sharpley and Syers, 1976; Wan and Wong, 2004), and to the differences between casts, burrows and bulk soil indicated by Le Bayon and Binet (2006). For the single-species treatments of Lr, Aca and Al, lack of sufficient cast material prohibited replicated PME activity measurements and statistical analysis. Replicates of PME activity in Ach and Lt casts showed that the maximum variation within a treatment was 1.6, whereas across all casts this variation was 4.6. This corroborates the presence of differences in PME activity among earthworm species (Satchell and Martin, 1984; Wan and Wong, 2004). As expected, the mixture showed an average PME activity of all species. The observed variation in PME activity among species did not clearly relate to earthworm functional groups, and for instance the two anecic species (Lt and Al) demonstrate both the lowest and the highest measured PME activity in casts. Despite the relatively large differences in PME activity among species, this did not result in significant differences among earthworm casts in the total pool of adsorbed P measured by Vos et al. (2019) for the same soil. Hence, any differences in the potential PME activity for mineralising organic P among earthworm species do not necessarily lead to variation in actual organic P mineralisation in soil. This difference between potential and actual PME activity depends on, among others, temperature, moisture and the availability of organic P as a substrate for mineralisation, which were not included as variables here.

A good correlation between PME activity measured at both pH-values was observed ($R^2=0.97$; $p<0.001$; Fig. 4.1b). Hence, the ambient pH employed during measurement did not affect the relative differences between treatments. However, most data points in Fig. 4.1b are situated below the 1:1 line, especially at higher enzyme activities. Consequently, measuring PME activity at the often assumed optimal pH 6.5 (Tabatabai and Bremner, 1969) would overestimate the potential PME activity in casts, as these often have a pH-H₂O of around 7.5 (Vos et al., 2022a; Vos et al., 2019).

The direct contribution of PME activity via organic P mineralisation to readily plant-available P was assessed by the relation between water-extractable ortho-P concentrations and PME activities. An inverse relation can be expected, since PME production by plants and microorganisms is regulated by their need for P relative to the supply of available P (Olander and Vitousek, 2000; Tadano et al., 1993; Toor et al., 2003). However, this inverse relationship between PME activity and water-extractable ortho-P was not observed in the bulk soil of the control treatments with differing P fertilisation (0EW0P, 0EW+Cast, 0EW+P; data not shown). This was caused by a high PME activity of the 0EW+P treatment which also had the highest water-extractable ortho-P concentration. As PME activities and ortho-P solubilities of the 0EW0P and 0EW+Cast treatments were equally low (indicating no contribution of surface applied casts to PME activity and water-extractable ortho-P in the bulk soil), this led to a trend in which water-extractable ortho-P increased with PME activity. However, while regular N fertilisation was applied, the N content of grass shoots pointed towards N-limited growth conditions in

the 0EW+P treatment, but not in the other treatments (Fig. S1). Under N-limiting conditions, N addition stimulates PME production by plants and microorganisms, as N is essential for enzyme synthesis and simultaneously increases the productivity of plants and microorganisms, increasing the P demand (Olander and Vitousek, 2000) and resulting in the observed elevated PME activities.

We did not observe differences in the ortho-P concentrations between earthworm burrows and bulk soil ($p=0.88$; Wilcoxon rank test), although the PME activity in the burrows was higher. 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). Hence, there is no additional effect of PME activity on water-extractable ortho-P in burrows compared to the bulk soil. Unfortunately, only two water extracts of casts (11.2 (Lr) and 5.9 (Lt) mg P L⁻¹ versus 0.09 mg P L⁻¹ for the 0EW0P treatment) could be made due to insufficient cast material, which impedes assessing the relation between PME activity and water-extractable ortho-P of casts for the present study. However, these two measured ortho-P concentrations were similar to those in Vos et al. (2019). Furthermore, our data on PME activity in casts show a negative trend with water-extractable ortho-P in Vos et al. (2019): for instance, high ortho-P solubility and low PME activity for Lr but low ortho-P solubility and high PME activity for Al. Thereby this trend among casts agrees with the expected inversed relationship between PME activity and water-extractable ortho-P. Additionally, since the PME activity for casts was much higher than for bulk soil as discussed above, direct mineralisation contributes to the increased level of the total pool of adsorbed P in casts compared to the corresponding bulk soil (Vos et al., 2019). Moreover, 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. 4.2). A similar relation was present for PME activity at pH 7.5, but because of the lower number of measurements this relation was weaker and not significant ($R^2=0.19$; $p=0.28$). Although these results should be interpreted with care as grass growth was not clearly P-limited (Fig. S1) and there was little variation in shoot P content, an increase in PME activity seems to lead to more hydrolysis of organic P, which, in turn, contributes to an increase in plant P uptake. As *A. longa* showed both the highest PME activity in its casts and the largest shoot P content, this species is likely the most instrumental of the studied species to affect P-availability by its direct contribution to mineralisation. This matches with observations from a field experiment in which especially *A. longa* contributed to P uptake of grass (Vos et al., 2022b). However, this direct earthworm-induced effect on grass P uptake through mineralisation of organic P is subtle, as the shoot P content does not increase to the same level as by mineral P fertilisation (Fig. 4.2).



Supporting information

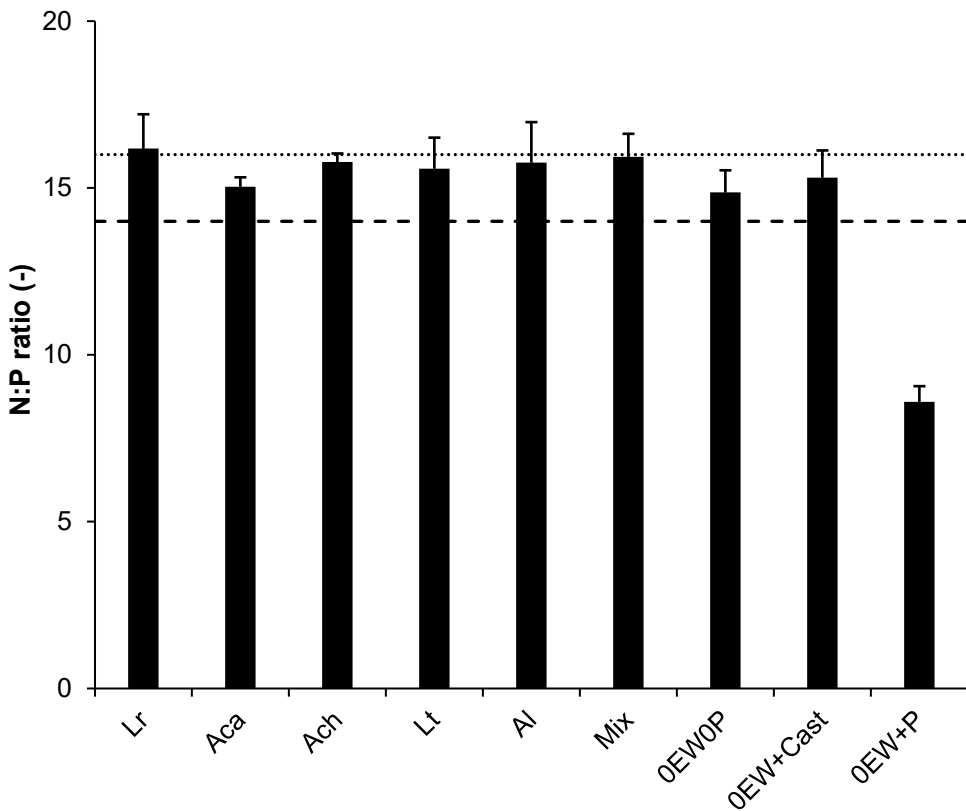
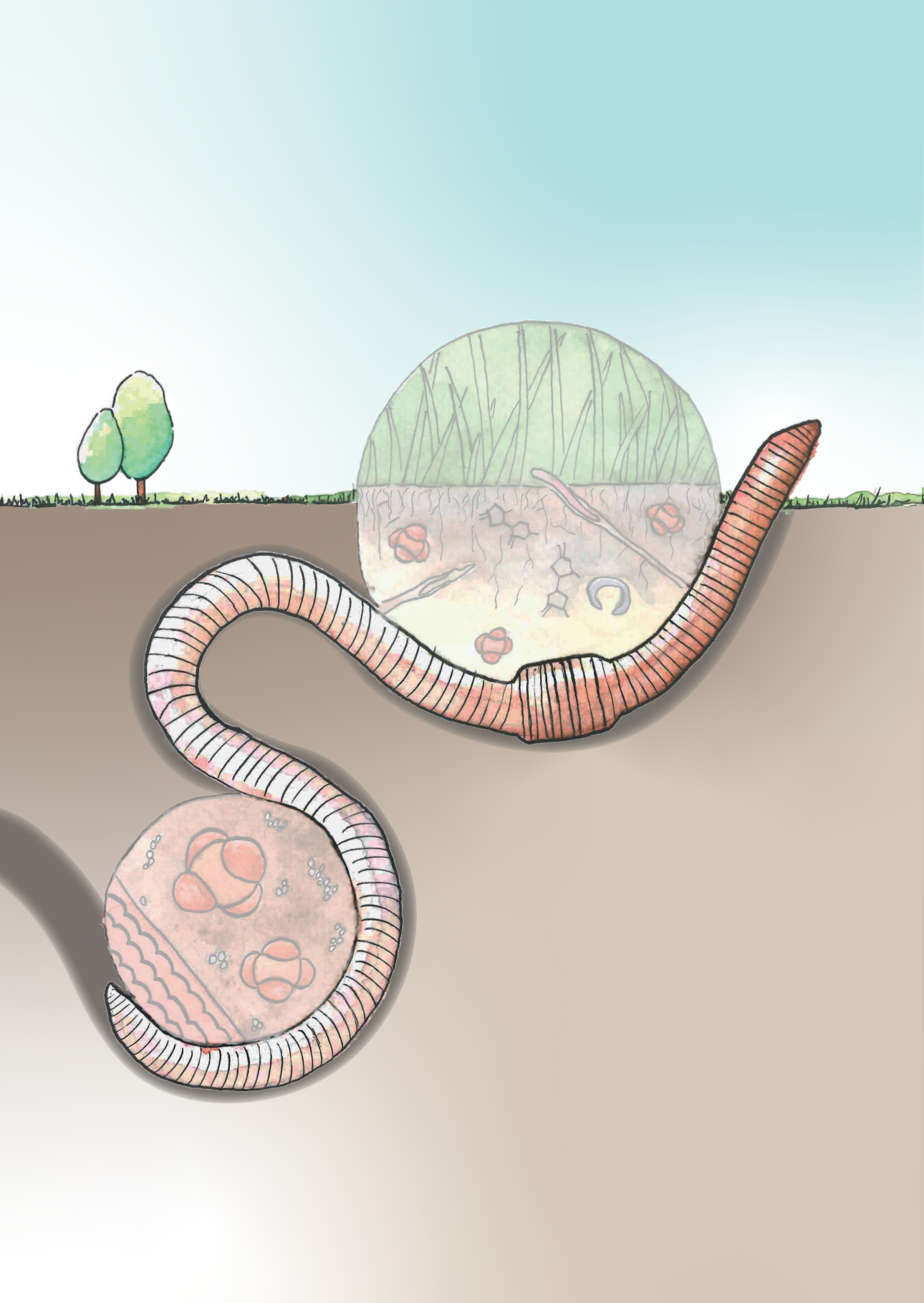


Figure S1 Ratio of N to P of the grass shoots. A N:P ratio above 16 (dotted line) is indicative of P-limited plant growth, while a ratio below 14 (dashed line) indicates N limitation (Koerselman and Meuleman, 1996). Error bars denote standard errors of four replicates.





Chapter 5

Do earthworms increase grass biomass production and phosphorus uptake under field conditions?

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Abstract

The key nutrient phosphorus (P) binds strongly to reactive soil particles, which makes it poorly available for plant uptake. In the search for sustainable ways to overcome a resulting P shortage, it has been shown that earthworms can increase the pool of plant available P and enhance plant P uptake under controlled (greenhouse) conditions. To validate these findings under field conditions and to study the effect of earthworm community composition, we conducted a two-year field experiment on grassland. Mesocosms containing a sandy soil with a low P-status and communities of five earthworm species common to the Netherlands (*Lumbricus rubellus*, *Aporrectodea caliginosa*, *Allolobophora chlorotica*, *Lumbricus terrestris* and *Aporrectodea longa*; monocultures, three- or five-species mixtures and controls without earthworms) were installed in a field and aboveground biomass production and P uptake of *Lolium perenne* were monitored. Earthworm community composition varied between the start and the end of the experiment, but multiple linear regression yielded strong indications that earthworms increased both biomass production ($R^2_{\text{adj}}=0.52$, $p<0.001$) and P uptake ($R^2_{\text{adj}}=0.48$, $p<0.001$). The species *A. longa* and *L. terrestris* were most instrumental for this earthworm effect. General relations between the number of earthworm species in a community and a P effect were not observed. Our results suggest that earthworms can indeed increase grass biomass production and P uptake on a low P soil in the field, and can thereby contribute to making the P nutrition of our agricultural systems more sustainable.

5.1. Introduction

Phosphorus (P) is an essential element for plant growth, which is taken up as orthophosphate (ortho-P) by plant roots from the soil solution (Hawkesford et al., 2012; Hinsinger, 2001). Phosphorus binds strongly to the soil mineral phase, and therefore the total amount of ortho-P in the soil solution of the top 30 to 60 cm of soil at any time is in general only 0.5% - 1.5% of the amount required by plants during a growing season (Hesterberg, 2010; Morel et al., 2000). The remainder is released by mineralisation of organic material containing organic P (George et al., 2018), or supplied by the soil mineral phase via buffering processes such as desorption of ortho-P from soil metal (hydr)oxides, over the course of the growing season (Nawara et al., 2017). Low mineralisation rates of organic P or a low ability of the soil to buffer the concentration of readily-available ortho-P at a sufficiently high level can result in P-limiting conditions for plant growth. To ameliorate this, P-availability can be increased by the application of animal manure or mineral fertiliser. However, global reserves of phosphate rock (i.e. the source of mineral P fertiliser) are limited, and P mining in its current form and amount is neither economically nor environmentally sustainable (Cordell et al., 2009; Sharpley et al., 2018). Additionally, P fertiliser is not always used optimally (Ros et al., 2020; Shepherd et al., 2016), and overapplication of P beyond plant needs can saturate the buffering capacity of the soil. This enhances P leaching or runoff, resulting in eutrophication of groundwater and surface waters (Carpenter et al., 1998; Sawyer, 1966; Schoumans and Chardon, 2015). This simultaneous deficiency and (over)abundance of P in soil has been labelled the world's "P paradox" (Shepherd et al., 2016).

The current need to reduce external mineral P fertiliser input to soils while ensuring a sufficient P supply to plants necessitates a search for new approaches to increase the sustainability of P use (Faucon et al., 2015; Rowe et al., 2016; Stutter et al., 2012). One of the explored approaches is the use of earthworms to increase soil P availability and thereby plant P acquisition in P-limited soils (see review of Le Bayon and Milleret (2009)). According to a meta-analysis of Van Groenigen et al. (2019), earthworm casts contain on average 84% more available P than the surrounding bulk soil, although this number varied among earthworm species. When earthworm casts act as hotspots of available P in soil, this P can be temporally and locally available for plant uptake. Such an earthworm-induced increase in plant available P has been linked to an increased P uptake and larger biomass production of grass (Mackay et al., 1982; Ros et al., 2017; Vos et al., 2014). Several mechanisms for this increase in available P have been proposed (Le Bayon and Milleret, 2009; Ros et al., 2017; Vos et al., 2019; Vos et al., 2014). Although the exact importance of each of these pathways is not yet completely understood, these studies identified i.a. the important roles of dissolved organic carbon (DOC) and pH in increasing the availability of P in casts.

Earthworms are especially abundant under grasslands (Didden, 2001; Yeates et al., 1998), which are of major importance to herbivores and the global food supply: more than

25% of the world's land area is covered by grasslands, of which about two-thirds is used for agriculture to produce forage for ruminants (FAO, 2017). Earthworms are conventionally classified into functional groups based on their feeding and burrowing behaviour. Three main categories are distinguished (Bouché, 1977): 1) *epigeic* earthworms which dwell in or on the topsoil and feed on surface litter; 2) *endogeic* earthworms which live deeper in the soil, make horizontal, non-permanent burrows, and feed on soil particles and soil-associated organic matter (OM); 3) *anecic* earthworms which create large permanent vertical burrows in which they drag down the surface litter they feed on. While distinguishing these functional groups is useful, earthworm species can show plasticity in their behaviour (Neilson et al., 2000), and a recent study suggests a less rigid separation between them (Bottinelli et al., 2020).

Functional groups do not seem to be the decisive factor in determining the observed large variation in readily-available ortho-P in the cast of different species (Vos et al., 2019). Yet, differences between earthworm species in readily-available ortho-P in cast likely influence the total earthworm effect of an earthworm population or community. Specifically in the field, where a variety of species in a community is encountered (Didden, 2001; Van Vliet et al., 2007), this can be relevant, as it is likely that different communities will not have the same earthworm effect. Additionally, synergistic or antagonistic effects of the cast properties of different earthworm species could affect the variation in P availability (e.g. what will be the overall effect of competition between DOC and ortho-P on P adsorption to soil minerals if DOC in some casts is more elevated than in others?). However, the role of multi-species interactions to plant P acquisition requires more attention (Mezeli et al., 2020), and the effect of earthworm communities on P uptake and biomass production could be essential to fully utilize the capacity of earthworms to increase soil P availability to plants (Vos et al., 2019).

Several studies have examined the effect of earthworms on soil nutrient (N, P, K) availability and uptake by plants in incubation studies or pot experiments in the greenhouse (Basker et al., 1992; Butenschoen et al., 2009; Kreuzer et al., 2004; Ros et al., 2017; Vos et al., 2014; Wurst et al., 2003). Such studies are often initially preferred above field experiments, as factors like soil moisture content, temperature, and light can be controlled during the experiment. However, more field studies are required in soil-ecological research (Kampichler et al., 2001), as greenhouse experiments have the obvious disadvantage that their results are often difficult to extrapolate to the field (De Vries, 1980; Kampichler et al., 2001), because effects observed in the greenhouse can be overruled by noise in plant growth conditions in the field. The complexity of field experiments is even enlarged when it concerns field experiments with earthworms. Earthworms digest only a few percent of the soil per year: the production of surface cast in temperate grassland systems varied between 2.2 and 91.6 ton ha⁻¹ yr⁻¹ according to a review by Feller et al. (2003), while the top 20 cm of soil weighs 2000 to 3000 ton ha⁻¹. On the other hand, surface cast may form only a small fraction of the total amount of soil

processed by the earthworms depending on earthworm species and time of the year (Lavelle and Spain, 2001), and extreme cases in which 100% of the surface soil was affected by earthworms within 19 days are reported as well (Mackay et al., 1983). However, at least several months to years are likely required before the earthworms have processed a sufficient amount of soil to expect an earthworm effect in the field. As long periods of time are required for field experiments with earthworms, dynamics in earthworm populations can also be expected. These can be related to the changing seasons, or can be the effect of intra- and interspecific competition between earthworms for resources like food and space. This makes field experiments on the effects of earthworms on soil nutrient uptake even more challenging. To date, some field studies have considered the dynamics of the earthworm communities that are naturally occurring in the field (e.g. Jiménez et al. (2003) and Choosai et al. (2010)) or studied soil structure effects of imposed earthworm treatments in an undisturbed field (Andriuzzi et al., 2015). However, field experiments on nutrient uptake with imposed earthworm communities that include a control without earthworms, are, to the best of our knowledge, not reported in literature.

This study aims to provide a controlled field study in which the effects of earthworms on biomass production and grass P uptake are validated under field conditions. Our novel approach consisted of a mesocosm-field experiment with imposed different earthworm communities on a soil with a low P-status and an experimental duration of more than two years. To reflect the earthworm variation encountered commonly in the field, we considered the effect of earthworm monocultures of five common earthworm species in the Netherlands, as well as their mixtures, when setting up our experiment. Possible dynamics in the earthworm communities were considered by creating as much as possible variation in the initial communities and analysing the variation after the final harvest. We hypothesised that (1) earthworm presence increases both grass biomass production and P uptake under field conditions and (2) this earthworm effect is larger for more diverse earthworm communities.

5.2. Materials and methods

5.2.1 Soil characteristics and pre-treatment

The topsoil (0-25 cm) of a non-calcareous sandy soil (plaggic podzol; IUSS Working Group WRB (2015)) was collected from Achterberg, the Netherlands (51°59'32"N, 5°35'2"E), in January 2017. This soil with a low P-status was selected, to be able to examine any enhancing effect of earthworms on P uptake and biomass production. The soil had been used for three decades as an extensively managed pasture, without applications of mineral fertiliser or animal manure. The pH of the soil (0.01 M CaCl₂) was 6.0 and the OM content was 4.2%. Total P content amounted to 343 mg P kg⁻¹, with 29% being present in the organic form. Based on the low P-AL value of this soil and the agronomic classification

of Reijneveld et al. (2010), the soil had a low P-status. The P loading of metal (hydr)oxides, $\alpha=0.12$, was low compared to an average value for non-calcareous sandy soils in the Netherlands of 0.22 (Koopmans et al., 2006) and maximum values ranging between 0.4 and 0.6 (Van der Zee et al., 1988; Van der Zee and Van Riemsdijk, 1988). Both this low P loading as well as the low P-AL value agreed with the absence of P fertilisation for a prolonged period of time. Additional soil physico-chemical characteristics can be found in SI Table S1.1 and the analytical methods used for soil characterisation are described in Vos et al. (2019).

After collection, the soil was sterilised by using 8 kGy γ -irradiation with the radioisotope cobalt-60 as energy source. This process eliminated all earthworms and cocoons present in the soil, as well as many of the other macro-, meso-, and microorganisms (Hu et al., 2022; McNamara et al., 2003).

5.2.2 Experimental setup

5.2.2.1 Treatments

A mesocosm-field experiment was set up at Wageningen University, the Netherlands (51°59'4"N, 5°39'57"E) as a randomised complete block design with earthworm community as treatment. Five earthworm species common to the Netherlands were used, which can be classified into the following functional groups: epigeic: *Lumbricus rubellus* Hoffmeister, 1843 (Lr); endogeic: *Aporrectodea caliginosa* (Savigny, 1826) (Aca) and *Allolobophora chlorotica* (Savigny, 1826) (green morphotype; Ach); and anecic: *Lumbricus terrestris* Linnaeus, 1758 (Lt) and *Aporrectodea longa* (Ude, 1895) (Al). The treatments consisted of earthworm monocultures of each of the five mentioned species, ten mixtures of three species (all possible combinations of these species), and a five-species mixture (all mentioned species). None of the earthworm treatments received P fertiliser. Furthermore, we included two controls without earthworms of which one did not receive P fertiliser (0EW0P) and one which was fertilised with mineral P (0EW+P). In total, this resulted in 18 treatments and each treatment was replicated five times, resulting in 90 columns.

Earthworm density was fixed at 15 individuals column⁻¹, which corresponds to 558 earthworms m⁻². This number is of the same order of magnitude as the earthworm density commonly observed in pastures in the Netherlands (Didden, 2001). In the monocultures, earthworms belonged to the same species, while for the combination treatments the number of individuals was divided equally over all concerning species.

Earthworms were collected from fields and park areas in and near Wageningen, the Netherlands, with the exception of *L. terrestris* which was purchased from Starfood Holland B.V., Barneveld, the Netherlands. Before addition to the columns, the earthworms were kept in dark mesocosms under controlled conditions (16 °C) in a sandy soil with leaves and grass litter. After their guts had been voided for 48 h (Dalby et al., 1996) to

remove soil from their intestines and minimise the introduction of foreign microbiomes, the earthworms were added to the columns during the first week the columns were in the field (see 2.2.2). Only adult or sub-adult specimens were used.

5.2.2.2 Column design

The columns used for this mesocosm-field experiment consisted of PVC tubes of 40 cm height and a diameter of 18.5 cm (see Fig. 5.1 for a schematic representation). They were open on top, and the bottom was closed with weed control fabric. This polypropylene fabric was tightly attached to the column and facilitated vertical transport of water and nutrients to the soil below, but should prevent earthworms from escaping. The bottom 35 cm of the columns were filled with soil and the surface of the soil within the columns levelled with the surrounding field surface. The top 5 cm of the columns were kept empty and remained above the field surface. On top of the soil in the columns, a 25 cm high wire structure was placed which supported a polyethylene net with a 1.5 mm mesh size, to cover the columns. The structure was flat on top in order not to hamper rainfall. It was high enough for the grass to grow freely and allowed light to pass through. However, earthworms were not able to escape the columns from the top, and neither were birds or other animals able to disturb the soil surface within the columns.

Each column was filled first with 12.25 kg of air-dried, γ -irradiated soil to a bulk density that mimicked the bulk density observed in the field. This soil had been sieved over 5 mm, homogenised and fertilised at the start of the experiment with N (133 kg ha^{-1} equally divided over N-NH_4 and N-NO_3), K (108 kg ha^{-1}), Ca (50 kg ha^{-1}), S (12 kg ha^{-1}), and micronutrients (SI S1.2). The P-fertilised control columns (0EW+P) were additionally amended with 87 kg P ha^{-1} . In all columns, an unfertilised germination layer for the grass seeds of 0.50 kg soil was placed on top of the fertilised soil. Furthermore, 0.40 kg inoculum was divided over the fertilised soil and the unfertilised germination layer. This inoculum was the same air-dried, sieved, and homogenised soil, but instead of being γ -irradiated, it was carefully sieved over a 1 mm sieve. Since earthworm cocoons of the smallest species found in the Netherlands are on average 3 mm in size (Edwards and Bohlen, 1996), this small mesh size prevented earthworm cocoons from passing through the sieve, but it did allow soil microorganisms to pass. Therefore, it was added to our columns to aid the recovery of the native microflora community. In total, each column was filled with 13.15 kg of air-dried soil. All columns were brought to a water holding capacity of 60% by adding de-ionised water.

Subsequently, 4.0 g of *L. perenne* seeds were spread on the germination layer of each column. During the germination of the seeds and early growth, with two subsequent preliminary grass cuts to stimulate tillering and root formation, the columns were kept in the greenhouse where they were watered every other day with de-ionised water and randomised every fortnight. After 44 days (April 2018), the columns were buried in the

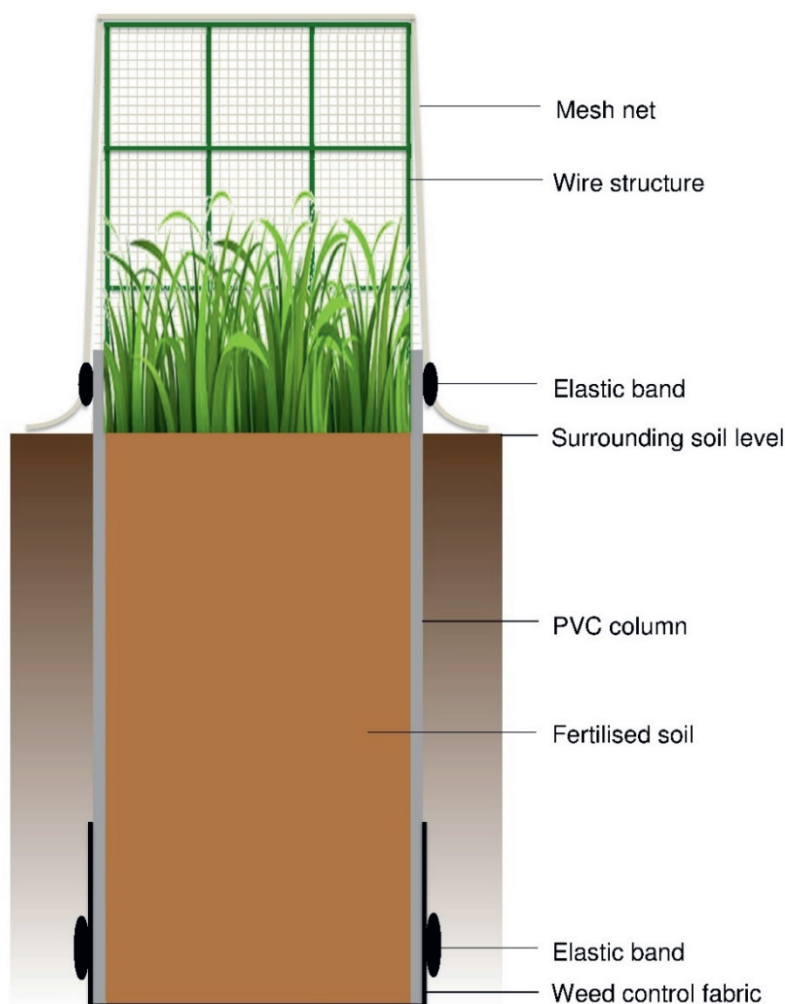


Figure 5.1 Schematic representation of a column.

field at 2 m distance from each other. This date was considered as the actual start of the experiment.

5.2.3 Management of the experiment and collection of samples

The field experiment ran for more than two years (April 2018 - June 2020). During the growing seasons, the grass was cut every three to four weeks (seven harvests in 2018, six in 2019, and two in 2020; the harvest dates are given in SI Table S1.3), which corresponds largely to the harvesting frequency of intensively managed grasslands in the Netherlands. At each harvest, the grass was cut at 5 cm above the soil surface, which is common practice for Dutch grasslands. The grass was dried for 48 h at 70 °C after which the dry weight was recorded. Nitrogen and K (and P for the 0EW+P control) fertilisers were applied

after each harvest, except for the last harvest of the season. At the start of the second and third growing seasons, N, K, (P), Ca, S, and micronutrients were added in equal amounts as at the start of the field experiment. Total amounts of added N appeared high in 2018, but the lower N additions led to N-limited grass growth in 2019, which was restored again in 2020. More information on the fertilisation scheme and the amounts of fertilisers added can be found in SI S1.2.

At the first harvest in the field as well as at the last harvest of each growing season (H1, H7, H13, and H15), soil samples were taken and the N and P contents of grass shoots were determined (at the end of the experiment, the N and P contents of the grass shoots of the 0EW0P and 0EW+P controls of all other harvests were additionally determined). For the soil sampling, two soil cores with a diameter of 1.5 cm and a depth of 10 cm were taken in each column and the holes were refilled with sterilised soil. The soil from the cores was mixed per column and used for preparing water extracts and for determining the fraction of root colonisation by arbuscular mycorrhizal fungi (AMF; see SI S2.3.7). Further, the presence of other animals in the columns, like occasional occurrence of ant colonies or crane fly larvae (*Tipula* spp.) were recorded once every growing season. Also the fraction *non-L. perenne* grasses present in the columns was then visually estimated (non-targeted grasses were not removed, as removal of these grasses and their roots could negatively impact the earthworms). These grasses likely originated from seeds dispersed by the wind, which germinated on the free spaces left when some *L. perenne* tillers died as a consequence of their sensitivity to high N fertilisation, heavy harvests, drought, and cold winters (Prins and Neeteson, 1982; Whitehead, 2000).

The presence of earthworm surface casts (as an indication of earthworm activity) was recorded two to five times per growing season. We observed an absence of earthworm activity in some of the columns to which earthworms had been added, at the end of the first growing season. Therefore the presence of the targeted earthworm species was secured by adding additional earthworms to each column during the second growing season (after H9 for most species, and several weeks later for *A. longa*; see SI S1.4). As earthworm survival could only be determined destructively, we do not have survival data at any time other than the final harvest. We could therefore not statistically test the effect of this mid-term earthworm replenishment.

Rainfall was the main source of water for the grass. During drought periods (summer of 2018, early summer of 2019, and spring of 2020; see SI Fig. S1.1), columns were watered by a sprinkler irrigation system with irrigation water that had a low P concentration (0.04 mg P L⁻¹).

At the final harvest of the field experiment (H15), the columns were removed from the field and the earthworm communities were determined. The top 10 cm of each column was searched by hand for earthworms hiding between the grass roots, while flushing the soil with water through a 0.5 mm sieve. The lower part of the soil was flushed with water through two large 1.2 mm sieves positioned misaligned on top of each other.

This method was tested in a preliminary experiment, in which 92% of all earthworms were retrieved. After collecting the earthworms from each column, they were counted and identified. Unfortunately, the weight of the recovered earthworms after defecation (Dalby et al., 1996) could not be measured due to limited laboratory access during the Covid-19 pandemic. Therefore it was estimated by using species-specific average weights of adults and juveniles based on previous unpublished experiments (*L. rubellus*: 0.50/0.15, *A. caliginosa*: 0.35/0.15, *A. chlorotica*: 0.30/0.15, *L. terrestris*: 3.50/not encountered, *A. longa*: 2.00/1.50 g earthworm⁻¹ for adults/juveniles).

5.2.4 Chemical analyses of grass and soil

The N and P contents of the grass shoots were determined according to Novozamsky et al. (1983). In short, grass was ground after drying for 48 h at 70 °C, and subsequently digested with a mixture of sulphuric acid, hydrogen peroxide, and salicylic acid with selenium as a catalyst. The N and P concentrations in the digest were measured by segmented flow analysis (SFA, Skalar, SAN⁺⁺).

The soil cores were sieved over a 2 mm sieve by which the soil and the roots were separated. The roots were cleaned with tap water and stained with the ink and vinegar method (Vierheilig et al., 1998). The fraction of root colonisation by AMF was determined with the standard magnified intersection method (McGonigle et al., 1990). The soil that passed through the 2 mm sieve was homogenised and a subsample was dried at 40 °C to determine the water content. To create a proxy for soil pore water, soil was extracted with ultra-pure water at a soil-to-solution ratio of 1:10 (w:v; based on dry weight) (Koopmans et al., 2006; Torrent and Delgado, 2001). Field moist soil was used for preparing these extracts, since drying and rewetting of soil has been shown to enhance the release of P and DOC to solution (Koopmans et al., 2006; Koopmans and Groenenberg, 2011). The extracts were shaken with 75 strokes min⁻¹ for 24 h on a reciprocal shaking machine, after which they were centrifuged for 10 min at 3750 rotations min⁻¹ and filtered through a 0.45 µm filter (NC45, Whatman). The pH, electrical conductivity (EC), and light absorbance at 254 nm (A254) of the soil water extracts were measured in subsamples of the filtrates. The EC was converted to ionic strength (Griffin and Jurinak, 1973) and A254 was used to calculate the fraction of aromatic DOC (Weishaar et al., 2003). The remainder of the filtrates was pooled by combining all replicates from the same treatment in equal volumes. In these pooled filtrates, ortho-P (SFA, molybdenum blue method; Murphy and Riley (1962)), DOC (SFA; Houba et al. (2000)), total dissolved P (TDP; high resolution - inductively coupled plasma - mass spectrometry, Element 2, Thermo Scientific for H1 and H7, and inductively coupled plasma - atomic emission spectroscopy, Varian Vista Pro for H13 and H15) were measured. For the final harvest (H15), only the soil samples of the non-P fertilised control (0EW0P) and the P fertilised control (0EW+P) could be analysed due to limited laboratory access during the Covid-19 pandemic. Further analytical details on the methods used for the water extracts can be found in Vos et al. (2014) and Vos et al. (2019).

5.2.5 Statistical analyses

Statistical analyses were performed in R version 4.0.2 (R core team, 2020). After evaluating the earthworm dynamics by determining the final earthworm communities as described in section 5.2.3, data were analysed by multiple linear regression (MLR). Based on the setup of this field experiment, data could be analysed with ANalysis Of Variance (ANOVA), but the encountered earthworm dynamics limited the usefulness of this approach (SI S1.6).

The effects of earthworm dynamics on biomass production and P uptake were determined for all non-P fertilised columns (which include all earthworm treatments as well as the 0EW0P control). The 0EW+P control was excluded from MLR analysis, because these columns received additional P fertilisation. Analysis by MLR started with a linear model of all included variables with the function *lm* (package stats) and subsequent selection of those variables that significantly contributed to model prediction by forward and backward model selection with the function *ols_step_both_aic* (package olsrr) (Hebbali, 2020). Differences between modelled and observed values were assessed by calculation of the root mean square error (RMSE) with the function *rmse* (package Metrics) (Hamner et al., 2018).

5.3. Results and discussion

5.3.1 Trends in biomass production and earthworm population

There was a normal pattern for grass growth in a temperate climate (Clark et al., 1997): aboveground biomass production and P uptake increased from spring to summer after which they decreased again (Fig. 5.2). The P content was higher in the grass of the P-fertilised control (0EW+P) compared to the non-P fertilised control (0EW0P), and depended on the amount of N applied (SI S2.4 and Fig. S2.1). Hence, our data demonstrated a positive effect of P fertilisation on P uptake. Total biomass production was higher in 2018 than in 2019: on average 53 g column⁻¹ (20 Mg ha⁻¹) in 2018 compared to 25 g column⁻¹ (9 Mg ha⁻¹) in 2019 for non-P fertilised columns. Likewise, P uptake was higher in 2018 than in 2019: 127 and 173 mg column⁻¹ in 2018 for the 0EW0P and 0EW+P controls, and 68 and 118 mg column⁻¹ for those controls in 2019, respectively. The main reason for these differences between years is the difference in N fertilisation (see SI S2.4 and Fig. S2.2). In 2020, total aboveground biomass production of all non-P fertilised columns was on average 10 g column⁻¹ (4 Mg ha⁻¹) for only the first months of the year.

The number of earthworms retrieved after the final harvest was 51% of the number introduced at the start, which was comparable to the 40% survival of the inoculated earthworms used in undisturbed mesocosms of the field study of Andriuzzi et al. (2015). However, earthworm survival varied strongly per species (34% for *L. rubellus*, 128% for *A. caliginosa*, 59% for *A. chlorotica*, 4% for *L. terrestris* and 29% for *A. longa*). Of all retrieved

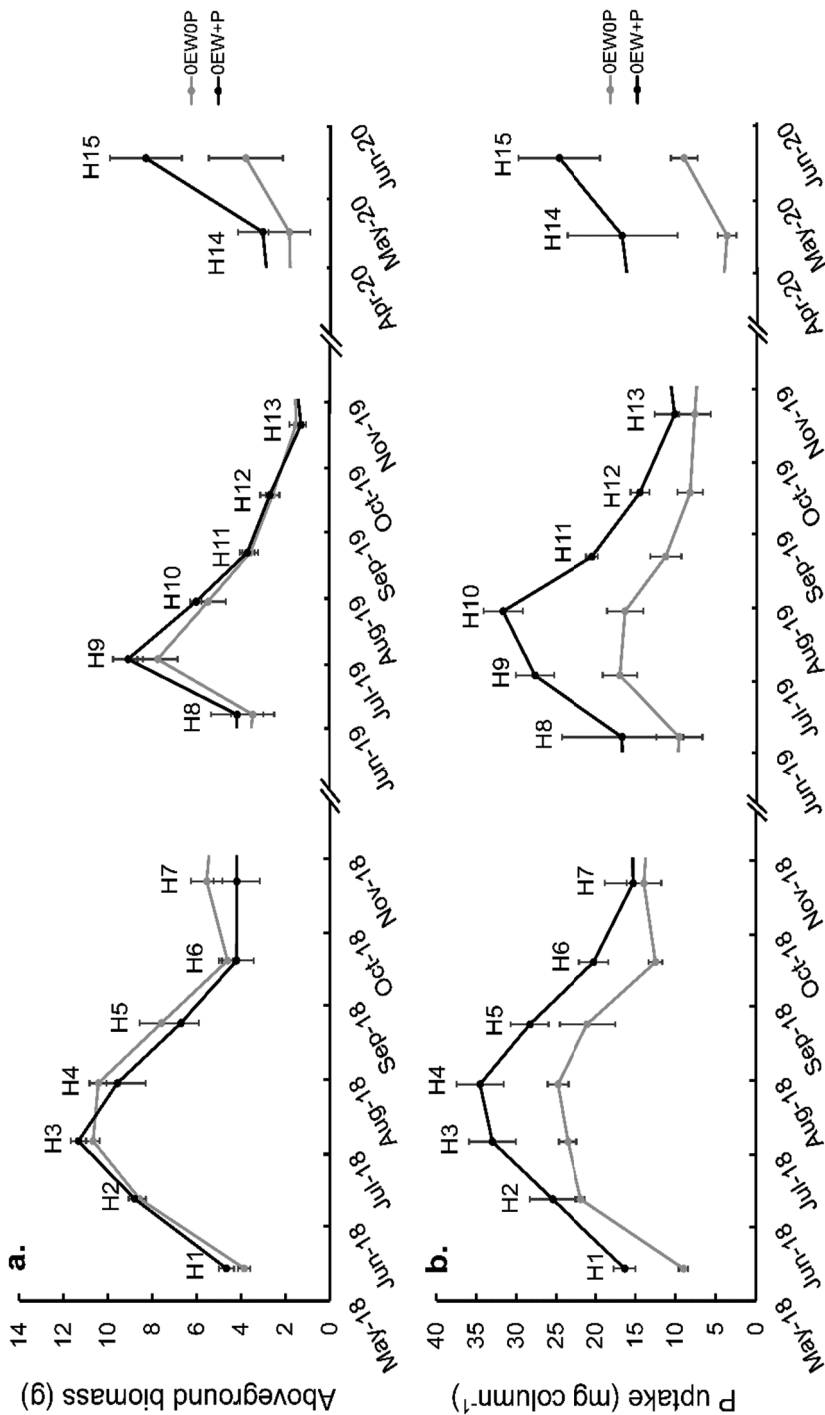


Figure 5.2 Average aboveground biomass production (a) and P uptake (b) for each harvest of the field experiment. For clarity only data of the non-P fertilised control (0EW0P) and the P fertilised control (0EW+P) are shown. Error bars denote standard errors.

earthworms, 30% were adults, while the other 70% were juveniles varying from recently hatched earthworms to sub-adults. Also cocoons were observed in most columns. Individuals of *A. chlorotica*, *L. terrestris*, and *A. longa* were either still present or absent in columns where they had been introduced, but they were never found in other columns. Individuals of *L. rubellus* and *A. caliginosa* on the other hand, were both found in columns where they had, and had not, been introduced, including in some controls (Fig. 5.3 and SI S2.1). Final earthworm communities therefore differed substantially from the introduced communities. Such differences could be expected in an experiment of more than two years, both in terms of population size and in the composition of the earthworm communities (e.g. as a consequence of intra- and inter-species competition, and possibly low reproduction and death of individuals or species). However, that some columns also contained non-introduced species after the final harvest (negative bars in Fig. 5.3) was intended to be prevented by the experimental setup. Because the composition of the earthworm communities had changed during the field experiment, data analysis according to ANOVA was not well-applicable. Therefore earthworm effects on biomass production and P uptake were analysed by MLR.

5.3.2 Earthworm effects on biomass production and P uptake

In 9 out of the 85 non-P fertilised columns the grass had died at the end of the experimental period (the cut off value to distinguish between present and absent grass cover was set at 0.5 g dry aboveground biomass), leaving a total of 76 columns. In 82% of those columns other grass species than *L. perenne* were observed. This non-*L. perenne* grass cover was on average 5.5% of the total column surface and consisted mainly of *Festuca arundinacea*, but *Poa pratensis*, *Phalaris arundinacea*, and some *Bromus* spp. were occasionally observed as well. Since these grass species produce different amounts of aboveground biomass, have different rooting patterns, and use different strategies to access a limited resource like P (Hill et al., 2006; Lynch, 2007; Ros et al., 2018), they affected biomass production and P uptake (p values of $p < 0.0001$; see SI S2.5). Therefore the fraction of non-*L. perenne* grasses was included as a variable in the MLR analysis. Chemical soil parameters were not included in the MLR analysis, as the observed variation in these parameters was mainly affected by the applied fertilisation scheme (SI S2.3 and S2.4). Also our biological soil parameter, the fraction of AMF colonisation of grass roots, was not included in the MLR analysis, as no significant differences between the non-P fertilised treatments were observed (SI S2.3.7).

Effects of the number of earthworms at the end of the experiment on biomass production (both harvests of 2020: H14+H15) and P uptake (H15) were included in our MLR analysis. We performed MLR with all earthworms encountered in the 76 columns with a present grass cover (SI Table S2.1), regardless whether they were present in a species monoculture or mixture. Because of the large difference in body mass of the earthworm

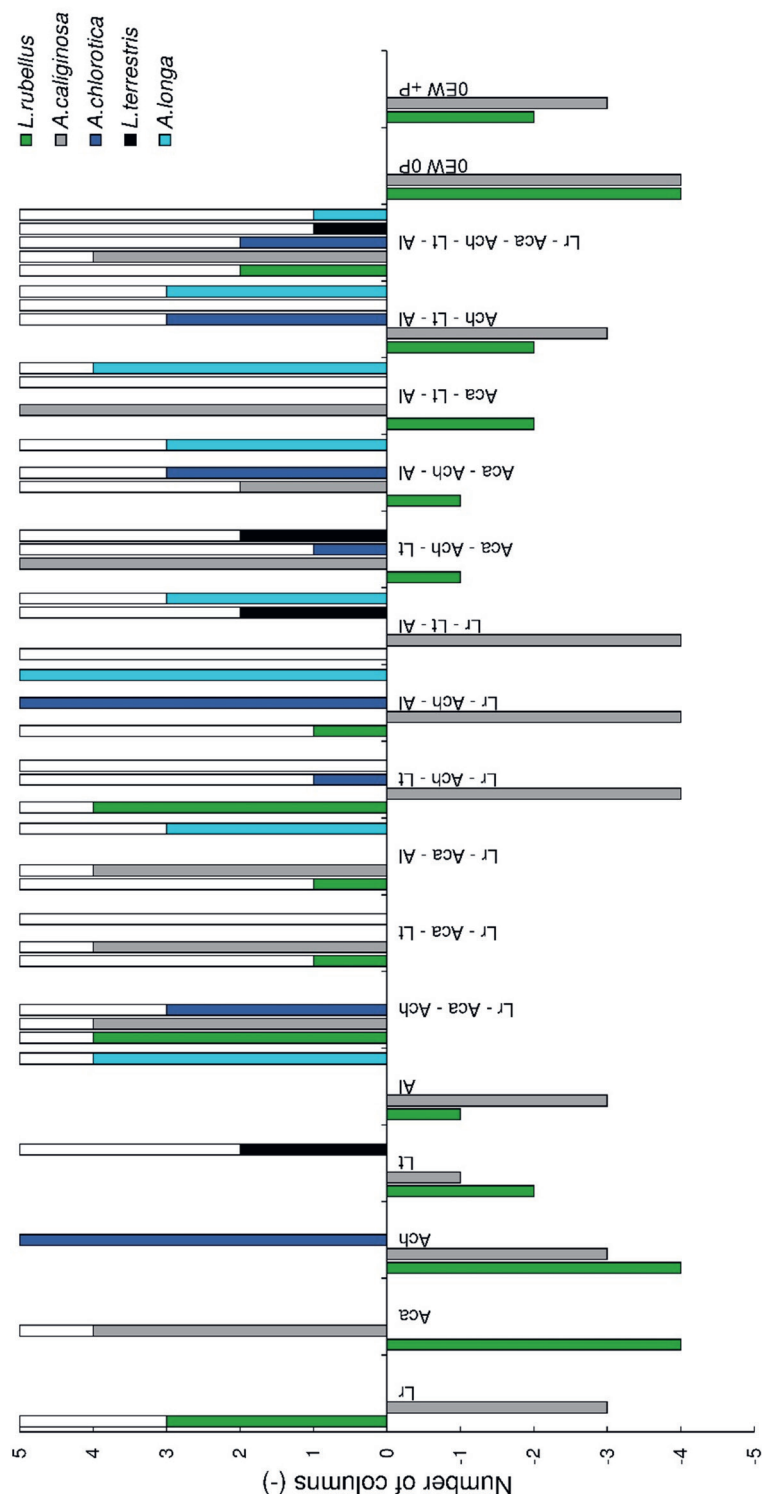


Figure 5.3 Earthworm species encountered after the final harvest. The value to which a positive bar is filled up signifies the number of columns in which an earthworm species initially added was still present. Since all treatments had five replicates, this is the maximum value. Bars with a negative value indicate the presence of an earthworm species which was not added at the start of the field experiment. Species abbreviations are: Lr - *Lumbricus rubellus*, Aca - *Aporrectodea caliginosa*, Ach - *Allolobophora chlorotica*, Lt - *Lumbricus terrestris* and Al - *Aporrectodea longa*.

species, the number of individuals per column was converted to grams using a species-specific estimated body weight (section 5.2.3).

Figures 5.4.a and 5.4.b show the total estimated earthworm body mass in a column in relation to the summed grass biomass production in 2020 and P uptake at the final harvest. When the estimated body mass of all earthworm species was used in the MLR model, forward and backward model selection resulted in a model in which only the fraction of non-*L. perenne* grasses and the estimated body mass of *A. longa* and *L. terrestris* affected the aboveground grass biomass ($R^2_{adj}=0.52$, $p<0.001$):

$$AGB = 6.37 (\pm 0.65) + 73.16 (\pm 8.95) * nLp + 0.34 (\pm 0.13) * AI + 0.60 (\pm 0.28) * Lt \quad (5.1)$$

in which AGB is the summed aboveground biomass of 2020 ($g \text{ column}^{-1}$), nLp the fraction non-*L. perenne* grasses at the final harvest (-), and AI and Lt the summed body mass ($g \text{ column}^{-1}$) of all *A. longa* and *L. terrestris* individuals encountered in each column after the final harvest, respectively. Values between brackets are standard errors.

The MLR model for P uptake at the final harvest was ($R^2_{adj}=0.48$, $p<0.001$):

$$P \text{ uptake} = 6.13 (\pm 0.80) + 86.89 (\pm 11.06) * nLp + 0.28 (\pm 0.16) * AI + 0.59 (\pm 0.34) * Lt \quad (5.2)$$

in which P uptake is the P uptake at the final harvest ($mg \text{ P column}^{-1}$) and the other variables as specified for Eq. 5.1.

The model predictions versus the observed data are distributed around the 1:1 line (Fig. 5.5), which emphasizes the importance of the present earthworm body mass for explaining grass biomass and P uptake. As the contributions of the earthworms in both MLR models were significant and positive, this indicated that these variables contributed to an increase of both aboveground biomass production and P uptake. The significantly increased aboveground biomass production and P uptake by *L. terrestris* agrees with results found in greenhouse pot experiments by Ros et al. (2017) and Vos et al. (2014). The latter study also investigated the presence of two other earthworm species (i.e. *A. caliginosa* and *L. rubellus*), but those species did not significantly increase biomass production and P uptake, which again agrees with the findings of our field experiment. Here we additionally studied two other species and also observed a biomass production and P uptake stimulating effect for *A. longa*.

The effects of *A. longa* and *L. terrestris* could be due to their relatively large size and body mass (Sims and Gerard, 1985) compared to the other species studied. Because they are larger, *A. longa* and *L. terrestris* produce more cast, which would make it more likely that plant roots benefit from the hotspots of plant available P created by these earthworms. The amount of cast with an elevated concentration of ortho-P might therefore be more important than the actual ortho-P concentration of those casts.

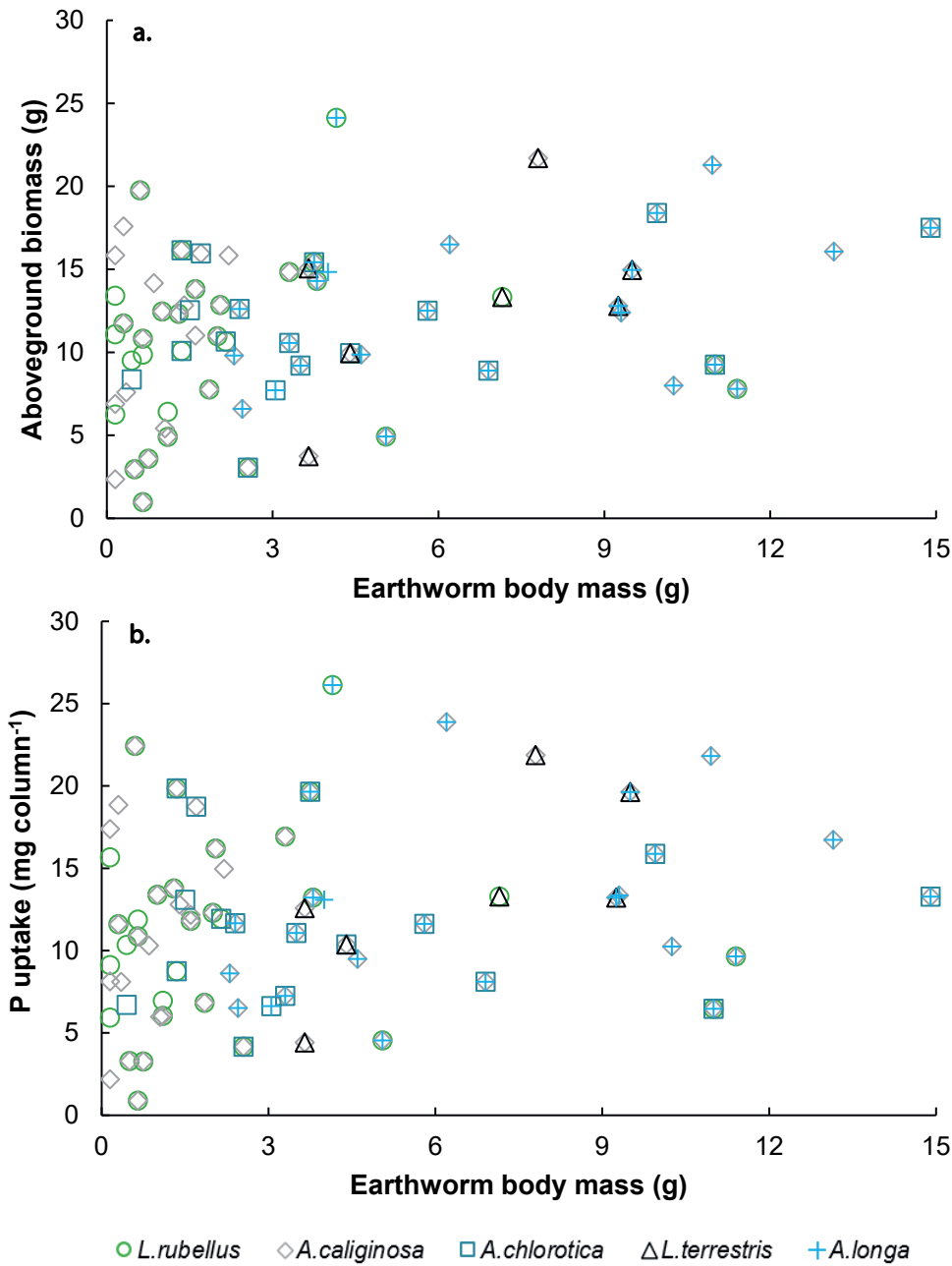


Figure 5.4 Scatter plot of total estimated earthworm body mass after the final harvest versus the cumulative aboveground biomass of 2020 (H14+H15) (a) and versus the P uptake at the final harvest (H15) (b). When differently coloured symbols overlap, this indicates that the total estimated earthworm body mass in that column was made up of different species.

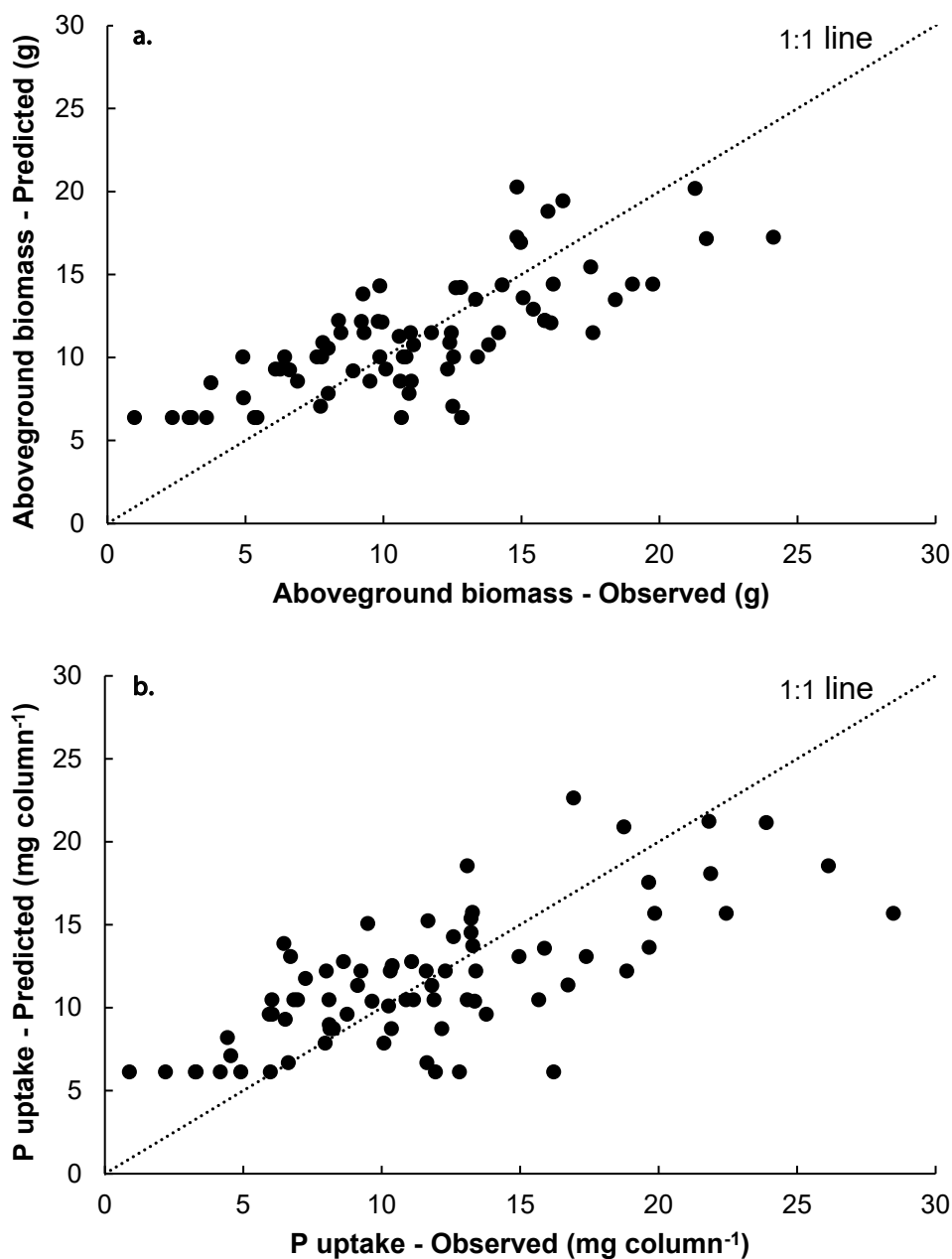


Figure 5.5 Scatter plot of the observed versus predicted results for aboveground biomass (a) and P uptake (b) at the final stage of the field experiment (H14+H15 for aboveground biomass and H15 for P uptake). Dotted lines are 1:1 lines which present perfect model fit.

Another possible reason for the importance of *A. longa* and *L. terrestris* might lie in the functional group of these earthworms. Both *A. longa* and *L. terrestris* are anecic species, that feed on fresh surface litter (as opposed to the lower quality soil-associated OM that endogeic species feed on), and drag this OM into the soil (as opposed to the OM the surface dwelling epigeic species feed on and which remains at the soil surface). Their contributions to plant available P may therefore be large through the mineralisation of high-quality OM, and because it is located at about the same location as plant roots. However, it should be noted that the effect of *A. longa* could also be related to the later re-introduction of this species (section 5.2.3).

After the final harvest, the number of earthworm species in a community varied from zero to four species. Both aboveground biomass production and P uptake did not show a relation with the number of earthworm species present ($R^2_{\text{adj}}=0.003$, $p=0.27$ for aboveground biomass and $R^2_{\text{adj}}=-0.003$, $p=0.38$ for P uptake; SI Fig. S2.12). This agrees with research that states that soil biodiversity or the abundance of species is not key for plant P acquisition (Mezeli et al., 2020), and studies that demonstrate that the number of species in a community is less important than the presence of keystone organisms (Heemsbergen et al., 2004; Hooper et al., 2005; Van der Plas, 2019). Our results indicate that *A. longa* and *L. terrestris* are therefore keystone organisms for aboveground biomass production and P uptake of grass.

As the presented MLR models on the effects of earthworms on biomass production and P uptake were based on the final stage of the field experiment, the validity of these models was tested for its initial conditions. Already at the early stage of the field experiment, an effect of *L. terrestris* in explaining grass biomass production and P uptake was shown (SI S2.7).

5.3.3 Validity of the results and practical implications

The presence of ant colonies and crane fly larvae ($p=0.29/0.61$ and $p=0.75/0.93$, for effects on grass biomass/P uptake), the variation in N fertilisation (SI S2.4), and the relatively warm and dry summers (SI Fig. S1.1) all contributed to the variation in biomass production and P uptake. Significant column-specific factors (e.g. invasions of non-*L. perenne* grasses in specific columns) were included in our MLR models, but non-significant factors (e.g. presence of ant colonies or crane fly larvae) and other factors that affected all columns simultaneously (e.g. low N fertilisation in 2019 and the relatively warm summers) were not included.

Despite the encountered variation, our results confirm the consistent earthworm effects that were observed earlier in greenhouse pot experiments (Mackay et al., 1982; Ros et al., 2017; Vos et al., 2014), but now under field conditions. While our models do not give insights on the underlying mechanisms of the observed earthworm effect, these mechanisms have been studied in detail in earthworm casts by Ros et al. (2017) and Vos et al. (2019). The variation in the concentration of ortho-P in the water extracts that were

prepared from the bulk soil samples was most likely related to variables like pH and ionic strength (Regelink and Koopmans, 2021), which, in turn, reflected the fertilisation scheme used in our experiment. As such, the observed variation in water-extractable ortho-P was not related to earthworm activity (see SI S2.4). This absence of any earthworm effects in the bulk soil agrees with results of Vos et al. (2014) and Ros et al. (2017).

Our results do not provide insights on the effects of earthworms on biomass production and P uptake over time, or on possible effects of the interactions of earthworm species in communities. The encountered earthworm dynamics made it impossible to investigate this and it is likely that such effects have to be studied under more controlled conditions.

Our MLR models explained about 50% of the observed variation in grass biomass production and P uptake in 2020. The question therefore remains what caused the remaining unexplained variation. Such explanations might be random effects, but can also relate to variations in other factors than earthworms that are affecting P-limitation (not all non-*L. perenne* grasses in our columns belonged to the same species which also caused variation). Further research should therefore also include plant specific adaptations to P-limited conditions, like changes in root architecture, root exudation or the plant microbiome (Lynch, 1995; Lynch, 2007; Marschner et al., 2006; Ramaekers et al., 2010; Ryan et al., 2001; Wang and Lambers, 2020).

The results of our study are first of all of interest for soils with a low P-status like the soils studied here and in Ros et al. (2017) and Vos et al. (2019). Apart from a low level of readily available P, those soils have a low P stock. This indicates that the beneficial effect of earthworms on grass growth and P uptake may not continue forever, as the P stock will be depleted when there is a continuous net withdrawal of P from soil by the harvested biomass. Hence, P additions will be needed at some stage to sustain optimal plant growth. However, also strongly P-fixing soils may occur under temperate grasslands (Schoumans et al., 2004; Vos et al., 2014). In such soils, P availability is low due to their strong P binding properties, although total P reserves can be considerable (Ramaekers et al., 2010; Schoumans et al., 2004). Here earthworm-enhanced P availability could therefore supply grass with P for a longer time. This holds as well for the so-called legacy P soils, i.e. soils that have a legacy of P from past P applications in excess of plant requirements (Kleinman et al., 2011). The availability of P in those soils is high, and enhancing it by means of earthworm activity is therefore irrelevant. Rather, a P mining strategy is needed here to decrease the risk of P losses to the aquatic environment (Buda et al., 2012; Kleinman et al., 2011), and to proceed towards a more sustainable nutrient management (Rowe et al., 2016). During P mining, soil P reserves can still be large when the P supply to grass or fast-growing crops becomes limiting for an optimal P uptake (Koopmans et al., 2004a; Koopmans et al., 2004b; Smolders et al., 2021). When earthworms can increase the concentration of readily available soil P in those situations, a larger drawdown of soil P

may be realised. Thereby earthworms contribute to making the P nutrition of our agricultural systems more sustainable.

5.4. Conclusions

Our results provide strong indications that the presence of earthworms, and particularly *A. longa* and *L. terrestris*, increased aboveground biomass production and P uptake by *L. perenne* in a field experiment on a soil low in available P. This finding confirms the results of experiments conducted previously under controlled (greenhouse) conditions. However, the imposed earthworm treatments in our field experiment were subject to variable natural environmental conditions and therefore exhibited significant changes over time. While no general relations between the number of earthworm species in a community and an earthworm effect were observed, our field experiment shows that specific earthworm species can enhance grass P uptake and biomass production. Thereby earthworms contribute to making the P nutrition of our agricultural systems more sustainable.

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Supporting information

S1 Materials and methods

S1.1. Soil characteristics

Table S1.1 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 ₂ O 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^{\dagger}	0.12

[†] P loading of reactive soil metal (hydr)oxides calculated according to Van der Zee and Van Riemsdijk (1986)

S1.2. Fertilisation

For the first growing season in 2018, the applied amounts of N, K, and P fertiliser were calculated from the removal of these nutrients with the harvested biomass in combination with an assumed nutrient use efficiency of 50%. For calculating nutrient removal, recorded biomass and assumed nutrient contents for *L. perenne* of 35 g N kg⁻¹, 25 g K kg⁻¹, and 3.5 g P kg⁻¹ (Reuter and Robinson, 1997) were used. The calculated amounts of N, K and P fertiliser were added as a solution, and split over two applications four days apart to prevent salt stress for the earthworms. De-ionised water was used to flush the solution into the soil. However, the large biomass production together with the assumed nutrient use efficiency of 50% resulted in excessively high nutrient gifts during the first growing season in 2018. These large nutrient applications are not representative for agricultural grasslands in the Netherlands (Schröder et al., 2007; Schröder and Neeteson, 2008) and excessive N fertilisation rates can lead to acidification and salinization of the soil (see SI S2.4). Therefore, N, K, and P fertiliser applications in the second growing season in 2019 followed the Dutch fertiliser recommendation scheme for agricultural grasslands (Commissie Bemesting Grasland en Voedergewassen, 2012). Since

these amounts appeared to be insufficient to avoid N-limited grass growth, N application during the third growing season in 2020 was in between the N applications of 2018 and 2019.

Table S1.2 Fertiliser application of N, K (all columns) and P (0EW+P columns) in kg ha⁻¹ after each harvest (H).

	N (kg ha ⁻¹)	K (kg ha ⁻¹)	P ‡ (kg ha ⁻¹)
Start growing season 1 (2018) †	271	108	101
H1	78	254	8
H2	278	-	22
H3	229	211	19
H4	252	-	20
H5	233	245	19
H6	141	-	11
H7	Not fertilised after this harvest (winter)		
Start growing season 2 (2019) †	133	108	87
H8	82	33	11
H9	58	33	9
H10	48	33	9
H11	33	33	9
H12	27	33	9
H13	Not fertilised after this harvest (winter)		
Start growing season 3 (2020) †	133	108	87
H14	180	33	11
H15	Not fertilised (experiment finished)		

† At the start of each growing season, fertiliser application also included 50 kg Ca ha⁻¹, 12 kg S ha⁻¹ and micronutrients (0.93 g B ha⁻¹, 0.93 g Mn ha⁻¹, 0.04 g Cu ha⁻¹, 0.09 g Zn ha⁻¹ and 0.02 g Mo ha⁻¹)

‡ Only the five 0EW+P columns

S1.3. Harvest dates

Table S1.3 Dates of the harvests (H) of the field experiment.

Growing season	Harvest	Date
2018	H1	14-05-2018
	H2	12-06-2018
	H3	06-07-2018
	H4	30-07-2018
	H5	24-08-2018
	H6	19-09-2018
	H7	22-10-2018
2019	H8	07-06-2019
	H9	03-07-2019
	H10	30-07-2019
	H11	22-08-2019
	H12	18-09-2019
	H13	21-10-2019
2020	H14	16-04-2020
	H15	18-05-2020

S1.4. Earthworm re-introduction

By the end of the first growing season in 2018, our observations of cast presence at the soil surface indicated an absence of casts in some of the columns to which earthworms were initially added. Since cast presence is only a proxy for earthworm activity, we dug out an additional column with five-species treatment at the end of the first growing season. This column was set up at the start of the field experiment and managed as all others. Earthworm presence in this column was determined by carefully hand-sorting all soil and roots. Out of the added 15 earthworms, 7 were found back, including individuals of all five species. Since some of the retrieved individuals were (small) juveniles, earthworm reproduction must have occurred in this additional column during the first growing season. As of some earthworm species only one individual was present, we wanted to secure the presence of the targeted species in each column of the actual field experiment. Therefore 7 additional earthworms were added to each column immediately after H9 (i.e., second harvest of the second growing season) (see Table S1.4 for the number of individuals per species). The species *A. longa* was added later in the second growing season, as for this species it took more time to collect a sufficient number of individuals in the field due to dry weather conditions.

Table S1.4 Number of individuals of each earthworm species added during the second growing season of the field experiment (July 2019 for Lr, Aca, Ach and Lt; October 2019 for Al). Species abbreviations are: Lr - *Lumbricus rubellus*, Aca - *Aporrectodea caliginosa*, Ach - *Allolobophora chlorotica*, Lt - *Lumbricus terrestris* and Al - *Aporrectodea longa*.

Treatment	Added earthworms				
	Lr	Aca	Ach	Lt	Al
Lr	7	-	-	-	-
Aca	-	7	-	-	-
Ach	-	-	7	-	-
Lt	-	-	-	7	-
Al	-	-	-	-	7
Lr - Aca - Ach	3	2	2	-	-
Lr - Aca - Lt	3	2	-	2	-
Lr - Aca - Al	3	2	-	-	2
Lr - Ach - Lt	3	-	2	2	-
Lr - Ach - Al	3	-	2	-	2
Lr - Lt - Al	3	-	-	2	2
Aca - Ach - Lt	-	2	3	2	-
Aca - Ach - Al	-	2	3	-	2
Aca - Lt - Al	-	3	-	2	2
Ach - Lt - Al	-	-	3	2	2
Lr - Aca - Ach - Lt - Al	2	1	2	1	1
0EW0P	-	-	-	-	-
0EW+P	-	-	-	-	-

S1.5. Climate data

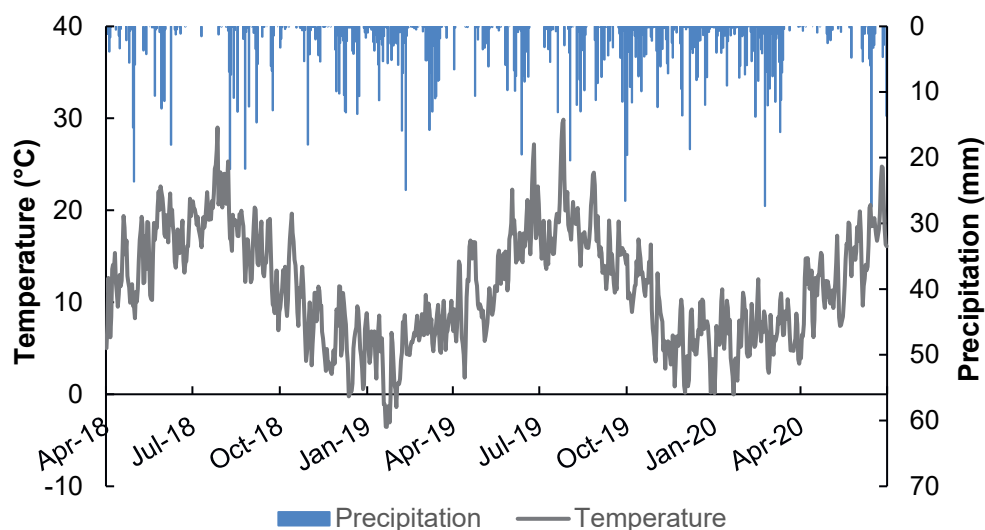


Figure S1.1 Average daily temperature in °C and total daily precipitation in mm during the experimental period. Data were recorded at weather station “De Veenkampen” which is situated less than 3 km from the experimental location (<http://www.met.wur.nl/veenkampen/data/>).

S1.6. Statistical analysis

Statistical analyses according to ANalysis Of Variance (ANOVA) were only used for data at the initial stage of the field experiment (SI S2.7). In the absence of earthworm dynamics, the effect of earthworm treatment (17 non-P fertilised treatments, as the 0EW+P control was excluded from this analysis because it received P fertilisation) was determined with linear mixed effects models by using the function *lme* (package *nlme*) (Pinheiro et al., 2020) after inclusion of block as a random factor. The models were tested with a type III SS ANOVA (package *base*) after testing for normality (Shapiro-Wilk test) and homoscedasity (Levene test) of the residuals. When the residuals were not homogeneously distributed, the variance structure *VarIdent* (package *nlme*) was included in the model and compared with the same model without the variance structure using ANOVA with the function *anova* (package *base*). The model with a significantly ($p < 0.05$) lower Akaike Information Criterion was used. When significant differences were observed with ANOVA, a Tukey’s honestly significant difference posthoc test was performed by using the functions *lsmeans* and *cld* (packages *emmeans* (Lenth et al., 2020) and *multcomp* (Hothorn et al., 2018)).

S2 Results and discussion

S2.1. Earthworm data

Table S2.1 Earthworm species encountered in each column after the final harvest (H15) of the field experiment. Data are presented according to the five replicates of each treatment. The grass in the columns presented in *italics* had died at H15, and therefore those columns were not included in the MLR analysis. Species abbreviations are: Lr - *Lumbricus rubellus*, Aca - *Aporrectodea caliginosa*, Ach - *Allolobophora chlorotica*, Lt - *Lumbricus terrestris* and Al - *Aporrectodea longa*.

Treatment	Total	Adults					Juveniles				
		Lr	Aca	Ach	Lt	Al	Lr	Aca	Ach	Lt	Al
Lr	5	1					4				
Lr	3	1	1				1				
Lr	5	1	1				1	2			
Lr	0										
<i>Lr</i>	2							2			
Aca	7	1	3					3			
Aca	8		2				1	5			
Aca	18		3				1	14			
Aca	2	1					1				
Aca	8		5					3			
<i>Ach</i>	17			5					12		
Ach	9	1		3			3		2		
<i>Ach</i>	21			9			1	4	7		
<i>Ach</i>	14			4			2	2	6		
Ach	7			2			1	3	1		
Lt	0										
Lt	2				1			1			
Lt	0										
Lt	1						1				
Lt	3				2		1				
Al	0										
Al	2					2					
Al	9					3		3			3
Al	13					1	1	11			
Al	10		1			4		2			3
Lr - Aca - Ach	8			2					6		
Lr - Aca - Ach	6	1	3					2			
Lr - Aca - Ach	10	1	1				3	5			

Treatment	Total	Adults					Juveniles				
		Lr	Aca	Ach	Lt	Al	Lr	Aca	Ach	Lt	Al
Lr - Aca - Ach	17						1	10	6		
<i>Lr - Aca - Ach</i>	<i>17</i>			<i>1</i>			<i>7</i>	<i>1</i>	<i>8</i>		
Lr - Aca - Lt	7							7			
Lr - Aca - Lt	0										
Lr - Aca - Lt	8		2					6			
Lr - Aca - Lt	4						1	3			
Lr - Aca - Lt	3		2					1			
Lr - Aca - Al	7					3		2			2
Lr - Aca - Al	0										
Lr - Aca - Al	14		2			2		10			
Lr - Aca - Al	11		1			1	1	7			1
Lr - Aca - Al	2							2			
Lr - Ach - Lt	5						2	3			
Lr - Ach - Lt	1							1			
<i>Lr - Ach - Lt</i>	<i>14</i>	<i>2</i>	<i>2</i>				<i>2</i>	<i>8</i>			
Lr - Ach - Lt	3		1				1	1			
Lr - Ach - Lt	7			2			1		4		
Lr - Ach - Al	35			6		4		3	21		1
Lr - Ach - Al	13	1		3		3		3	1		2
Lr - Ach - Al	12			5		4		3			
Lr - Ach - Al	8			3		1		1	3		
Lr - Ach - Al	7			1		1			5		
Lr - Lt - Al	8		1					7			
Lr - Lt - Al	6					2		4			
Lr - Lt - Al	6		1		1	2		1			1
Lr - Lt - Al	0										
Lr - Lt - Al	12		2		1	2		7			
Aca - Ach - Lt	9		1	1				7			
Aca - Ach - Lt	1		1								
Aca - Ach - Lt	2				1			1			
Aca - Ach - Lt	2		1				1				
Aca - Ach - Lt	6		1		2			3			
Aca - Ach - Al	19		1	1		2		15			
Aca - Ach - Al	2			1					1		
Aca - Ach - Al	0										
Aca - Ach - Al	22		1	3		1		17			
Aca - Ach - Al	3					2	1				

(Table S2.1 continues on next page)

(Table S2.1 continued)

Treatment	Total	Adults					Juveniles				
		Lr	Aca	Ach	Lt	Al	Lr	Aca	Ach	Lt	Al
Aca - Lt - Al	4					1		3			
Aca - Lt - Al	3		1				1	1			
Aca - Lt - Al	19					4		15			
Aca - Lt - Al	3					1		2			
Aca - Lt - Al	21					3	7	9			2
Ach - Lt - Al	0										
Ach - Lt - Al	5			2				2			1
Ach - Lt - Al	10			1		1	1	6			1
Ach - Lt - Al	11			2				8			1
Ach - Lt - Al	1						1				
Lr - Aca - Ach - Lt - Al	3						3				
Lr - Aca - Ach - Lt - Al	1							1			
Lr - Aca - Ach - Lt - Al	1							1			
Lr - Aca - Ach - Lt - Al	6			1	1			4			
Lr - Aca - Ach - Lt - Al	9		2	1		1	4	1			
0EW0P	1						1				
0EW0P	2						1	1			
0EW0P	1							1			
0EW0P	6	2					3	1			
0EW0P	6		1				1	4			
0EW+P	1							1			
0EW+P	2							2			
0EW+P	5	1	2					2			
0EW+P	0										
0EW +P	4	1					3				

S2.2. Aboveground data

S2.2.1 P content

The P content of grass was relatively constant during the first growing season in 2018 for the non-P fertilised control (0EW0P) (2.5 ± 0.1 g P kg⁻¹) and the P fertilised control (0EW+P) (3.4 ± 0.4 g P kg⁻¹) (Fig. S2.1). This changed in 2019, when the P content increased and peaked at H13. This is most likely a consequence of N-limited grass growth, as the N/P ratio of the aboveground biomass was below 14, which denotes N limitation (Koerselman and Meuleman, 1996) (Fig. S2.2). This N-limited grass growth is probably the result of the altered fertilisation scheme (see SI S2.4). According to the N/P ratio in the third growing season, which was above 16, grass growth of the non-P fertilised control (0EW0P) was again limited by P in 2020 (Fig. S2.2)

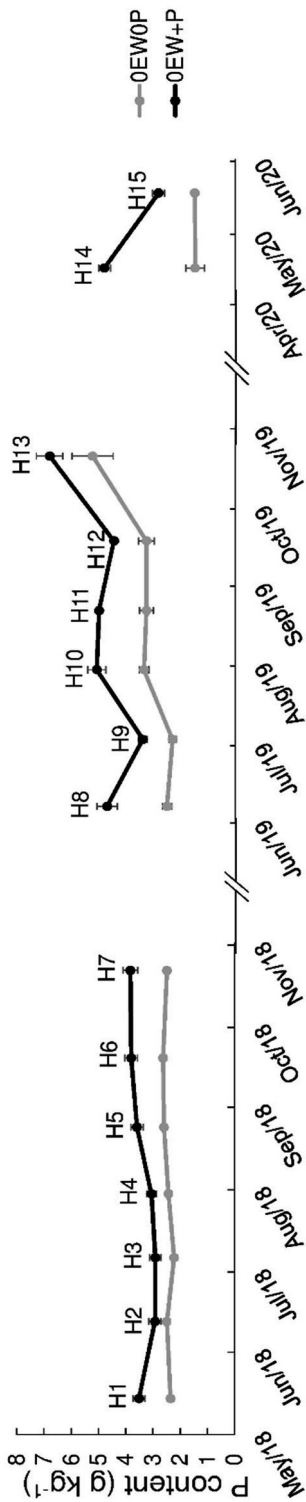


Figure S2.1 Phosphorus content of grass shoots of each harvest of the field experiment for the non-P fertilised control (0EW0P) and the P fertilised control (0EW+P). Error bars denote standard errors.

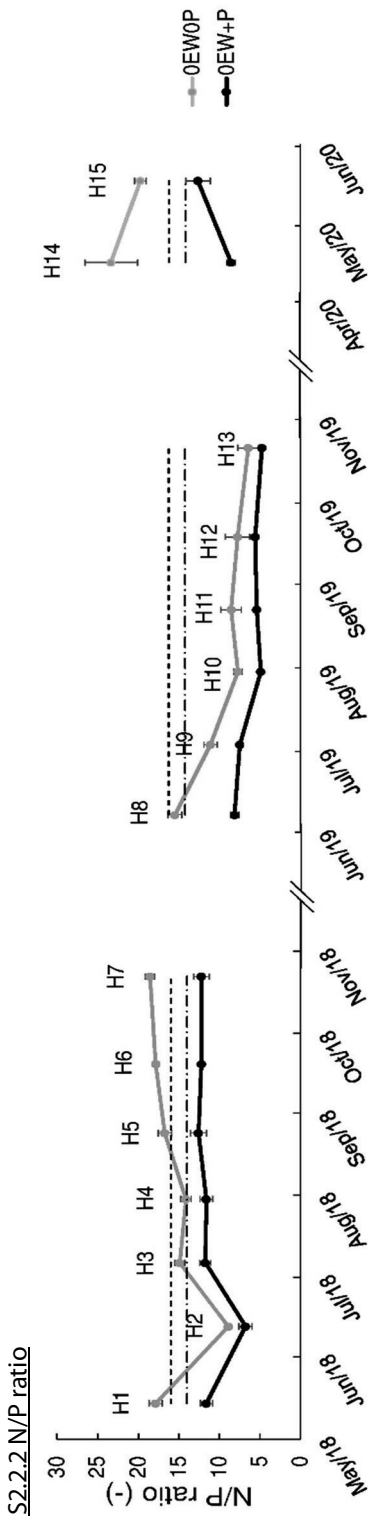


Figure S2.2 Ratio of N/P for each harvest of the field experiment for the non-P fertilised control (0EW0P) and the P fertilised control (0EW+P). Error bars denote standard errors. The dashed line indicates a N/P ratio of 16, above which grass growth is limited by P, while the dash-dotted line indicates a N/P ratio of 14, below which grass growth is N-limited (Koerselman and Meuleman, 1996).

S2.3. Belowground data

S2.3.1 pH

The pH values of the water extracts of the soil (pH~6-7; Fig. S2.3) were similar as those in another experiment with the same soil (Vos et al., 2019). The differences that were observed were mainly present between harvest times and caused by the differences in fertilisation as discussed in more detail in SI S2.4.

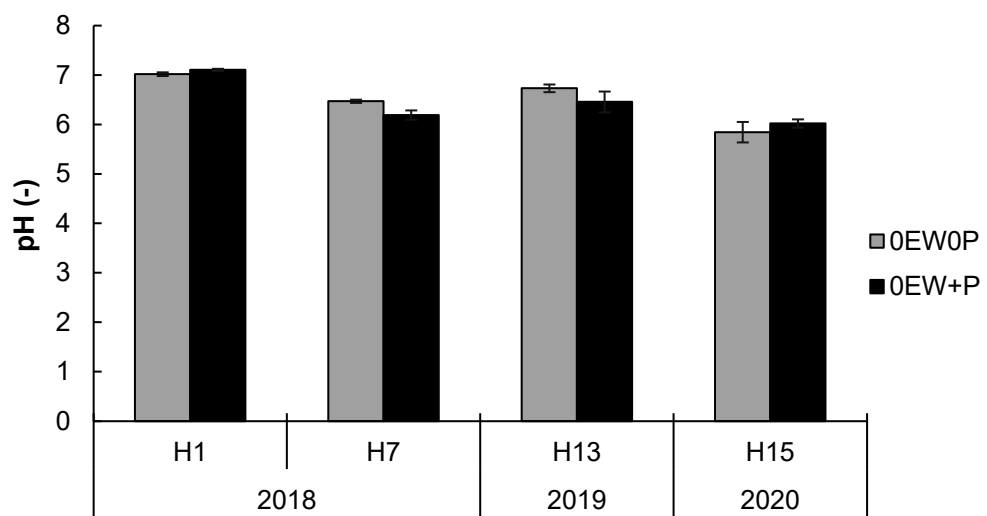


Figure S2.3 pH of the water extracts of the soil samples for the four harvests of the field experiment at which those samples were taken. Only data of the non-P fertilised control (0EW0P) and P fertilised control (0EW+P) are shown. Error bars denote standard errors.

S2.3.2 Ionic strength

The range of observed ionic strength values was in general between 0.1 and 0.6 mmol l⁻¹ (Fig. S2.4), which agreed with observations of the ionic strength in similar water extracts of the same soil (Vos et al., 2019). The only exceptions were the higher ionic strength values for the non-P fertilised control (0EW0P) and P fertilised control (0EW+P) at H15. For this specific harvest only the controls, and not any other columns, were measured, because of the limited laboratory access due to the Covid-19 pandemic. However, the observed elevated ionic strength values are probably not specific only for the controls. They may have been true for all columns, as they were probably caused by the relatively short time between the large fertiliser application at the start of the third growing season in 2020 and the soil sampling at H15.

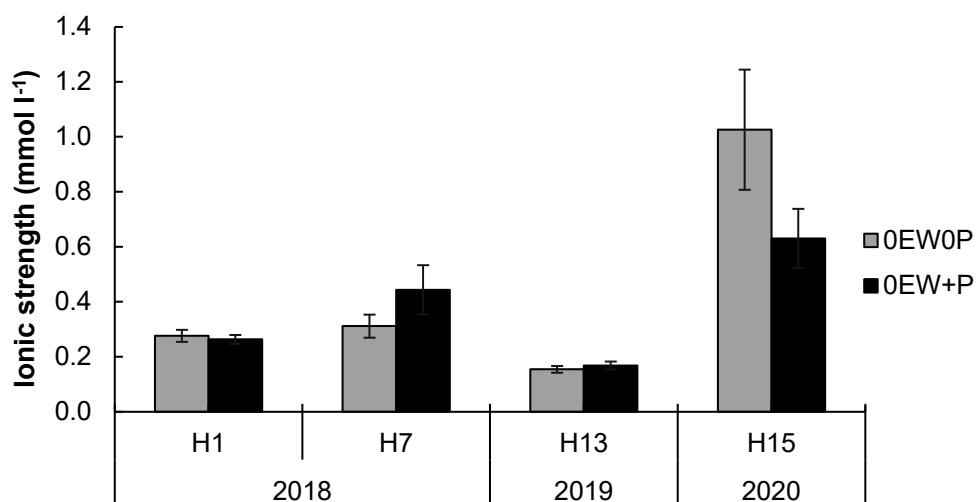


Figure S2.4 Ionic strength of the water extracts of the soil samples for the four harvests of the field experiment at which those samples were taken. Only data of the non-P fertilised control (0EW0P) and P fertilised control (0EW+P) are shown. Error bars denote standard errors.

S2.3.3 Ortho-P

Apart from the fluctuations in ortho-P concentrations over time (Fig. S2.5) as caused by the fertilisation scheme and discussed in SI S2.4, the ortho-P concentrations in the soil water extracts did not indicate any differences between all non-P fertilised treatments (data not shown). However, previous studies also reported the absence of an earthworm effect on the concentrations of readily-available ortho-P in the bulk soil (Ros et al., 2017; Vos et al., 2019; Vos et al., 2014). Therefore the significant effect of *A. longa* and *L. terrestris* on P uptake (and thereby biomass production) that was observed in our study, was probably too small to result in measurable differences in the equilibrium between soluble P in the soil water extracts and the soil solid phase.

The elevated concentration of ortho-P in the soil water extracts of the P fertilised control (0EW+P) compared to all non-P fertilised columns, is caused by P fertilisation. The strong increase in the ortho-P concentration at H13 and H15 can be explained by the application of mineral P fertiliser exceeding the removal of P by harvesting, which led to an accumulation of P in soil and as a consequence to an increase in the concentration of ortho-P in solution.

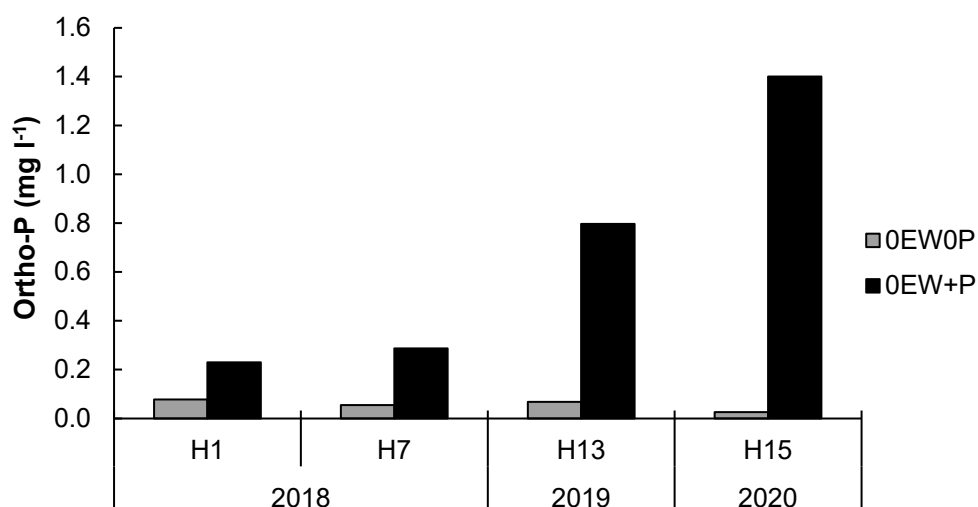


Figure S2.5 Concentration of ortho-P in the water extracts of the soil samples for the four harvests of the field experiment at which those samples were taken. Only data of the non-P fertilised control (0EW0P) and P fertilised control (0EW+P) are shown.

S2.3.4 Fraction ortho-P to TDP

Since total dissolved P (TDP) was relatively constant over time for all non-P fertilised columns (data not shown), the fraction ortho-P to TDP (Fig. S2.6) decreased when the ortho-P concentration decreased (Fig. S2.5). Like the ortho-P concentration (SI S2.4.1), the contribution of ortho-P to TDP therefore decreased with a decrease in pH ($R^2_{\text{adj}}=0.24$; $p<0.001$) and increase in ionic strength ($R^2_{\text{adj}}=0.58$; $p<0.001$). As discussed in more detail in SI S2.4.1, the ortho-P concentration is sensitive to changes in pH and ionic strength. Organic P compounds and colloidal P species, which form the difference between ortho-P and TDP, may be less sensitive to such changes in pH and ionic strength than ortho-P. This would explain the lower fluctuations over time in the concentrations of TDP than of ortho-P, and therefore the similar behaviour of ortho-P and the fraction ortho-P to TDP.

The contribution of ortho-P to TDP was relatively low in all non-P fertilised columns (~40-65%) compared to the P fertilised control (0EW+P) (~70-95%). This is characteristic for extensively managed soils where ortho-P is a less important part of TDP than in heavily fertilised soils (Dou et al., 2009; Koopmans et al., 2007; Lehmann et al., 2005). The higher contribution of ortho-P to TDP for the P fertilised control (0EW+P) is the result of the application of mineral P fertiliser and its value largely corresponds to the 68-70% observed for 1:2 (w:v) water extracts of fertilised non-calcareous sandy soils under grassland in the Netherlands (Koopmans et al., 2006).

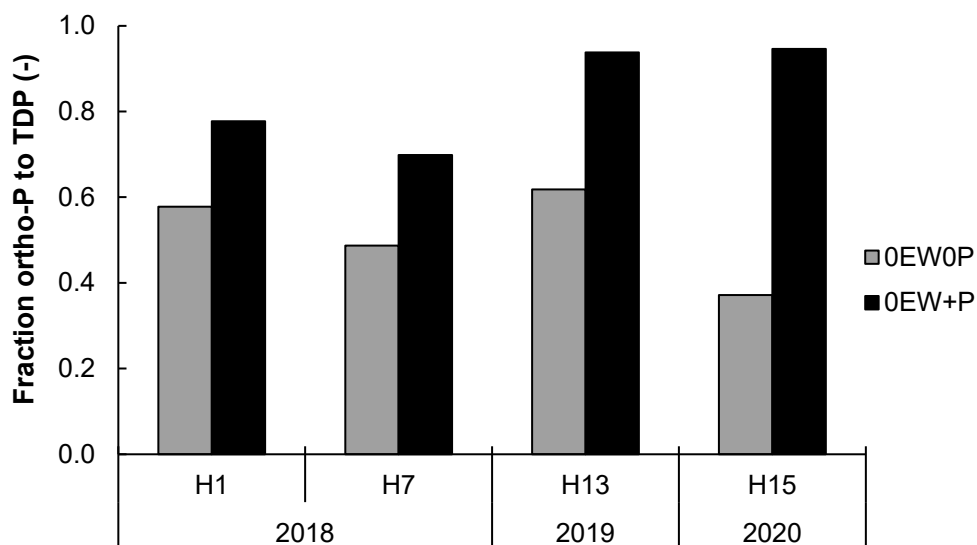


Figure S2.6 Ortho-P concentration expressed as a fraction of the TDP concentration in the water extracts of the soil samples for the four harvests of the field experiment at which those samples were taken. Only data of the non-P fertilised control (0EW0P) and P fertilised control (0EW+P) are shown.

S2.3.5 DOC

The dissolved organic carbon (DOC) concentration in the soil water extracts showed overall a decrease over the experimental period for all non-P fertilised columns (Fig. S2.7). As ortho-P, DOC is negatively charged, and therefore a higher net positive surface charge of the soil metal (hydr)oxides as a consequence of the decreasing pH (SI S2.4.1), could lead to an increase in the adsorption of DOC. Furthermore, the humic substances in DOC are sensitive to coagulation and flocculating with decreasing pH and increasing ionic strength (Oste et al., 2002; Römkens and Dolfing, 1998), which can cause a decrease in the DOC concentration in solution as well. Both mechanisms could explain an effect of the applied fertilisation scheme on the observed decrease in DOC over the first growing season in 2018. However, while pH had increased and ionic strength had decreased again by the end of the second growing season in 2019 (H13), DOC had further decreased. Therefore, the constant removal of harvested grass, resulting in limited input of fresh OM acting as a source for DOC (Kalbitz et al., 2000), in combination with the mineralisation of OM present as DOC, likely contributed to the decrease of the concentration of DOC over time. The higher concentration of DOC in the water extracts of the P fertilised control (0EW+P) compared to the non-P fertilised control (0EW0P) could point towards a larger input of OM by grass roots in the P fertilised control (0EW+P) which overall had a higher aboveground biomass production (Fig. 5.2.a and section 5.3.1).

Apart from the decreasing DOC concentrations over time, no clear differences in the DOC concentration between the earthworm treatments were present (data not shown). The absence of an earthworm effect on the concentrations of DOC in the bulk soil agrees with the results of Vos et al. (2014).

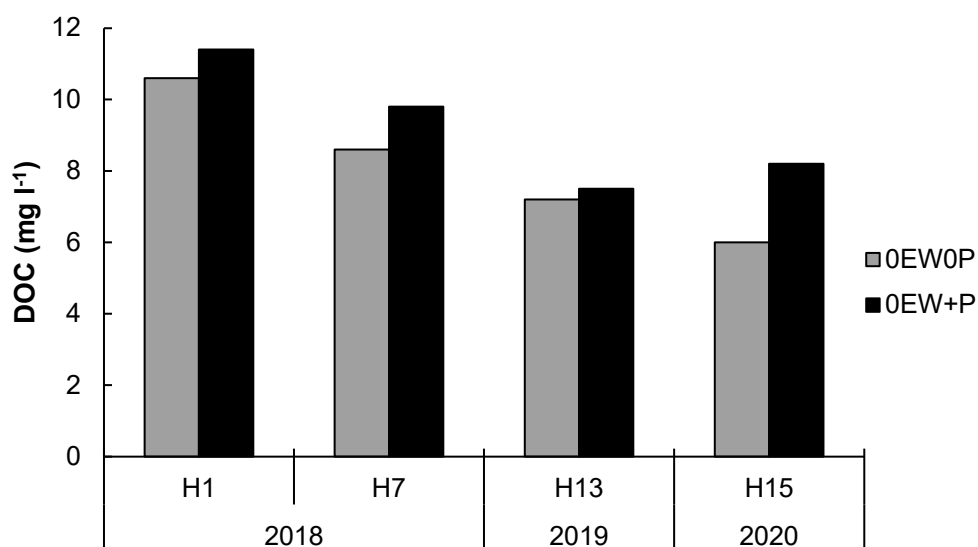


Figure S2.7 The DOC concentration in the water extracts of the soil samples for the four harvests of the field experiment at which those samples were taken. Only data of the non-P fertilised control (0EW0P) and P fertilised control (0EW+P) are shown.

S2.3.6 Aromaticity of DOC

The fraction of aromatic DOC as indicated by the light absorbance at 254 nm can be used as measure of the chemical reactivity of DOC (Weishaar et al., 2003). A lower degree of aromaticity is further associated with a more hydrophilic character of DOC and a lower molecular weight, with therefore a higher susceptibility towards mineralisation than larger humic substances (Swietlik and Sikorska, 2006). According to two of our previous greenhouse pot experiments (Vos et al., 2019; Vos et al., 2014), the fraction of DOC with a lower susceptibility towards mineralisation decreased in the earthworm casts compared to the bulk soil.

Low but significant correlations with pH (positive correlation; $R^2_{\text{adj}}=0.04$; $p=0.0011$) and ionic strength (negative correlation; $R^2_{\text{adj}}=0.24$; $p<0.001$) were present for the aromaticity of DOC, which showed the same directions as ortho-P did with these soil parameters. This is in agreement with literature, where the larger humic acid molecules are reported to adsorb more strongly to soil metal (hydr)oxides and flocculate more easily than the smaller fulvic acids which remain in solution when the pH decreases and the ionic

strength increases (Römken and Dolfing, 1998; Weng et al., 2007). An increase in adsorption and/or flocculation of DOC as suggested in section S2.3.5, would thereby mainly result in adsorption and/or flocculation of the larger and more aromatic DOC molecules, leading to a decrease in the aromaticity of DOC when pH decreases and ionic strength increases.

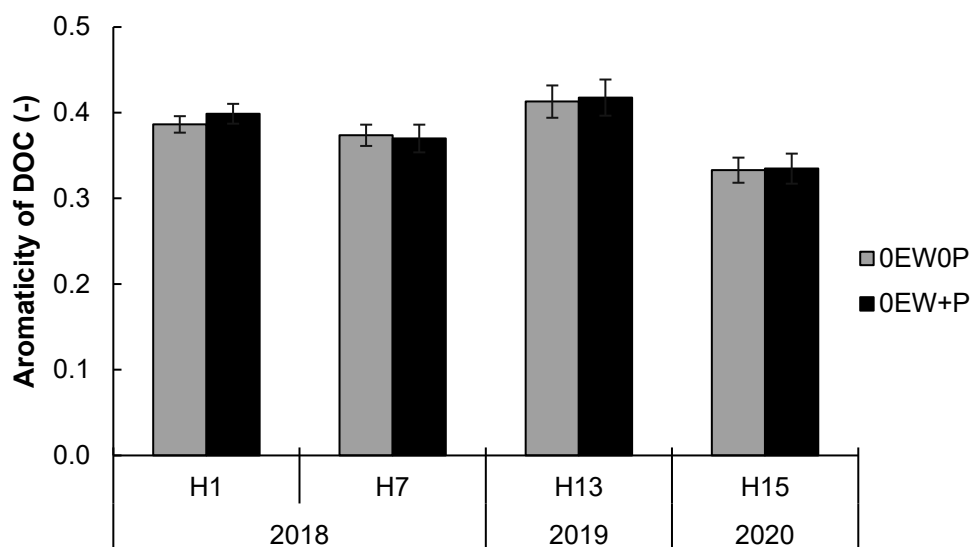


Figure S2.8 Aromaticity of DOC in the water extracts of the soil samples for the four harvests of the field experiment at which those samples were taken. Only data of the non-P fertilised control (0EW0P) and P fertilised control (0EW+P) are shown. Error bars denote standard errors.

S2.3.7 AMF

Arbuscular mycorrhizal fungi (AMF) are the soil biota probably most often associated with increasing plant P uptake under P-limiting conditions, since they effectively extend the volume of soil exploited by plants (Parniske, 2008; Richardson et al., 2009). How AMF interact with earthworms with respect to plant P availability is unclear and contradictory results have been found (Le Bayon and Milleret, 2009). On the one hand, earthworms have been reported to decrease AMF colonisation by direct grazing (Bonkowski et al., 2000) or by disruption of the AMF mycelium because of their burrowing (Tuffen et al., 2002). On the other hand, this grazing and burrowing can facilitate the dispersal of AMF spores, which may lead to increased root colonisation (Milleret et al., 2009; Zaller et al., 2013; Zarea et al., 2009). The interplay of these contradicting effects can be responsible for the observed absence of differences in the fraction of roots colonised by AMF between all non-P fertilised columns (data not shown).

Our results showed a low fraction of root colonisation by AMF at the start of the experiment (Fig. S2.9). This was expected as γ -irradiation eliminates soil life to a large extent (Hu et al., 2022; McNamara et al., 2003) and AMF colonisation had to recover after the filling of the columns. During the first growing season in 2018, AMF colonisation increased and seemed to have reached a stable level of approximately 0.7 in all non-P fertilised columns by the end of this season, as this fraction was again observed at the end of the second growing season in 2019. The overall increase of the root colonisation from 0.7 to above 0.9 at H15 might be due to the timing of sampling, as the soil samples of H7 and H13 were taken in autumn and those of H15 in summer. Colonisation by AMF is known to be especially high during the summer months when plant growth, and therefore P uptake, is at its maximum (Staddon et al., 2003).

The fraction of root colonisation of the P fertilised control (0EW+P) at H1, H7, and H13 was considerably lower than the fraction of root colonisation of the non-P fertilised control (0EW0P) (Fig. S2.9). This is in agreement with the lower dependency of plants on AMF when P is amply available (Liu et al., 2016).

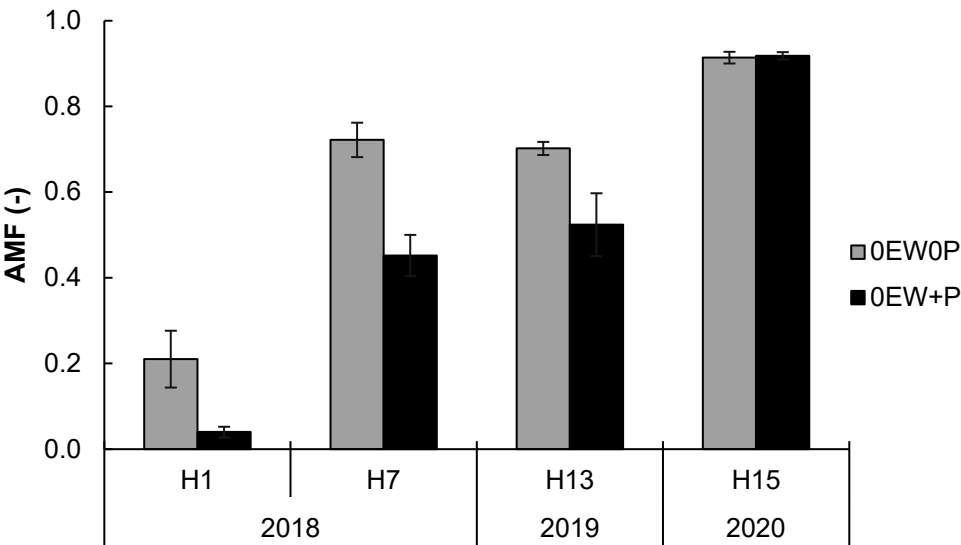


Figure S2.9 Fraction of AMF colonisation of grass roots collected at the four harvests of the field experiment at which soil samples were taken. Only data of the non-P fertilised control (0EW0P) and P fertilised control (0EW+P) are shown. Error bars denote standard errors.

S2.4. Effects of fertilisation

S2.4.1 pH and ionic strength

In 2018, the first year of this field experiment, the amount of maintenance fertiliser was based on the estimated nutrient uptake by the produced biomass, taking into account a presumed nutrient use efficiency for N and K of 50% (SI S1.2). Aboveground biomass production during this first growing season was high: on average over all non-P fertilised columns 53 g column⁻¹, which corresponds to 20 Mg ha⁻¹, compared to an average yield of approximately 12 Mg ha⁻¹ for fertilised pastures in the Netherlands (Sibma and Ennik, 1988). This large biomass production together with the assumed low nutrient use efficiency resulted in excessively high nutrient gifts in 2018. This can lead to strong increases in the salinity of the soil, as well as acidification of the soil due to preferential NH₄⁺ uptake (and subsequent proton excretion by plant roots), nitrification (production of protons which can remain in the soil in case of nitrate leaching instead of denitrification) (Bolan et al., 1991) or the desorption of protons due to the higher ionic strength as a consequence of fertilisation (Houba et al., 1990). We indeed observed both a pH decrease, as well as an ionic strength increase in the soil water extracts for H7 compared to H1 (Fig. S2.3 and S2.4).

Such changes in pH and ionic strength are undesirable, because both may increase the net positive surface charge of the Al- and Fe-(hydr)oxides in the soil (Hiemstra and Van Riemsdijk, 1996), thereby increasing ortho-P adsorption and reducing the concentration of readily-available ortho-P in the soil solution (Regelink and Koopmans, 2021; Weng et al., 2012). Indeed, a lower concentration of ortho-P in the soil water extracts was observed at H7 compared to H1 for the non-P fertilised control (OEWO_P) (Fig. S2.5). A similar decrease in the ortho-P concentration was present for all other non-P fertilised columns (data not shown). When plotting pH and ionic strength data of H1, H7, H13, and H15 against the ortho-P concentration for all non-P fertilised treatments (Fig. S2.10.a; $R^2_{\text{adj}}=0.47$, $p<0.001$ for pH and Fig. S2.10.b; $R^2_{\text{adj}}=0.56$, $p<0.001$ for ionic strength), the observed correlations confirm the direction of the effects described for pH and ionic strength on ortho-P solubility by Weng et al. (2012) and Regelink and Koopmans (2021).

Because of the importance to keep soil pH and ionic strength constant over time, we decreased the amounts of N and K for all treatments and P for the P-fertilised control (OEW+P) applied as a maintenance fertiliser for the second growing season in 2019. For this purpose, we followed the Dutch fertilisation recommendation for grassland (Commissie Bemesting Grasland en Voedergewassen, 2012) (SI S1.2). This led to a large extent to a restoration of pH and ionic strength at H13 towards their original values as measured at H1 (Fig. S2.3 and S2.4).

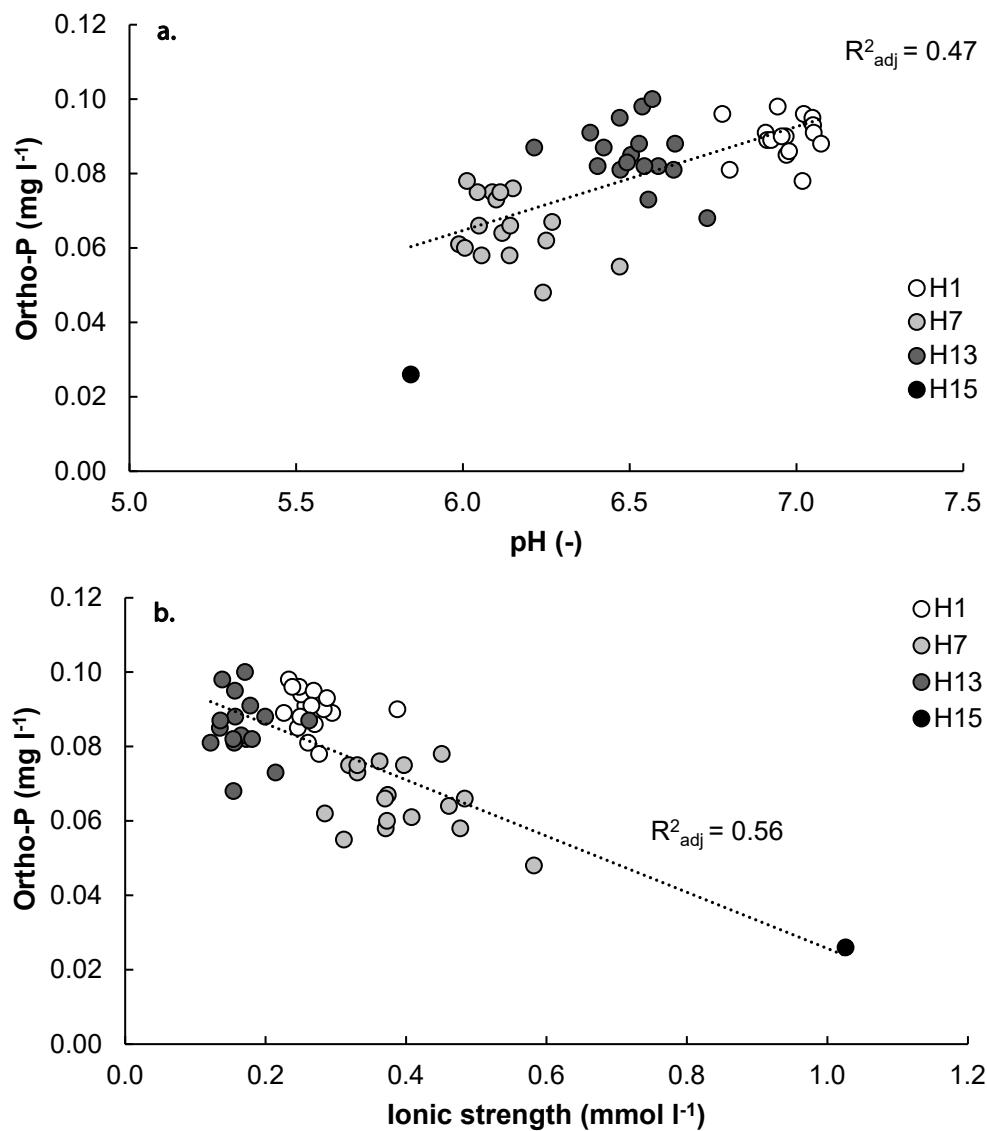


Figure S2.10 Scatter plot of pH (a) or ionic strength (b) versus the concentration of ortho-P in the water extracts of the soil samples of all non-P fertilised treatments. The dotted lines are regression lines for all harvests.

S2.4.2 Nutrient content of grass

By the end of the second growing season in 2019 (H13), when the N and P contents of the grass shoots were again determined, much higher P contents of all columns were observed than at H1 and H7 (Fig. S2.1 shows this for only the 0EW controls). The lower input of maintenance fertiliser during the second growing season compared to the first growing season had strongly decreased grass growth (Fig. 5.2a), which, in turn, had led to

less P dilution in the aboveground biomass and thereby caused a strong increase in the P content of grass. The observed values were indicative for luxury uptake of P and strongly exceeded the recommended 3 g P kg^{-1} dry grass, which is advised for healthy and high yielding dairy cows (Valk and Šebek, 1999). However, because the aboveground biomass production in the second growing season was much lower than in the first season, it led to a lower total P uptake for 2019 than for 2018 (Fig. 5.2b). Additionally, the N/P ratio calculated from the measured N and P contents of the shoots of all non-P fertilised columns harvested at H13 pointed towards N-limited grass growth (Fig. S2.2), because the N/P ratio was below 14 (Koerselman and Meuleman, 1996). Since it is not possible to determine the effect of earthworms on P uptake by grass if N is limiting grass growth, N fertilisation was again increased for the third growing season in 2020 to a level in between the fertiliser gifts of 2018 and 2019 (SI S1.2).

Due to the application of P fertiliser to the P-fertilised control (0EW+P), the N/P ratio of this control was at all times lower than the ratio of the non-P fertilised control (0EW0P) (Fig. S2.2). This demonstrates that P fertilisation had been successful. Our data of the P fertilised control (0EW+P) showed a mild (first and third growing season) or more severe (second growing season) N limitation of grass growth. The non-P fertilised control (0EW0P) showed, with the exception of H2 (N limitation), H3 and H4 (co-limitation of both N and P), P-limited grass growth in the first and third growing season, because the N/P ratio was above 16 (Koerselman and Meuleman, 1996). Also in all other non-P fertilised columns P limitation was observed at H1 and H7, although, as described for the 0EW controls, at H13 grass growth had been limited by N (data not shown). However, this had again changed to P-limited grass growth at H15. Hence, at the final stage of the experiment nutrient limitation was again as intended at the experimental setup.

S2.5. Non-*L. perenne* grasses

The fraction of non-*L. perenne* grasses at the final harvest (H15) correlated strongly with total aboveground biomass production in 2020 (H14+H15) and the P uptake at H15 for all non-P fertilised columns with a present grass cover (76 columns). Our data showed for a higher fraction of non-*L. perenne* grasses larger values for both biomass production (Fig. S2.11.a; $R^2_{\text{adj}}=0.46$, $p<0.001$) and P uptake (Fig. S2.11.b; $R^2_{\text{adj}}=0.45$, $p<0.001$). This is understandable as *F. arundinacea* was the non-targeted plant species present most in this field experiment. Although comparison of biomass production between grass species is complicated by effects of environmental conditions and species varieties, this grass species has been observed to exhibit a larger aboveground biomass production than *L. perenne* (Van Eekeren et al., 2010). In addition, our columns may have experienced drought during both warm and dry summers (SI S1.5), as the irrigation system could not always compensate sufficiently for the large evapotranspiration. This created favourable growth conditions for *F. arundinacea* because it forms deep roots, thereby making it more tolerant to drought as it can access water from deeper soil layers (Anderson, 1982).

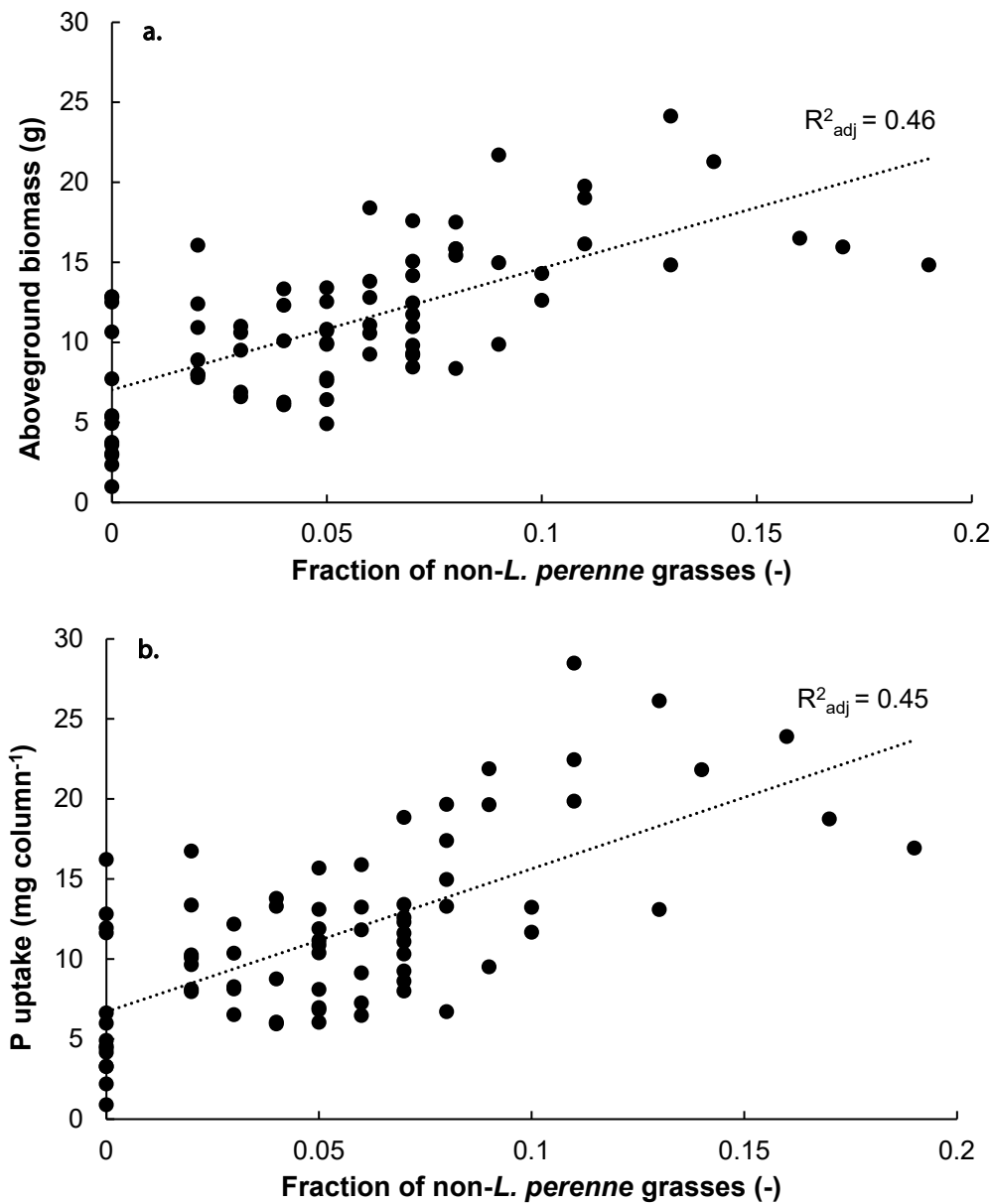


Figure S2.11 Scatter plot of the fraction of non-*L. perenne* grasses versus the cumulative aboveground biomass production of 2020 (H14+H15) (a) and versus P uptake at the final harvest (H15) (b). Dotted lines are regression lines.

S2.6. Earthworm diversity

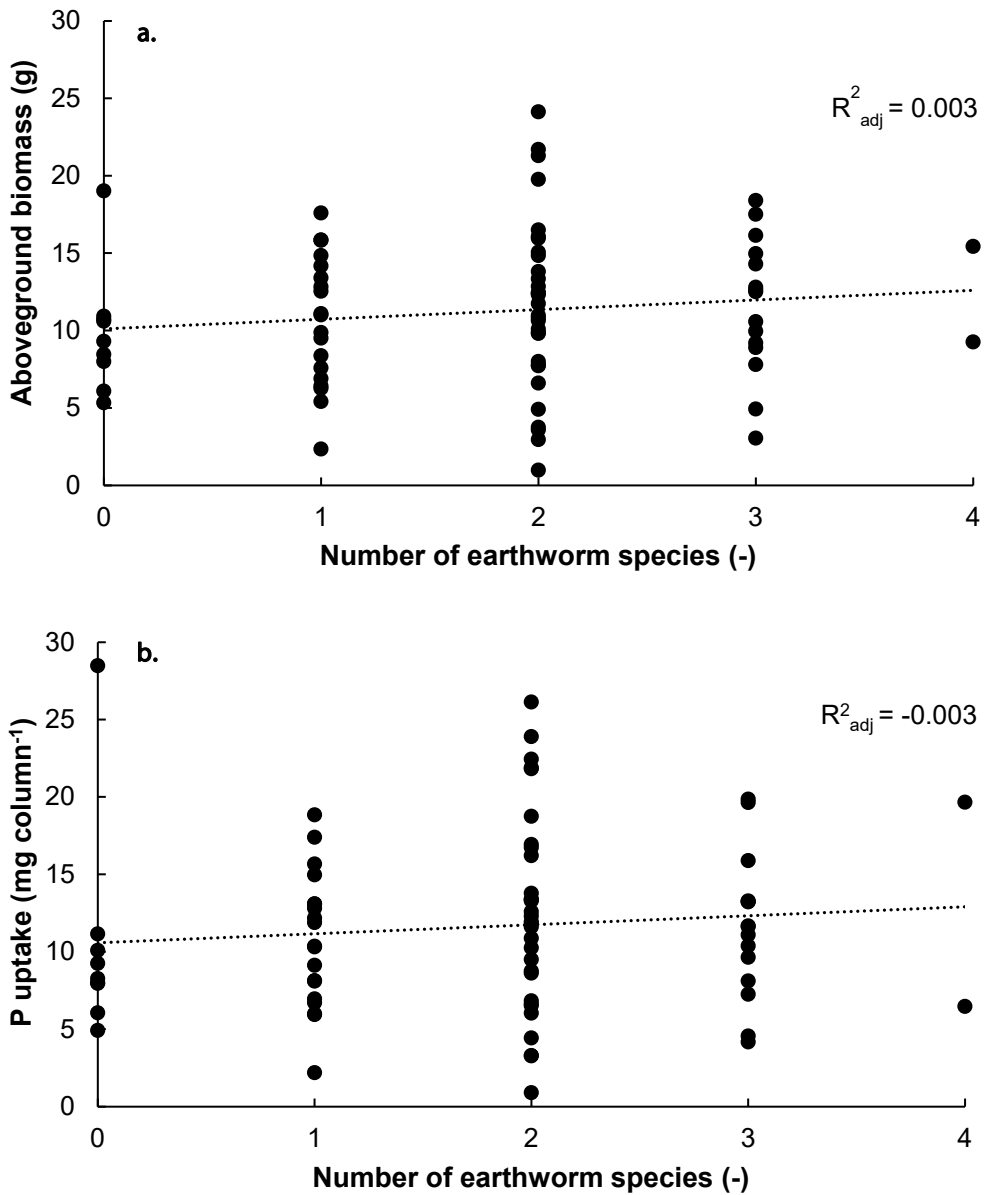


Figure S2.12 Scatter plot of the number of earthworm species observed in each column after the final harvest versus the cumulative aboveground biomass of 2020 (H14+H15) (a) and versus the P uptake at the final harvest (H15) (b). Dotted lines are regression lines.

S2.7. Initial earthworm effects

The effects of earthworms on biomass production and P uptake at the initial stage of this field experiment were assessed on similar (number and time of the year) harvests as the final earthworm effects (e.g. the first two harvests of the growing season, H1+H2, for biomass production, and H1 for P uptake). As the experimental setup had most likely not changed much shortly after the start of the experiment, both the approaches of ANOVA and testing the obtained MLR models were used:

- ANOVA of all 85 non-P fertilised columns (which include all earthworm treatments as well as the 0EW0P control) was performed as described in SI S1.6. This yielded a significantly ($p < 0.0001$) larger biomass production for the monoculture of *L. terrestris* and the three species-mixtures of Aca-Lt-Al, Lr-Aca-Lt, Ach-Lt-Al, and Lt-Ach-Lt compared to the non-P fertilised control (0EW0P). Similar analysis resulted in a significantly ($p = 0.0008$) larger P uptake for the monoculture of *L. terrestris* and the two three-species mixtures of Aca-Lt-Al and Lr-Aca-Lt compared to the non-P fertilised control (0EW0P). As all these treatments contained *L. terrestris*, this earthworm species was probably responsible for the observed effects.
- For the prediction of the grass biomass production and P uptake at the initial stage of the field experiment, the fraction of non-*L. perenne* grasses inserted in eq. 1 and eq. 2 was set at zero and the estimated body mass of different earthworm species was calculated from the number of earthworms initially added. In Fig. S2.13, predicted values are plotted against measurements (85 datapoints). The data of the initial stage of the experiment showed a large spread and a clear inconsistency with the 1:1 line that represents perfect model fit: RMSE=6.56 for biomass production and RMSE=7.59 for P uptake (the RMSE for the final data that were used to create the models and are shown in Fig. 5.5 are RSME=3.23 for biomass production and RMSE=3.99 for P uptake). Better models for the initial conditions would be obtained if the aboveground biomass and P uptake solely depended (with different coefficients) on the initially added weight of *L. terrestris* (models not shown).

Both approaches show the importance of *L. terrestris* for the initial stage of the field experiment. While this can be a genuine effect of this species on P availability in this P-limited soil, other factors may have played a role here. At the start of the field experiment, the earthworms had to settle in the columns after their addition. As the used *L. terrestris* earthworms were not accustomed to living under natural conditions, they were more probable to die shortly after introduction to the field. Subsequently, the decaying earthworm bodies could have acted as a source of additional P (on average 0.7-0.9% of dry earthworm biomass; Syers and Springett (1984)) in this soil with a low soil P status. Nutrients from earthworm tissue have been shown to become quickly taken up by plants (Whalen et al., 1999), which could have resulted in the observed initial effects of *L. terrestris* on biomass growth.

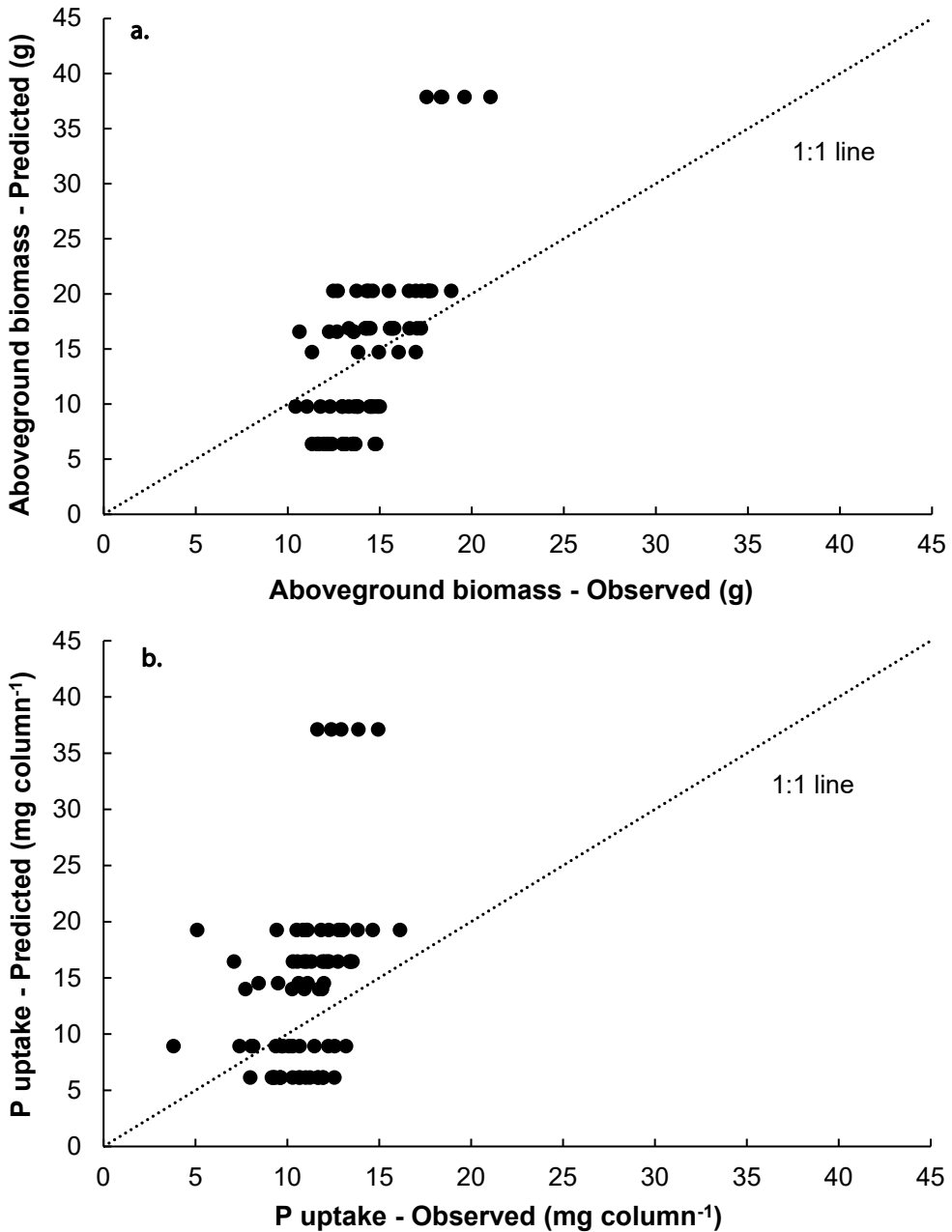
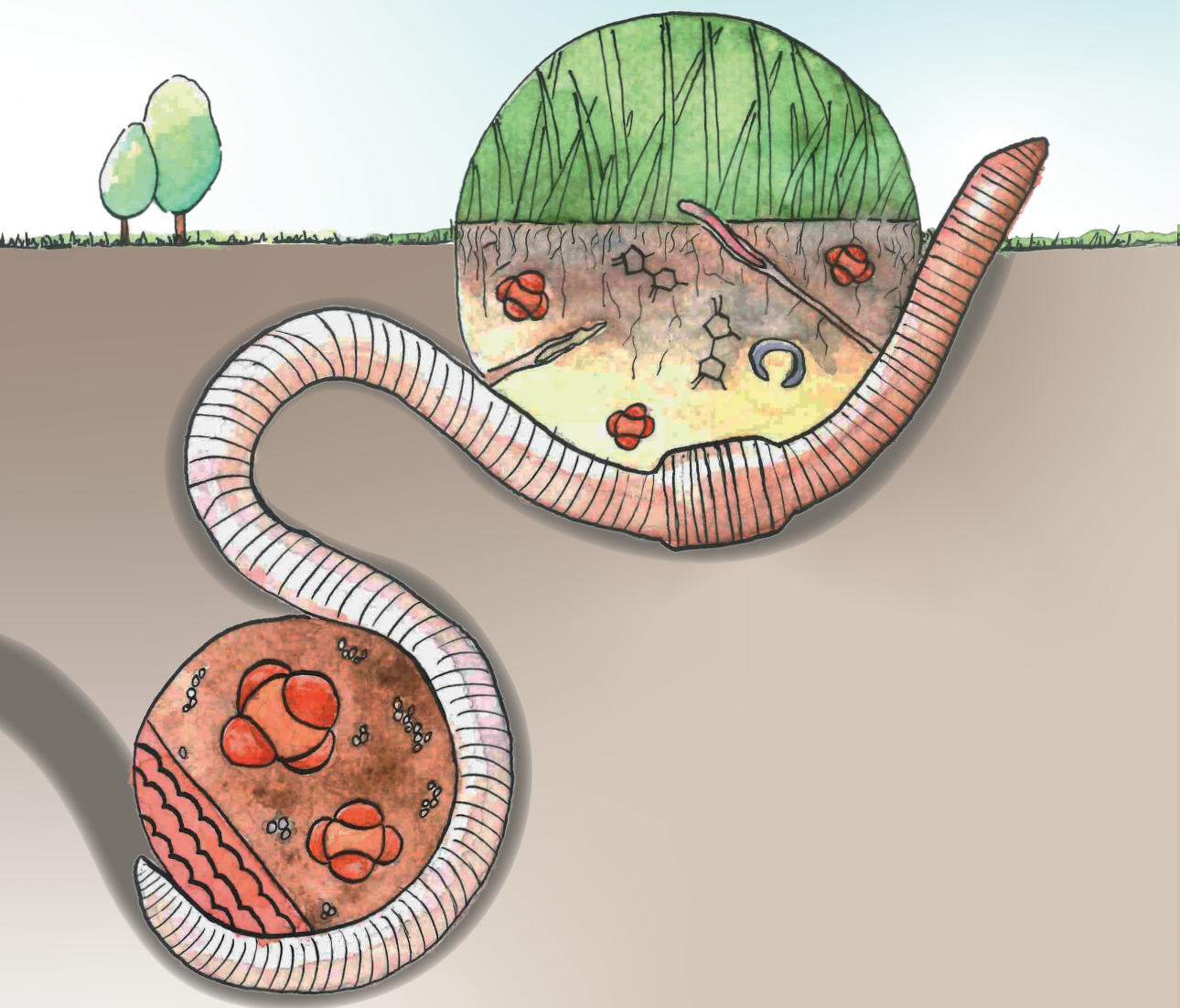


Figure S2.13 Scatter plot of the observed versus predicted results for aboveground biomass (eq.1 in the main text) (a) and P uptake (eq. 2 in the main text) (b) at the initial stage of the field experiment (H1+H2 for aboveground biomass and H1 for P uptake). Dotted lines are 1:1 lines which represent perfect model fit.

Additionally, the discrepancy in the fit of eq. 1 and eq. 2 to the data from the initial stage of the field experiment was largely caused by the monocultures of *L. terrestris* (the five highest datapoints in Fig. S2.13.a and S2.13.b). Especially for this species the model therefore did not fit the initial data. A reason for this could be incorrect assumptions for model input for the weight of *L. terrestris* if less individuals were still present in the columns than used in the model input, because some of them had died. Furthermore, the large spread can be a consequence of the early phase of the experiment in which an effect of earthworms had not established yet.

Apart from the initial and final stages of the experiment, no data on the presence of earthworms were available.





Chapter 6

General discussion

Hannah M.J. Vos

6.1 Introduction

Phosphorus (P) is essential for plant growth, but most of the P in soils is not available for plant uptake as P binds strongly to mineral soil constituents. Additionally, the use of P in agriculture is challenged by the current increase of the world population and changing human diet, resulting in a growing demand for food and P (Godfray et al., 2010; Sattari et al., 2012; Tilman et al., 2002). Furthermore, the reserves of phosphate rock as source of mineral P fertiliser are declining, while areas that have historically received excessive P additions face environmental risks that are related to chronic dissolved P losses from legacy P soils to ground- and surface waters (Buda et al., 2012; Carpenter et al., 1998; Sawyer, 1966; Schoumans and Groenendijk, 2000). These challenges associated with agricultural P use enforce us to increase productivity while using P fertiliser in a more sustainable manner (Ros et al., 2020). This requires a search for more sustainable and efficient forms of plant P nutrition. Sustainable agricultural intensification will require adequate P fertiliser inputs (Sattari et al., 2016), overcoming global P imbalances (MacDonald et al., 2011; Steffen et al., 2015) and finding ways to improve the utilisation of P already present in soil (Rowe et al., 2016; Sattari et al., 2012). This last strategy requires finding new ways to access soil P pools that are less available to plants. Exploring the role of earthworms in sustaining optimal P acquisition for plants can therefore contribute to solving the P challenges we are currently facing. In this thesis, I explored this **role of earthworms for improving soil P utilisation**. Thereby my research primarily focussed on the effect of earthworms in grassland systems in the Netherlands without additional P fertilisation on P uptake and grass yield to elucidate the earthworm-related mechanisms that contribute to a better utilisation of existing soil P pools.

This thesis contributes to a better understanding of the effect of earthworms on plant-available P for various earthworm species and across different soils. In this final chapter, I will summarise my main findings and discuss these in the context of my research objectives (section 6.2). Subsequently, I will discuss some considerations regarding the experimental approach that I have chosen in my research (section 6.3), place my results in a broader perspective and discuss the implications of my findings for practice (section 6.4). Finally, I propose future research opportunities based on my research (section 6.5) and give some final concluding remarks (section 6.6).

6.2 Results of this research

6.2.1 Synthesis of the main findings

As stated in Chapter 1, this research considered the contribution of the effect of earthworms to a more sustainable P nutrition of grass. Thereby the main objective was **to elucidate the effect of earthworms on plant-available P in soil and P uptake by grass, and to explore its potential to increase the sustainability of P nutrition of grass**. The

primary goal of this research was addressed by specific research objectives that are dealt with in the different research chapters. These specific objectives were:

1. To assess the effect of different earthworm species on plant-available P and relate this to a) physico-chemical soil properties and b) earthworm ecology.
2. To determine the controlling mechanisms of earthworm-enhanced P-availability and quantify the relative importance of all contributing mechanisms.
3. To assess the effect of earthworm diversity on plant-available P.
4. To evaluate if the effect of earthworms on plant-available P results in an increased grass P uptake and biomass production under realistic field conditions.

These research objectives form the basis of the discussion of the main findings of this research.

The **variation among earthworm species** was assessed in Chapter 2 in a greenhouse pot experiment on a non-calcareous sandy soil with a low P-status. Earthworm-enhanced P-availability varied considerably among eight earthworm species that are common in the Netherlands. All measured P pools (water-extractable ortho-P, P-Olsen and P_{ox}) were increased in casts compared to the bulk soil, and water-extractable ortho-P was up to two orders of magnitude larger in casts. While the variation among species was not significant for P_{ox} and small (but significant) for P-Olsen, it varied strongly for water-extractable ortho-P. This showed that while all investigated species increased the total pool of reversibly adsorbed P measured by P_{ox} , a large variation exists in the ability of earthworm species to alter the extent to which P is readily-available to plants. Furthermore, all species showed a large pH increase in water extracts of casts compared to bulk soil (up to 1.6 pH units) and strongly elevated dissolved organic carbon (DOC) concentrations (up to 15 times). This variation was not related to classical **earthworm ecological classification** in earthworm functional groups, and species from the same functional group showed large variation in **cast properties** like pH and DOC as measured in water extracts. In turn, this variation could be linked to the existing variation in water-extractable ortho-P as proxy for readily plant-available P. There was a clear positive correlation between water-extractable ortho-P and DOC, emphasizing the importance of the link between both variables. In addition, DOC, P_{ox} , total carbon (C) content as well as dissolved inorganic C were important predictors for the concentration of water-extractable ortho-P in a multiple linear regression model. It was therefore concluded that multiple pathways contributed to earthworm-enhanced P-availability.

In Chapter 3, these pathways were quantified further, and their relative importance was assessed. The probe-ion method of Hiemstra et al. (2010a) in combination with surface complexation modelling was used to determine the **controlling mechanisms** of earthworm-enhanced P-availability by assessing the mechanisms that affect water-extractable ortho-P. This revealed a mechanism not previously considered: the decrease

of the reactive surface area (RSA) of metal-(hydr)oxides in casts by particle growth, resulting in a decrease of the surface area that is available for P adsorption. We concluded that the contribution of the controlling mechanisms of earthworm-enhanced P-availability in earthworm casts consists of (Fig. 6.1): a) a relatively small effect of the increase of pH in casts; b) a major contribution of P addition through stimulated mineralisation inside the earthworm gut; c) a potentially major effect of a decrease in the RSA of casts by particle growth which is catalysed by the formation of Fe^{2+} during the reducing conditions caused by mineralisation; and d) a decrease in the competition between natural organic matter (NOM) and P for binding sites on metal-(hydr)oxides in casts compared to the bulk soil. The decrease of the RSA was only observed for Fe-(hydr)oxide-dominated soils, whereas it was absent or minor in Al-(hydr)oxide-dominated soils. This demonstrates that soil mineralogy influences earthworm-enhanced P-availability and suggests that earthworms have the largest potential to improve the sustainability of P use in Fe-(hydr)oxide-dominated soils.

All controlling mechanisms of earthworm-enhanced P-availability are directly (mechanism b) or indirectly related to the capacity of an earthworm to mineralise the organic material it ingests. Therefore, the pathway of **mineralisation of organic P** (controlling process B in Figures 1.2 and 6.2) was investigated in more detail in Chapter 4. This showed that the potential activity of phosphatase enzymes that catalyse the mineralisation process of monoester P compounds was considerably increased by earthworms in the order casts>burrows>bulk soil. Phosphatase activity varied among earthworm species, but this variation was not related to earthworm functional groups. Additionally, phosphatase activity in casts correlated with the P content of grass shoots, suggesting a direct earthworm-induced effect on grass P uptake through mineralisation of organic P.

After establishing the influence of individual earthworm species on P-availability in controlled conditions, Chapter 5 considered the effect of **earthworm diversity** on grass biomass production and P uptake in a mesocosm field experiment. Here a non-calcareous sandy soil with a low P-status was used. The changes in the composition of earthworm communities over time made it impossible to investigate the effect of species interactions in communities (section 6.2.2). An effect on grass biomass production or P uptake of increasing the number of earthworm species in the established community at the end of the experiment was not observed. However, using a multiple linear regression model on established communities, *A. longa* and *L. terrestris* were identified as keystone species to increase grass P uptake and biomass production. This experiment encompassing over two years, showed that studying imposed earthworm populations under field conditions is highly challenging. Nonetheless, it demonstrates that the effect of earthworms on P uptake was not only present under controlled greenhouse conditions but can also be observed at the larger scale of more **realistic field conditions**.

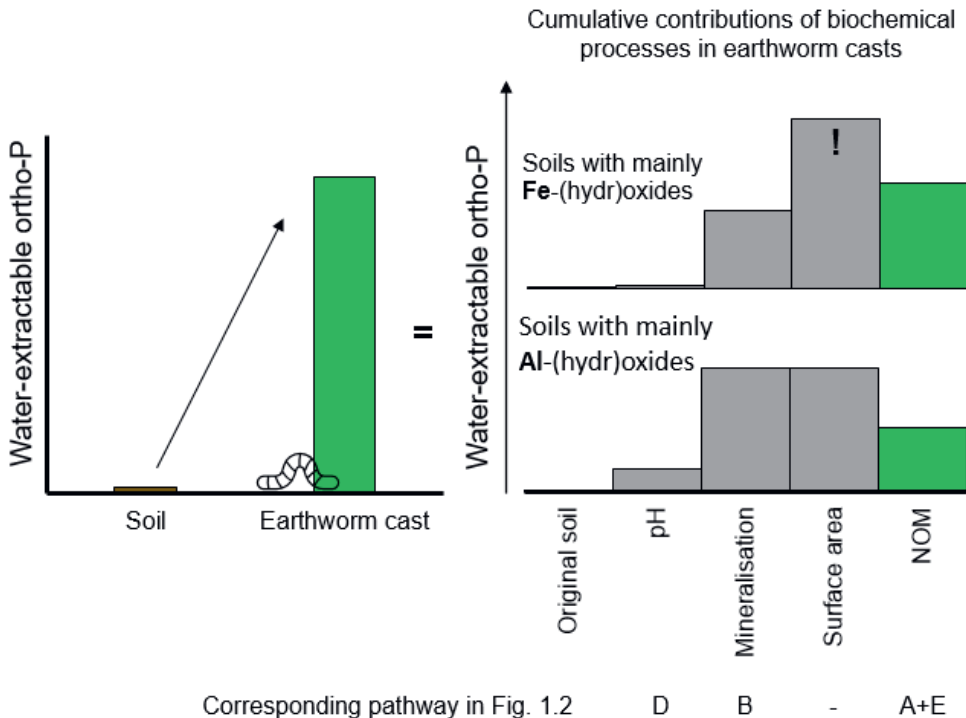


Figure 6.1 A semi-quantitative impression of the effect of earthworms on water-extractable ortho-P in bulk soil and cast as proxy for plant-available P (left graph). This effect of earthworms consists of multiple controlling mechanisms of which the cumulative contribution of each is shown in the graphs on the right (i.e., the bar above e.g. 'Surface area' includes the previously named effects of pH and Mineralisation, and the green bar above 'NOM' is the net effect). Starting point for the two graphs is the condition of the controlling mechanisms of earthworm-enhanced P-availability in the bulk soil (original soil). The controlling mechanisms indicated on the x-axis are: change in pH (pH), additional P input from increased mineralisation of organic matter (Mineralisation), a decrease in RSA increasing water-extractable ortho-P as there is less surface area available for P adsorption (RSA), and a decrease in competition for adsorption sites between water-extractable ortho-P and NOM (NOM) as the NOM loading becomes lower in earthworm casts. To correspond with the pathways described in Fig. 1.2 of Chapter 1, the letters used there to describe the initially proposed pathways of earthworm-enhanced P-availability are given below the graphs. The contribution of these controlling mechanisms varies depending on the composition of the metal-(hydr)oxide fraction of the soil as indicated by the difference between the upper and lower right graph for two contrasting soils.

Based on the findings in my thesis, an updated version can be made of Fig. 1.2 in Chapter 1. Figure 6.2 shows the controlling mechanisms of earthworm-enhanced P-availability in a soil where the fraction of metal-(hydr)oxides is dominated by Fe-(hydr)oxides.

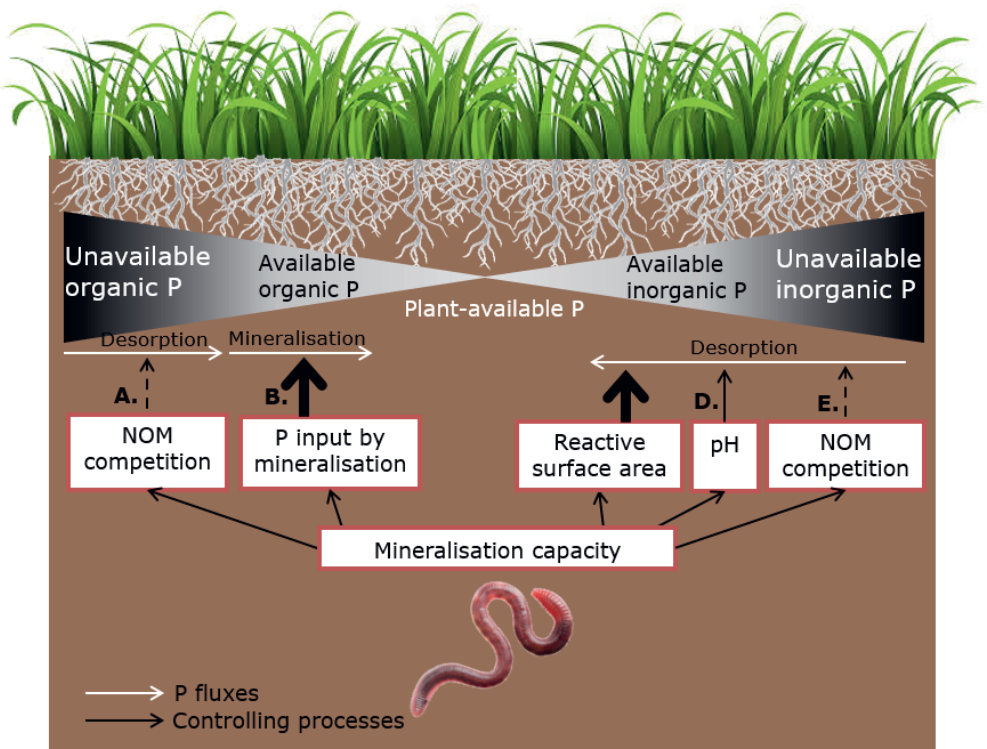


Figure 6.2 Controlling mechanisms of earthworm-enhanced P-availability in a soil where the fraction of metal-(hydr)oxides is dominated by Fe-(hydr)oxides. The letters of the controlling processes correspond to the processes described in Fig. 1.2, and the thickness of the black arrows demonstrates the contribution of each controlling mechanism to earthworm-enhanced P-availability: thick arrows from ‘P input by mineralisation’ and ‘Reactive surface area’ indicate major contributions, the thin arrow from ‘pH’ demonstrates a minor contribution, and the dashed arrows from ‘NOM competition’ indicate a negative effect on plant-available P.

6.2.2 The effect of earthworm diversity

The **third research objective** aimed at addressing the effect of earthworm diversity on plant-available P. Literature has demonstrated a positive effect of earthworm diversity on grass biomass production (Schon et al., 2017), and an effect on P uptake by grass might be expected as well. As a large variation among earthworm species in cast properties that influence P solubility exists (Chapter 2), and some species affect a controlling mechanism

of earthworm-enhanced P-availability in absolute terms more than others (Chapter 3), species interactions may be expected in a community if the included species stimulate different controlling mechanisms. This may further affect the level of plant-available P in soil.

In Chapter 5 we considered the possible effects of interactions among earthworm species in communities, but we did not observe any effect on biomass production and P uptake of the number of species in a community (Chapter 5. SI Fig. S2.12). The changes in earthworm communities over time made it impossible to investigate this in detail. Therefore, we recommended to study the effect of earthworm diversity **under controlled conditions**. This is what we did in the greenhouse pot experiment eventually published as Chapter 4. Although not reported in Chapter 4, the experiment included two- and three-species combinations besides the monocultures and five-species mixture. However, no effect of earthworm diversity on grass biomass production and P uptake was detected in that experiment either (SI Fig. S1).

The extended results of the experiment in Chapter 4 and those of the mesocosm field experiment of Chapter 5 both indicate that an effect of species diversity on grass P uptake or biomass production is absent. However, both experiments **do not enable us to address our research objective on the effect of earthworm diversity as well as we intended**. A first reason for this is that it was practically impossible to create and control specific earthworm communities in a field experiment in which all individuals of all introduced species survived. For short-term greenhouse pot experiments like the study presented in Chapter 4, this was still largely possible (i.e., earthworm survival was 78% after 84 days), but under more realistic conditions in long(er)-term field experiments this is extremely challenging. Secondly, the effect of earthworms on plant-available P exists clearly in casts, but it is difficult to link this effect to (an increase in) grass growth. This is primarily caused by the difficulty to separate the effect of earthworms on readily plant-available P by added cast fertility, from the many other beneficial effects that earthworms can have on plant growth, for instance via an improved soil structure because of earthworm activity (see also 6.4.2) (Edwards and Lofty, 1980). As the effect of earthworms on plant growth via increasing plant P-availability is therefore difficult to demonstrate for individual earthworm species, this is even more so for the interactions between various species.

6.2.3 *The effect of selective feeding*

In the initial phase of this research project, much emphasis was put on the pathway of selective feeding of earthworms on small oxide particles resulting in an increased intake of P (pathway C in Fig. 1.2). This pathway was replaced in Fig. 6.2 by the pathway of a decrease in the RSA of casts by particle growth, which was initially unknown, but resulted from the measurements and modelling exercises reported in Chapter 3. That study indicated that by including the important contribution of a decrease in the RSA, the

elevated concentrations of water-extractable ortho-P in casts could be explained without the contribution of selective feeding by earthworms. The underlying assumption was that all additional P measured as $\text{PO}_4\text{-ox}$ that was present in earthworm casts, compared to the bulk soil, was attributed to the release of P by stimulated mineralisation inside the earthworm gut. This assumption was underlined by the strong correlation observed between various dissolved constituents of organic matter (DOC, dissolved organic N, sulphur, and dissolved organic P). The omission of the pathway of selective feeding in my later results does, however, not mean that selective feeding by earthworms could not, in part, be responsible for the observed increase in $\text{PO}_4\text{-ox}$ (and P_{ox} measured in Chapter 2). Literature shows that earthworms actively select the organic C in their food (Curry and Schmidt, 2007; Doube et al., 1997; Lee, 1985; Sharpley and Syers, 1976). This can either be an external source of organic matter in the form of grass (roots) and organic residues, or a soil-related source of organic C in a matrix of organic material with embedded metal-(hydr)oxide nano-particles that are smaller than the mean oxide particle (Chapter 2). However, this research **cannot distinguish the different mechanisms** (e.g., selective feeding and stimulated mineralisation of organic P) contributing to the increase in $\text{PO}_4\text{-ox}$ and P_{ox} . Therefore, I did not include selective feeding as a separate pathway in Fig. 6.2, but, if present, its effect is included in the pathway of 'P input by mineralisation' (pathway B in Fig. 6.2).

6.3 Consequences of the chosen experimental approach

At the start of this research, several important decisions were made that largely affected my results. In retrospect, I would like to discuss the consequences of the chosen experimental approach.

6.3.1 The choice of soil

Since my research focussed on Dutch grassland soils, a set of representative soils **typical for grasslands in the Netherlands** that covered a broad range in relevant soil properties was selected. I considered soil texture, pH, soil organic matter content and the amount of non-crystalline metal-(hydr)oxides when selecting soils. Soil texture was considered because not all soil types are equally important for grassland (Table 1.1). Furthermore, these soil properties were included as they influence the solubility of P. Any effect of earthworms on plant-available P and P uptake by grass can best be detected on soils with a low P-status and P-deficient growth conditions (Ros et al., 2017). Therefore, I additionally considered soil P-status as selection criterion. This strongly limited the choices for selecting soils: as discussed in Chapter 1, the P loading of most agricultural soils in the Netherlands is relatively high, and almost half of the Dutch agricultural soils are considered P-saturated when a soil type specific critical threshold is used to calculate the degree of P saturation (Schoumans and Chardon, 2015). From this perspective, it was

challenging to identify soils with a low P-status. The set of four selected soils consists of three non-calcareous sandy soils and a calcareous clay, as these are important soil types for grasslands (Table 1.1) and they are known to sustain large earthworm populations (Van Eekeren et al., 2014). Details on the selected soils are given in Chapter 3, Table 3.1.

One of the soils is used in all experiments, including the three **experiments with only one soil type** (Chapters 2, 4 and 5). This specific soil is a non-calcareous sandy soil with a low P-status in which the fraction of metal-(hydr)oxides is dominated by Al-(hydr)oxides. When selecting soils, I was not yet aware of the importance of the type of metal-(hydr)oxide (e.g., dominantly Fe- or Al-(hydr)oxides), which resulted from my findings in Chapter 3. In retrospect, this raises the question of the relevancy of using a non-calcareous sandy soil in which the fraction of metal-(hydr)oxides is dominated by Al-(hydr)oxides as the main soil for studying earthworm-enhanced P-availability in my experimental work. This question is two-fold as it: a) relates to the relevance of Al-(hydr)oxide-dominated sandy soils for grasslands in the Netherlands and b) to the potential of earthworms for increasing plant-available P on this soil type. Both aspects will be discussed separately.

How common are grasslands in the Netherlands on non-calcareous sandy soils dominated by Al-(hydr)oxides?

The contribution of Fe- and Al-(hydr)oxides in soils used for grasslands in the Netherlands can be estimated from national datasets of soil characteristics. One of such datasets consists of range of 19 agricultural topsoils from the Netherlands selected by Hiemstra et al. (2010a). Although land use was not reported in this study, the dataset consisted for 95% of soils where the metal-(hydr)oxide fraction was dominated by Fe-(hydr)oxides. Another dataset is reported by Koopmans et al. (2006), and comprises 78 agricultural (arable and grassland) soils from all major soil types in the Netherlands (Table 6.1). Overall, 76% of the investigated soils were dominated by Fe-(hydr)oxides and 24% by Al-(hydr)oxides. These fractions were comparable when the 31 grasslands were considered (81% dominated by Fe-(hydr)oxides and 19% by Al-(hydr)oxides). All Al-(hydr)oxide-dominated soils were non-calcareous sands. When grasslands on non-calcareous sandy soils were considered specifically, 11 soils were present in which for 6 of these soils the fraction of metal-(hydr)oxides consisted predominantly of Al-(hydr)oxides. A third dataset of 74 organic matter-rich (3-15%) non-calcareous sandy soils from the Netherlands (Schoumans et al., 1991) consists for 36% of Fe-(hydr)oxide-dominated soils and for 64% of Al-(hydr)oxide-dominated soils. While this dataset mainly comprises arable land, 11 of the 16 investigated grasslands were dominated by Al-(hydr)oxides. Hence, the soil most frequently used in my experimental work is **relevant** for grasslands on non-calcareous sands in the Netherlands.

Table 6.1 Number of soils in the study of Koopmans et al. (2006) on the major soil types in the Netherlands. The data are separated by dominant metal-(hydr)oxide and agricultural land use, and the values between brackets indicate the range of the percentage of the dominating metal-(hydr)oxide. A metal -(hydr)oxide was considered as dominant when it contributed more than 50% of the total sum of metal-(hydr)oxides, expressed in moles per unit soil mass. Soil samples from grassland were taken from the 0-5 cm soil layer, whereas those from arable land were taken from the plough layer.

Soil type	Fe-(hydr)oxides		Al-(hydr)oxides	
	Grass	Arable	Grass	Arable
Non-calcareous sand	5 (53-87%)	0	6 (50-86%)	13 (56-87%)
Calcareous clay	3 (76-80%)	17 (77-83%)	0	0
Non-calcareous clay	5 (77-88%)	0	0	0
Peat	7 (53-66%)	0	0	0
Calcareous sand	5 (73-87%)	17 (51-78%)	0	0

What is the potential of earthworm-enhanced P-availability in non-calcareous sandy soils dominated by Al-(hydr)oxides?

Chapter 3 showed that one of the important pathways contributing to earthworm-enhanced P-availability, a decrease of the RSA of casts, is only present in soils in which the metal-(hydr)oxide fraction is dominated by Fe-(hydr)oxides. It is not, or only to a limited extent, present in soils which are dominated by Al-(hydr)oxides. This would suggest that the important contribution of a decrease of the RSA to P solubility played less of a role in the Al-(hydr)oxide-dominated non-calcareous sandy soil used in the studies outlined Chapters 2, 4 and 5. Table 6.2 shows the concentration of water-extractable ortho-P in casts for various studies that used the same methods of cast collection and preparation of water extracts and subsequent ortho-P measurement. The data do not indicate that the concentration of water-extractable ortho-P measured in Fe-(hydr)oxide-dominated soils is higher compared to Al-(hydr)oxide-dominated soils in absolute terms. However, as different soils are used, comparison of the increase of water-extractable ortho-P in casts compared to the bulk soil (values between brackets in Table 6.2) is a better indicator for the potential of a soil for earthworm-enhanced P-availability. This shows that for the two Fe-(hydr)oxide-dominated soils the increase in water-extractable ortho-P was much higher than in the Al-(hydr)oxide-dominated soil. This supports the suggestion made in Chapter 3 that the largest potential for earthworm-enhanced P-availability lays in Fe-(hydr)oxide-dominated soils. Moreover, it suggests that the choice for a soil in which the fraction of metal-(hydr)oxides is dominated by Al-(hydr)oxides as main soil used in the experimental work for this thesis may have resulted in a **smaller effect than can be expected for many other soils** in the Netherlands. This also implies that the effects of

earthworms on plant-available P observed for this Al-(hydr)oxide-dominated soil, are likely larger in Fe-(hydr)oxide-dominated soils.

Table 6.2: Concentrations of water-extractable ortho-P in the casts of *L. rubellus* and *L. terrestris* according to various studies on non-calcareous sandy soils with a low P-status (total of three soils; grey-scaled colours indicate when the same soil was used). Values between brackets represent the factor by which water-extractable ortho-P in earthworm casts was increased relative to the bulk soil.

Study	Dominating metal-(hydr)oxide	Water-extractable ortho-P (mg L ⁻¹)	
		<i>L. rubellus</i>	<i>L. terrestris</i>
Vos et al. (2014)	Fe-(hydr)oxide	8.6 (>428) [†]	3.6 (>180) [†]
Ros et al. (2017)	Fe-(hydr)oxide	-	9.4 (472)
Chapter 3 S2	Fe-(hydr)oxide	8.8 (>439) [†]	3.1 (>156) [†]
Chapter 2	Al-(hydr)oxide	6.2 (105)	3.4 (57)
Chapter 3 S3	Al-(hydr)oxide	10.8 (106)	8.0 (79)
Chapter 4	Al-(hydr)oxide	11.2 (112)	5.9 (68)

[†] The >sign indicates that the concentration of ortho-P measured in the water-extracts of the bulk soil was below the detection limit. Calculations are in that case based on the detection limit of the segmented flow analyser.

6.3.2 The choice for a proxy for plant-available phosphorus

This research primarily focussed on the use of **water-extractable ortho-P** as a proxy for plant-available P. While this P intensity method is used regularly in literature to estimate plant-available P in the soil solution (Koopmans et al., 2006; Sonneveld et al., 1990; Torrent and Delgado, 2001), an extraction with 0.01 M CaCl₂ is more common. The latter method mimics soil solution conditions more closely because: a) it has an ionic strength close to that of the soil solution in many soils (i.e., 0.03 M); and b) it uses calcium (Ca) as cation, which is the primary cation on the soil adsorption complex (Houba et al., 2000). However, this extraction method can result in ortho-P concentrations which are below the detection limit of a common laboratory measurement apparatus like the segmented flow analyser (SFA) if the P loading of a soil is low. This is because of the presence of Ca in solution, which facilitates ortho-P adsorption to metal-(hydr)oxides (Mendez and Hiemstra, 2020; Rietra et al., 2001; Weng et al., 2012). Furthermore, the relatively high ionic strength of the 0.01 M CaCl₂ solution causes an increase in the net positive surface charge of the metal-(hydr)oxides in soil (Hiemstra and Van Riemsdijk, 1996), thereby increasing the ortho-P adsorption. Hence, both processes decrease the ortho-P concentration in solution (Regelink and Koopmans, 2021; Weng et al., 2012). For soils with a low P loading, a different extractant than 0.01 M CaCl₂ should therefore be used to prevent detection limit problems and allow for the comparison of treatments. As this was the case for one of the non-calcareous sandy soils used in this research, an extraction with water at a solution-to-

soil ratio of 10 L kg⁻¹ was used throughout this research to approximate plant-available P. However, as ortho-P in a 0.01 M CaCl₂ extract is an established agronomic P test and is used as a P intensity method in combination with P-AL as a P quantity method for P fertiliser recommendations in the Netherlands, I recommend using a 0.01 M CaCl₂ extraction as proxy for plant-available P when possible in future research. As this might result in detection limit problems for some soil samples, the standard SFA setup for ortho-P measurement could be equipped with a 50-cm liquid waveguide capillary cell and a LED lamp operating at 880 nm to largely improve the sensitivity of the SFA (Gimbert et al., 2007; Koopmans et al., 2020) (e.g., for the SFA used in this research, the detection limit would decrease from ~0.02 mM to ~0.06 µM ortho-P).

Additionally, **drying and rewetting** of soil has been shown to increase the release of P and DOC in water and weak salt extracts (De Troyer et al., 2014; Koopmans et al., 2006; Koopmans and Groenenberg, 2011; Turner and Haygarth, 2001), and therefore water extracts were prepared from moist material throughout my research. However, often soil samples are dried before processing in the laboratory because this facilitates representative subsampling and sample storage and minimises microbial activity that may alter the soil nutrient status (Van Erp et al., 2001). Although it would be preferable to use fresh material whenever possible, premoistening a dried soil prior to extraction may be an option when drying is inevitable, as remoistening can restore the original soil properties (Van der Paauw, 1971).

The choice for water-extractable ortho-P as a proxy for P in soil solution that is linked to readily plant-available P, is a static measurement and does not reflect the dynamic process of plant P-availability on the **long-term**. Bioavailability of P is a dynamic process that does not only depend on the short-term diffusion flux of P from the bulk soil solution to the root surface (P intensity) where uptake of P occurs. Ultimately, this flux is linked to the total amount of reversibly adsorbed P in soil (P quantity) since on the long-term P in soil solution has to be replenished by buffering of P from the soil solid phase (Koopmans et al., 2004a). When the P flux to the root surface decreases below the rate that is required for an optimal plant growth, plants will start to experience P deficiency and P fertiliser should be applied to overcome this. This raises the question what the static increase in water-extractable ortho-P in earthworm casts means for earthworm-enhanced P-availability on the long-term. My field experiment showed that plants can still benefit from the presence of earthworms on the medium-term (Chapter 5), although the experiment did not allow to ascribe this with certainty to the effect of earthworms on raising readily plant-available P in casts. However, it is very difficult to relate static soil P tests to a dynamic bioavailability process, and further research is required to determine how long grass will be able to benefit from earthworm activity that leads to an increase in the level of readily plant-available P.

6.4 Agronomic implications of this research

6.4.1 Global validity of my findings

Because of the additional pathway of a decrease of the RSA for soils where the fraction of metal-(hydr)oxides is dominated by Fe-(hydr)oxides, Chapter 3 concludes that earthworms may have the **largest potential** to increase the level of readily plant-available P in Fe-(hydr)oxide-dominated soils. However, this still needs to be tested for the effect of earthworms on grass P uptake in such contrasting soils. Furthermore, the potential to increase readily plant-available P in a soil by earthworms is additionally determined by the abundance of earthworms in that soil. It is therefore the presence of Fe-(hydr)oxides, combined with the likelihood of the occurrence of earthworms that determines the potential of a soil to raise the level of readily plant-available P.

In **the Netherlands**, many agricultural soils exist where the fraction of metal-(hydr)oxides is dominated by Fe-(hydr)oxides. In the three datasets discussed in section 6.3.1, this fraction varied between 95, 76 and 36% of the investigated soils. Additionally, the abundance of earthworms in the Netherlands is high, and ranges among the highest in Europe (Rutgers et al., 2016) as well as the world (Phillips et al., 2019). This suggests that there is a large potential for the Netherlands to increase the level of readily plant-available P in soils by employing earthworms (see section 6.4.3 for a discussion on managing earthworm populations). However, the P loading of many Dutch agricultural soils is relatively high (Koopmans et al., 2006; Schoumans and Chardon, 2015). This makes the large potential for earthworm-enhanced P-availability in the Netherlands mostly important in the future, as restrictions in P use through the Dutch manure policy in the form of equilibrium P fertilisation can result in a decline of grass yield and P content on the long-term (Van Middelkoop et al., 2016). In those conditions, employing earthworms to increase readily plant-available P in soil could contribute to compensate for this loss of yield and P content. Already today, the large potential of earthworms can be relevant for soils where a P mining strategy is adopted to decrease the risk of dissolved P losses to the aquatic environment from heavily P fertilised soils (legacy P soils) (Koopmans et al., 2004b; Van der Salm et al., 2009). For such soils it has been shown that the P supply to grass or fast-growing crops can become limiting for optimal P uptake while total P reserves are still relatively high (Koopmans et al., 2004a; Koopmans et al., 2004b; Smolders et al., 2021). When, in those situations, earthworms can help to make P that remains in soil better accessible to plants through enhancing P solubility, a larger P draw-down could potentially be realised (Rowe et al., 2016). As such, earthworms could provide a viable strategy to proceed towards a more sustainable P nutrition in those soils.

Globally, P-limitation to crops is widespread in agriculture, especially in tropical regions (Hou et al., 2020). Here the expected impact of P fertilisation will be largest (Ros et al., 2020). Simultaneously, many of those areas face large (economic) restrictions to P fertiliser application. This means that the effect of earthworm-enhanced P-availability

could be very relevant for the tropics as employing earthworms can be a more economically feasible option than applying P fertiliser. Since my research considered soils and earthworm species common to the Netherlands, it is essential to validate my findings for tropical soils and earthworm species. Such research could potentially include other ecosystem engineers, like termites, which can largely affect mineralisation similar to earthworms, but can be present in ecosystems that are less suitable for earthworms (Jeffery et al., 2010). However, a potential effect of termites on readily plant-available P is likely smaller than that of earthworms, as it could only be caused by the enrichment of their excreta with P released by mineralisation. Because in general termites do not ingest soil particles, a change in the adsorption equilibrium on soil metal-(hydr)oxides is absent. However, including termites in this research, could be interesting because in general earthworms are less abundant in the tropics than in the temperate region of the Netherlands, with exception of the wet tropics of particularly South America and South East Asia, and regions in China (Phillips et al., 2019). Therefore, the potential of earthworm-enhanced P-availability seems to be lower in the tropics from the perspective of earthworm abundance. Meanwhile, a conclusion on the potential of earthworms to enhance readily plant-available P from the perspective of the dominating metal-(hydr)oxide in soils should be given with care. Both Fe- and Al-(hydr)oxides are common in the tropics (Kirsten et al., 2021; Qafoku et al., 2004; Xu et al., 2016) and a global analysis of clay minerals shows that Fe-(hydr)oxides are largely present in the weathered tropical soils from South America and Africa (Ito and Wagai, 2017). However, these data are indirectly obtained from literature on spectroscopic analyses and, to my knowledge, a database on the type of metal-(hydr)oxides based on soil extractions of soils around the world does not exist. In the absence of a worldwide database, the dominating type of metal-(hydr)oxides in tropical soils can be estimated from regional studies that report the required data. An example of such a study is Mendez et al. (2022), who performed ammonium oxalate extractions on 18 soils from sub-Saharan Africa. In 94% of the soils these authors investigated, Al-(hydr)oxides dominated the fraction of metal-(hydr)oxides. However, in 56% of the investigated soils Fe-(hydr)oxides comprised 30% or more of the total fraction of metal-(hydr)oxides, and thereby still had an important contribution to total reactivity. Another example includes a dataset of five soils from South America, of which four were dominated by Fe-(hydr)oxides and one by Al-(hydr)oxides (Anna Favaro and Wietse Wiersma, personal communication). Overall, these data suggest a large contribution of Al-(hydr)oxides in tropical soils. Therefore, also in this respect the potential for earthworm-enhanced P-availability is lower in the tropics compared to the Netherlands. Furthermore, many tropical soils have low P loadings, which limits the potential of earthworm-enhanced P-availability on the longer term. However, earthworm-enhanced P-availability can be relevant for specific areas of the (wet) tropics, as earthworms might speed up the cycling between organic and inorganic P in soils with low P loadings, and elevated P-availability in cast of tropical earthworm species has been

observed (Brossard et al., 1996; Chapuis-Lardy et al., 1998; Chapuis-Lardy et al., 2011; Lopez-Hernandez et al., 1993). In those regions, this option can be more economically feasible and can additionally circumvent problems associated to P (over)fertilisation. Thereby employing earthworms to increase the level of readily plant-available P could contribute to sustainable development of such regions as it allows for a direct change towards a more sustainable P nutrition of agriculture.

6.4.2 Quantity versus quality of earthworm casts

Water-extractable ortho-P concentrations in casts vary considerably among earthworm species (Chapter 2). Based on Fig. 2.1, this concentration, i.e., the **quality of earthworm casts** in this respect, is highest for *L. rubellus*, followed by *L. terrestris* and *A. caliginosa*. The lowest ortho-P concentrations were measured in the water extracts of casts of *A. chlorotica* and *A. longa*. These five species were also used in the mesocosm field experiment discussed in Chapter 5 where we considered the effect of earthworms on grass biomass production and P uptake. Our analyses show that *A. longa* and *L. terrestris* (Eq. 5.2) exerted a positive effect on P uptake of grass in the field. These results agree with the study of Vos et al. (2014), who investigated the effect of three earthworm species (*L. rubellus*, *A. caliginosa* and *L. terrestris*) in a greenhouse pot experiment and only observed a significantly increased grass P uptake in pots with *L. terrestris*. However, Mackay et al. (1982) did observe a positive effect on P uptake for a mixture of *L. rubellus* and *A. caliginosa*, but their greenhouse pot experiment did not include other earthworm species.

Chapters 2 and 5 demonstrate that different earthworm species have the highest water-extractable ortho-P concentrations in casts (*L. rubellus*), and a significantly increased grass P uptake (*A. longa* and *L. terrestris*). This suggests that water-extractable ortho-P cannot be translated directly in plant P uptake. However, besides the quality of casts, earthworm species also vary considerably in the amount and location of the casts they produce, i.e., the **quantity of earthworm casts** in this respect. As suggested in Vos et al. (2014) and in Chapter 5, the larger body mass (Sims and Gerard, 1985) and thereby larger cast production of *A. longa* and *L. terrestris* compared to the other species studied, could be the reason my data suggest an important role for those species. Since these species produce larger casts, this increases the likelihood of plant roots benefitting from hotspots of readily plant-available P created by earthworms. The amount of cast with an elevated concentration of readily plant-available P might therefore be more important for total P uptake than the actual water-extractable ortho-P concentration of those casts.

Besides nutrient availability, grass biomass production also depends on factors like soil moisture content and soil aeration, and **earthworm activity** can strongly affect these parameters (Bottinelli et al., 2017; Edwards et al., 1992; Schon et al., 2017; Schrader et al., 2007). Additionally, earthworm burrowing can affect microbial activity and thereby the contribution of microbes to plant-available P (Brown et al., 2000). As epigeic species, like *L. rubellus*, mainly dwell on the soil surface, they affect the structure of a soil less. In

particular anecic species, like *A. longa* and *L. terrestris*, affect water infiltration and soil aeration (Capowiez et al., 2014; Springett, 1983). Despite my attempts to prevent water limiting growth conditions by irrigating my experiment, this might explain why the anecic species contributed significantly to the models in Chapter 5, as I cannot rule out that other, unmeasured conditions like the soil moisture content, limited grass growth rather than P-availability. Additionally, an effect of earthworms on increasing soil moisture levels can affect P-availability to plants if sufficient P is available for plant uptake, but uptake is limited by the diffusion of P towards the plant roots. In such conditions, the flux of P towards the plant roots can be increased by the increased water-filled pore space due to higher soil moisture levels. This demonstrates that disentangling the effects of earthworm activity and increased nutrient availability by earthworms is complex (Edwards and Lofty, 1980). While the effects of other nutrients can be excluded in experiments by supplying ample amounts of these nutrients, it is practically impossible to eliminate the effects of earthworm activity.

6.4.3 Managing earthworm populations

My thesis shows that earthworms can contribute to increasing the sustainability of P nutrition of grasslands on low P-status soils by increasing the level of readily plant-available P. To realise the potential benefits of earthworms, earthworm densities can be stimulated. Earthworm densities under Dutch grasslands are relatively high, especially in grasslands used for dairy production (Deru et al., 2018; Rutgers et al., 2016). However, both in- and outside the Netherlands areas with low earthworm densities occur. One possibility to increase their numbers is to **inoculate** soils that have low earthworm densities with earthworms (Marashi and Scullion, 2003). This can be achieved by adding grass sods that contain earthworm individuals and cocoons, and can be effective for epigeic and endogeic species (Van Eekeren et al., 2014). However, this research showed that especially anecic species have a positive effect on biomass production and P uptake. Stimulating those populations can therefore be of particular interest, and could be achieved by introducing individuals with for instance the Earthworm-Inoculation-Unit method (Butt et al., 1997).

Earthworm inoculation can speed up colonisation, but its success depends on the suitability of the environment for earthworms. Soil moisture content and food supply for earthworms are especially important, as habitat colonisation and earthworm survival primarily depend on those factors (Curry, 2004). In the absence of sufficient **soil moisture**, earthworms lose weight, enter diapause or even die (Eriksen-Hamel and Whalen, 2006; Gerard, 1967; Holmstrup, 2001; Lavelle and Spain, 2001). Maintaining a moist soil is, therefore, extremely important. In practice, for many soils this discourages stimulating dry soil conditions by lowering the groundwater level via drainage. However, in soils with a groundwater level relatively close to the soil surface, like many peatlands, drainage can be encouraged, as high groundwater levels can result in undesired anoxic conditions for

earthworms (Deru et al., 2018; Plum and Filser, 2005). In arable systems retention of crop residues can prevent soil water evaporation by forming a protective layer (Curry, 2004). Moreover, crop residues can supply additional **food** for earthworms (Curry, 2004; Van Eekeren et al., 2014). In grasslands large food inputs can be achieved by maintaining a healthy and species diverse grass cover, thereby maximizing root biomass. Especially the inclusion of clovers in the species mix can stimulate earthworm abundance (Van Eekeren et al., 2009b). Furthermore, the presence of plants is additionally beneficial for the effect of earthworms as they increase the fertility of earthworm casts (Van Groenigen et al., 2019). That fertilisation stimulates the growth of plants and thereby increases the food supply of earthworms has been recognised for a long time. In general, a positive relation is observed between judicious fertiliser input and the number of earthworms (Andersen, 1983; Standen, 1984; Syers and Springett, 1984). Both the application of inorganic and organic fertiliser can have these effects on grass growth. Whether a difference between the two is observed on the number of earthworms primarily depends on weather conditions (Timmerman et al., 2006). However, often organic fertilisation is preferred by earthworms because of the acidifying effect of some inorganic fertilisers (Ma et al., 1990) (see below) and because manure additionally contains large amounts of organic C (Koopmans et al., 2007) which can serve as a food source (Van Eekeren et al., 2009a; Whalen et al., 1998). In general, more organic material is beneficial for earthworms (Bouché, 1977) and additions of other organic soil amendments like compost (Leroy et al., 2008) or the solid fraction of cattle slurry (Deru, 2021) can have positive effects on earthworm populations as well. The timing of the addition of soil amendments is thereby crucial for the effect on earthworm abundance, as earthworms especially require additional food inputs during times of high activity (spring and autumn) (Eggleton et al., 2009; Gerard, 1967; Lavelle and Spain, 2001). Additional food inputs can also be realised by ruminant grazing, as many ruminants leave grass residues and their excrements can be both a food source and a refuge for earthworms (Chardon et al., 2007; Curry, 2004; Van Eekeren et al., 2014). However, the feasibility of all these management practices will depend on the specific conditions of the farm. Furthermore, management practices that can increase food inputs for earthworms can also increase P inputs to soil (e.g., by fertiliser addition), which should be considered when assessing the effect of such management practices on readily plant-available P.

Besides soil moisture and the presence of food, earthworms are affected by **soil temperature** (Curry, 2004; Eggleton et al., 2009; Gerard, 1967; Whalen et al., 1998), and earthworm numbers can considerably decrease during cold winters (Timmerman et al., 2006). **Soil pH** is also an important parameter determining the quality of earthworm habitat (Curry, 2004; Edwards and Bohlen, 1996). Although earthworm species vary in their sensitivity to soil acidity, in general they do best in soils with a neutral pH (Baker and Whitby, 2003; Curry, 2004; Didden, 2001). Increasing the soil pH by liming therefore stimulates their numbers in acidic soils, while the use of acidifying fertilisers can reduce

their population (Curry, 2004; Ma et al., 1990; Pfiffner, 2014; Syers and Springett, 1984). Furthermore, soil **tillage** also affects earthworm populations. Intensive tillage can physically kill earthworms, expose them to harsh environmental conditions or predators, remove their food source and destroy the burrows of anecic earthworms (Briones and Schmidt, 2017). Agricultural management of Dutch grasslands can comprise the application of manure, mowing, renewing grasslands, or converting them to or from arable land. In studies on the effects of manure application on earthworm abundance in grassland soils, the effects of manure itself, its location and its application method (e.g., broadcasting or slit injection) are often intertwined, and results of attempts to separate them are largely affected by seasonal influences (Van Vliet and de Goede, 2006).

6.4.4 Increasing the sustainability of phosphorus nutrition of grasslands

In practice, the results of this research are particularly relevant for the **production of grass and its P content** in dairy farming. My research focusses on soils with a low P-status and might therefore be less applicable to the present-day situation in the Netherlands where grasslands often have a neutral to high P-status (Reijneveld et al., 2010). This results in grass growth not frequently being limited by P. However, due to the restrictions on P application rates posed by the Dutch manure policy aimed at realising equilibrium P fertilisation, there is a risk that grass yields and P contents will decline in the future (Van Middelkoop et al., 2016). The data of our field experiment (Chapter 5) demonstrate that specific earthworm species have a positive effect on grass biomass production and P uptake in the absence of P fertilisation. However, Chapter 5 SI Fig. S2.1 shows that the P content of grass shoots of the non-P fertilised treatment without earthworms (0EW0P) remained for most harvests well below 3 g P kg⁻¹ dry matter. This might be insufficient to meet the P requirements of lactating and high-yielding dairy cows (Valk and Šebek, 1999). As the earthworm population changed over the duration of my field experiment and earthworms invaded the 0EW0P control columns, the shoot P content of the 0EW0P treatment can be interpreted as representative for all non-P fertilised columns including earthworms. Since my field experiment was conducted on a soil where the fraction of metal-(hydr)oxides was dominated by Al-(hydr)oxides, the effect of earthworms might have been larger in a Fe-(hydr)oxide-dominated soil (section 6.3.1). However, my results suggest that while earthworms can strongly increase the level of readily plant-available P in casts, their contribution on low P-status soils is likely not large enough to result in grass P contents that are required for lactating and high-yielding dairy cows.

Another limitation of the effect of earthworm-enhanced P-availability relates to the fact that P cannot be synthesized. Earthworms can, therefore, not create P where there is none. They can only increase soil P-availability to plants when P is present in less plant-available forms, and for agricultural soils with a correct or low P loading, P inputs are essential to counterbalance P removal through its produce (Jordan-Meille et al., 2012; Sattari et al., 2016). To increase its efficiency, this P fertilisation should be aimed at the 4R

nutrient stewardship framework (Right source, Right rate, Right place, Right time) (Grant and Flaten, 2019; Mikkelsen, 2011). The aspect of **timing** is also important for the practical application of my findings on earthworm-enhanced P-availability. Phosphorus uptake by grass is particularly limited in spring when temperatures are relatively low and low growth rates result in limited P uptake (Jacob Goelema, personal communication). In the Netherlands, this can result in large fluctuations in the P content of the first cut of grass (often in May) depending on the weather conditions in spring (CBS (2020) and Jacob Goelema, personal communication). Stabilising these fluctuations by increasing P-availability in spring could possibly be a contribution of earthworms, although further research on this topic is required. If earthworm abundance could be particularly stimulated in spring, earthworm-enhanced P-availability could potentially contribute to increased P uptake of grass at the time of the first grass cut. Naturally, earthworm activity has a peak in spring and autumn (Eggleton et al., 2009; Gerard, 1967; Lavelle and Spain, 2001; Whalen et al., 1998), so this already coincides with the period that is possibly of interest. However, management could be focussed on the height and precise timing of this peak in earthworm activity. A possibility could be to apply manure (i.e., a food source for earthworms) early in the year in order to stimulate earthworm populations. Thereby high earthworm abundances could be reached well before the first grass cut, allowing the fresh grass to benefit from earthworm-enhanced P-availability. Yet, early fertilisation may increase the risk of P leaching to groundwater (Liu et al., 2017) and based on the current legislation in the Netherlands manure can often not be applied before February (Rijksdienst voor Ondernemend Nederland, 2022). However, earlier application has likely only limited effects on earthworm populations, as cold winters can largely decrease earthworm numbers (Timmerman et al., 2006).

As the challenges associated with P use are likely too large to solve by a single approach, agriculture should focus on a **combination of several approaches**. All of those should contribute to (timely) enhanced P uptake by grass on low P-status soils for retaining high production and quality in grasslands. Stimulating earthworm populations to increase the level of readily plant-available P could be one of those approaches. However, other mechanisms to optimise P uptake by plants should be adopted simultaneously. For instance, the study of Mackay et al. (1982) showed that earthworms have an increasing effect on the effectivity of P fertiliser for grass yield and P uptake. A viable strategy for a sustainable intensification of agriculture could therefore be to combine required P applications to grasslands (Sattari et al., 2016) with stimulation of earthworm populations. This approach is currently being studied at Wageningen University by Laura Ferron. Her PhD research is aimed at the use of circular P fertilisers like struvite, and investigates the role of earthworms to their applicability in agriculture.

6.5 Future research opportunities

Throughout this chapter, I have identified research gaps to be addressed in the future. The most important future research opportunities are:

- My research concerns several earthworm species and soils that are typical for the Netherlands. However, globally many more soil types and earthworm species exist which I have not studied. Additionally, environmental conditions like temperature and humidity differ across the globe, implying that my findings cannot be translated directly to other agroecosystems. However, section 6.4.1 elaborates on the potential of my findings for areas outside the Netherlands, particularly in the wet tropics. Therefore, I would recommend to investigate whether my findings also apply to **earthworm species and soils from different areas around the world**. Such experiments should focus on soils where the fraction of metal-(hydr)oxides is dominated by Fe-(hydr)oxides, as my research showed that those soils have the largest potential for earthworm-enhanced P-availability.
- Section 6.3.2 demonstrates that when earthworm-enhanced P-availability is found on the short (e.g., my greenhouse pot experiments) to medium-short term (e.g., the two growing seasons of my mesocosm field-experiment), this does not indicate the presence of this effect on the long-term. It is therefore important to study the **temporal dynamics of earthworm-enhanced P-availability**. This includes investigating if earthworms can contribute to better utilise the pool of legacy P when by P mining the P flux from the soil solid phase towards the roots becomes too low to sustain the demand of the plant. This requires long-term experiments, which could be pot or field experiments. Pot experiments could be similar to those of Koopmans et al. (2004b) and Nawara et al. (2018), but special attention should be paid to the size of the pots, which should be large enough to sustain a healthy and active earthworm population. For field experiments, a running long-term P mining field experiment could be joined, like the experiments on grasslands in Zegveld and the Waiboerhoeve (Ehlert et al., 2018), where the addition and stimulation of earthworms could be studied for its contribution to maintaining grass biomass production and P uptake during P mining.
- Furthermore, it is important to validate and **update** my findings, in particular the field experiment reported in Chapter 5, and use the insights gained on maintaining and controlling imposed earthworm communities in the field. Field experiments on earthworm-enhanced P-availability are required to determine the significance of the mechanisms observed under controlled greenhouse conditions for more realistic semi-controlled conditions in the field. Finally, consistent results can be translated into management strategies for farmers and evaluated in practice.
- Besides the potential of earthworm-enhanced P-availability, future research should also consider possible **negative effects** associated to it. Chapter 3 showed that the redox sensitivity of Fe-(hydr)oxide-dominated soils contributed to earthworm-

enhanced P-availability by resulting in a decrease of the RSA in casts. However, this could also be relevant for the solid-solution partitioning of other oxyanions like arsenate (As(V)) (Stachowicz et al., 2008; Verbeeck et al., 2017). In As(V)-contaminated soils, a decrease of the RSA caused by earthworms might lead to increased levels of soluble As(V), translating into higher toxicity. This negative effect of earthworm presence was shown by Sizmur et al. (2011), although not yet investigated in the context of a decrease of the RSA.

- My research demonstrates that earthworms can increase the level of readily plant-available P and can thereby contribute to increasing the sustainability of agricultural P use. However, it also made clear that the challenges associated to agricultural P use are likely too large to be solved by a single approach. Therefore, future research should focus on a **combination of several approaches** that aim to increase P uptake from soils with a low P-status. Examples could be experiments on combining earthworms with circular P fertilisers (section 6.4.4) or with selecting grass species for increased P uptake (Ros et al., 2018).

6.6 Concluding remarks

The main objective of this research was to elucidate the effect of earthworms on plant-available P in soil and P uptake by grass, and to explore its potential to increase the sustainability of P nutrition of grass. My research presents new insights on the identification and quantification of the mechanisms controlling earthworm-enhanced P-availability (Fig. 6.2). By spectacularly increasing the concentrations of water-extractable ortho-P in their casts on a soil with a low P-status, earthworms are able to contribute to increasing the level of readily plant-available P. Earthworms can increase grass biomass production and P uptake, implying that stimulating their numbers can increase the sustainability of P nutrition of grass. Earthworm-enhanced P-availability has potential to contribute to a more sustainable P nutrition for agricultural grasslands in (the future of) the Netherlands. However, the challenges associated with agricultural P use are likely too large to be solved by a single approach. A combination of several approaches is needed by agriculture to provide an adequate P nutrition to grass on low agronomic P-status soils. My research shows that stimulating earthworm populations to raise the level of readily plant-available P could be one of those approaches.

Supporting information

S1 Additional analyses earthworm diversity

Although not reported in Chapter 4, the experiment included two- and three-species combinations besides the reported monocultures and five-species mixture. These species combinations were: *A. caliginosa* + *A. chlorotica*, *L. terrestris* + *A. longa*, *L. rubellus* + *A. caliginosa*, *L. rubellus* + *L. terrestris* and *L. rubellus* + *A. caliginosa* + *L. terrestris*. These treatments were not discussed in Chapter 4 because enzyme analyses were not conducted on these treatments. However, we did measure grass biomass production of all four harvests and grass P content at the final harvest for those treatments. Analysis of these data showed no significant effect of any of the non-P fertilised treatments (which include the earthworm-free controls 0EW0P and 0EW+Cast) on cumulative aboveground biomass production ($p=0.71$) or P uptake at the final harvest ($p=0.75$). Nor could any effect of the number of added earthworm species be observed ($p=0.57$ and $p=0.28$ for cumulative aboveground biomass production and P uptake at the final harvest, respectively; Fig. S1). In part, this absence of significant differences could be explained by the absence of a strong P-limitation for plant growth experienced in that experiment.

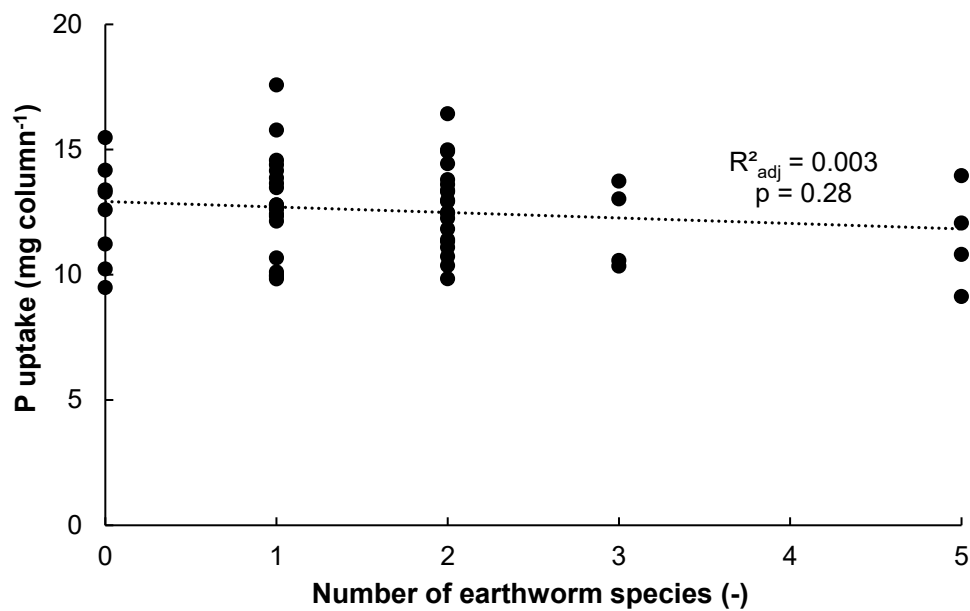
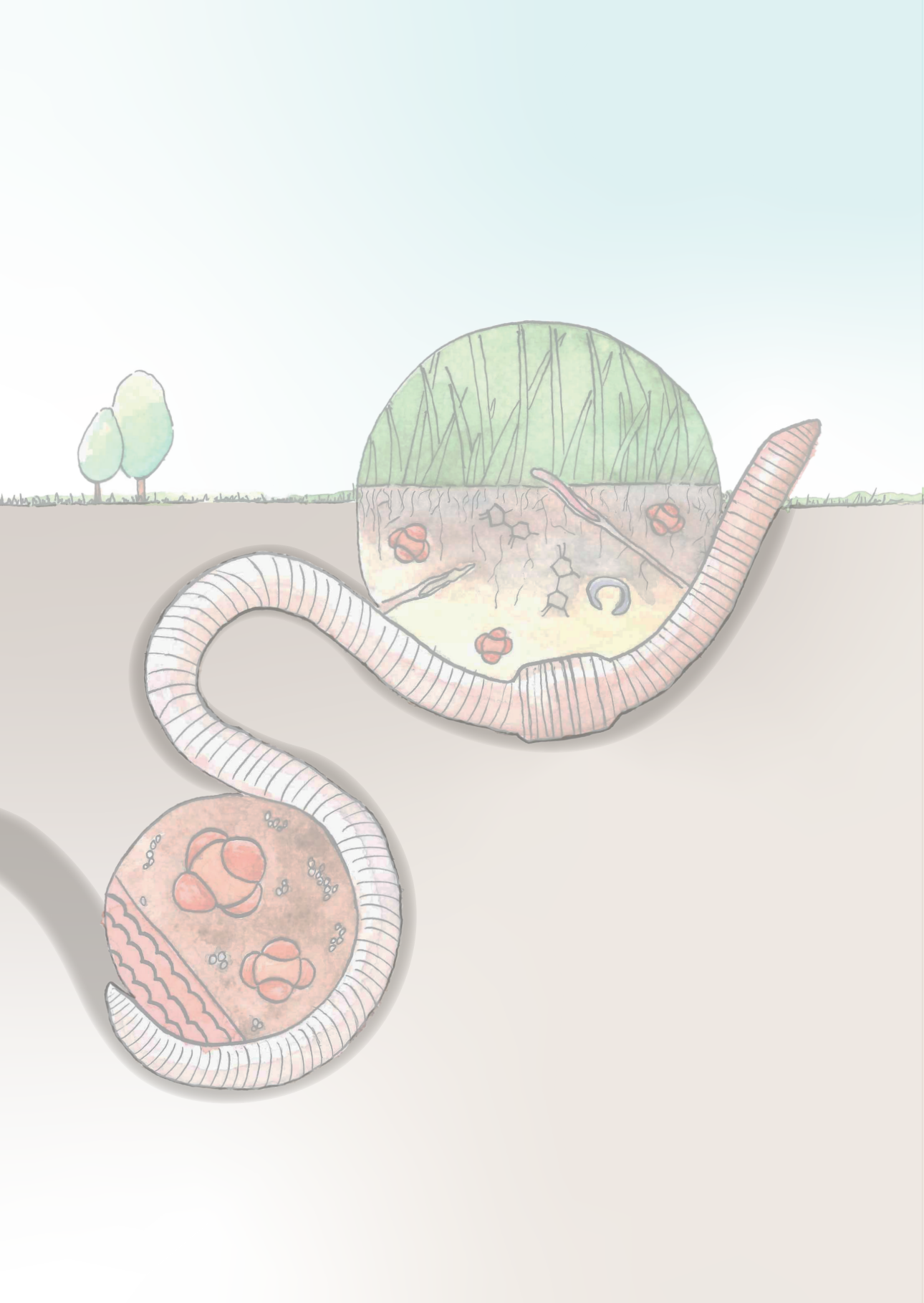


Figure S1 Scatterplot of the number of earthworm species added to each pot versus the P uptake at the final harvest of the experiment reported in Chapter 4. The dotted line represents the regression line.





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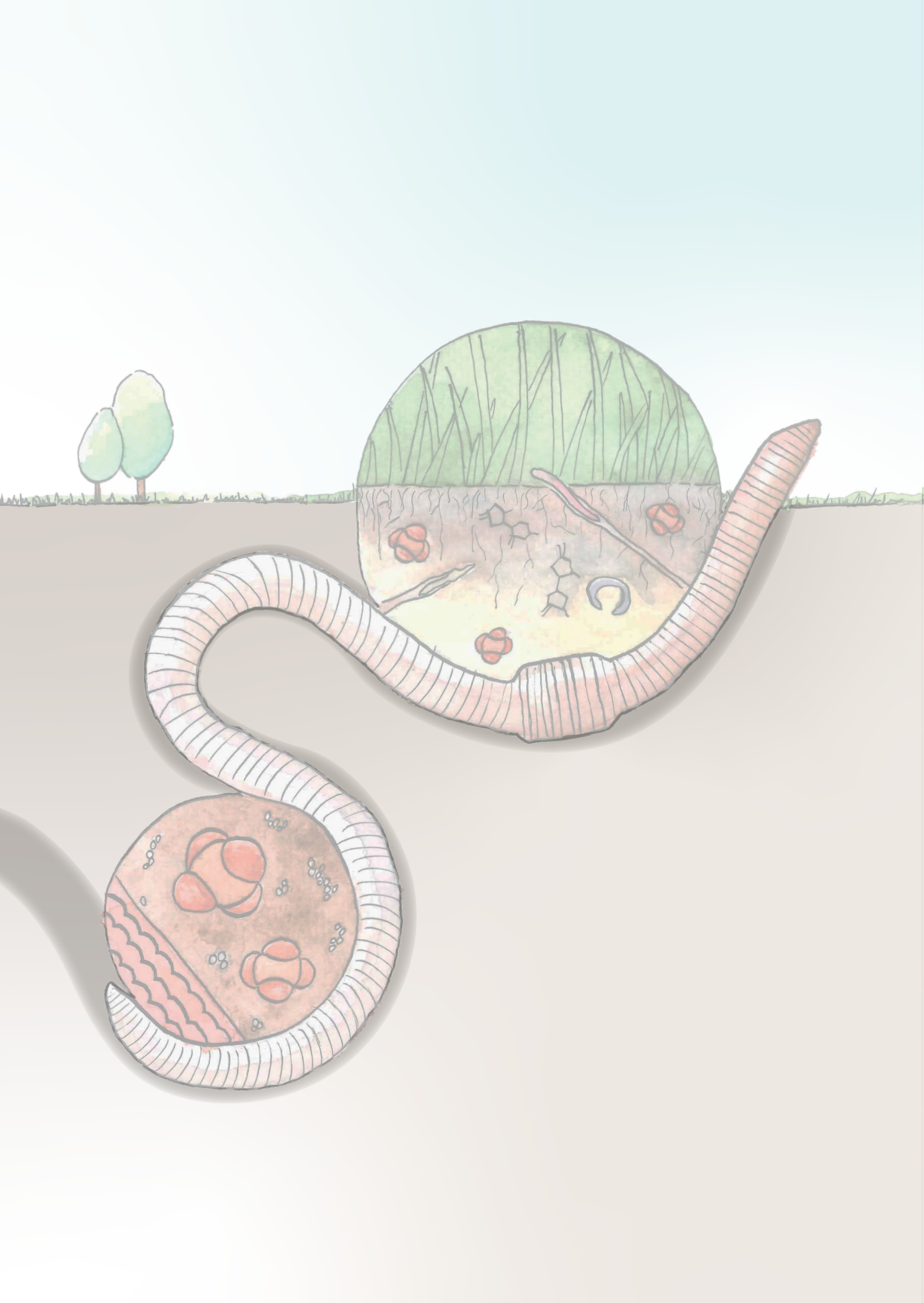
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Summary

Samenvatting

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Summary

Phosphorus (P) is essential for plant growth, but most of the P in soils is not available for plant uptake as it binds strongly to mineral soil constituents. Additionally, the use of P in agriculture is challenged by both the current increase of the world population as well as a changing human diet towards more consumption of animal products. This results in a growing demand for food and P. Furthermore, the reserves of phosphate rock as source of mineral P fertiliser are declining, while areas that have historically received excessive P additions face environmental risks that are related to chronic dissolved P losses from legacy P soils to ground- and surface waters. These challenges associated with agricultural P use enforce us to increase productivity while using mineral P fertiliser in a more sustainable manner. Sustainable agricultural intensification will require adequate P fertiliser inputs, overcoming global P imbalances and finding ways to improve the utilisation of P already present in soil. This last strategy requires finding new ways to access soil P pools that are less available to plants. Exploring the role of earthworms in sustaining optimal P acquisition for plants can therefore contribute to solving the P challenges we are currently facing. In this thesis, I explored the role of earthworm-enhanced P-availability for improving soil P utilisation. My research primarily focussed on the effect of earthworms in grassland systems from the Netherlands, as grasslands are important to global food production, and can sustain large earthworm populations.

The main objective of my thesis was to elucidate the effect of earthworms on plant-available P in soil and P uptake by grass, and to explore its potential to increase the sustainability of P nutrition of grass. The specific research objectives were (1) to assess the effect of different earthworm species on plant-available P and relate this to a) physico-chemical soil properties and b) earthworm ecology; (2) to determine the controlling mechanisms of earthworm-enhanced P-availability and quantify the relative importance of all contributing mechanisms; (3) to assess the effect of earthworm diversity on plant-available P; and (4) to evaluate if the effect of earthworms on plant-available P results in an increased grass P uptake and biomass production under realistic field conditions. These objectives are addressed in four studies (Chapters 2-5).

Chapter 2 showed that while all investigated species increased the total pool of reversibly adsorbed P, a large variation exists in the ability of earthworm species to alter the extent to which P is present in readily plant-available forms. This variation was not related to classical earthworm ecological classification, and species from the same functional group showed large variation in cast properties like pH and dissolved organic carbon as measured in water extracts. In turn, this variation could be linked to the existing variation in ortho-P solubility measured in the same extracts. It was therefore concluded that multiple pathways contributed to earthworm-enhanced P-availability.

In Chapter 3, these multiple pathways were quantified further, and their relative importance was assessed. The probe-ion method, which derives the reactive surface area (RSA) of a soil, in combination with surface complexation modelling revealed a so far

unconsidered mechanism: the decrease of the RSA of metal-(hydr)oxides in casts by particle growth, resulting in a decrease of the surface area that is available for P adsorption. The contribution of the controlling mechanisms of earthworm-enhanced P-availability in earthworm casts consists of: a) a relatively small effect of the increase of pH in casts; b) a major contribution of P addition through stimulated mineralisation inside the earthworm gut; c) a potentially major effect of a decrease in the RSA of casts by particle growth which is catalysed by the formation of Fe^{2+} during the reducing conditions caused by mineralisation; and d) a decrease in the competition between natural organic matter and P for binding sites on metal-(hydr)oxides in casts compared to the bulk soil. The reduction of the RSA was only observed for Fe-(hydr)oxide-dominated soils, whereas it was absent or minor in Al-(hydr)oxide-dominated soils. This demonstrated that soil mineralogy influences earthworm-enhanced P-availability and suggests that earthworms have the largest potential to improve the sustainability of P use in Fe-(hydr)oxide-dominated soils.

All controlling mechanisms of earthworm-enhanced P-availability are directly or indirectly related to the capacity of an earthworm to mineralise the organic material it ingests. Therefore, the pathway of mineralisation of organic P was investigated in more detail in Chapter 4. This showed that the potential activity of phosphatase enzymes that catalyse the mineralisation process of monoester P compounds was considerably increased by earthworms in the order casts>burrows>bulk soil. Phosphatase activity varied among earthworm species, and correlated with the P content of grass shoots, suggesting a direct earthworm-induced effect on grass P uptake through mineralisation of organic P.

The mesocosm field experiment of Chapter 5 considered the effect of earthworm diversity on grass biomass production and P uptake. We did not observe an effect of a larger number of earthworm species on P uptake at the end of the experiment. However, using a multiple linear regression model on established communities, *A. longa* and *L. terrestris* were identified as keystone species to increase grass P uptake and biomass production. Furthermore, this study showed that the effect of earthworms on grass P uptake was not only present under controlled greenhouse conditions but can also be observed at the larger scale of more realistic field conditions.

In the final chapter of this thesis (Chapter 6), I summarised my main findings, placed my results in a broader perspective and discussed the implications of my findings for practice. Earthworm-enhanced P-availability has potential to contribute to a more sustainable P nutrition for agricultural grasslands in (the future of) the Netherlands. However, the challenges associated with agricultural P use are likely too large to be solved by a single approach. A combination of several approaches is needed by agriculture to provide an adequate P nutrition to grass on low agronomic P-status soils. My research shows that stimulating earthworm populations to increase the utilisation of P in soil could be one of those approaches. Future research should focus on assessing the effect of

earthworm-enhanced P-availability on a global scale and its contribution to the utilisation of soil P over longer periods of time. Furthermore, future research should particularly focus on combining earthworms with other approaches that aim to increase P uptake from soils with a low P-status.

Samenvatting

Fosfor (P) is essentieel voor plantengroei, maar de meeste P in de bodem is niet beschikbaar voor opname door planten omdat P sterk bindt aan minerale bodembestanddelen. Daarnaast staat het gebruik van P in de landbouw onder druk door de toename van de wereldbevolking en de per capita consumptie van meer producten van dierlijke oorsprong. Dit betekent een toenemende vraag naar voedsel en daarmee P. Tegelijkertijd nemen de reserves van fosfaaterts, de bron van minerale P-meststof, af, terwijl gebieden waar in het verleden overmatig P aan de bodem werd toegevoegd, nu geconfronteerd worden met milieurisico's vanwege chronische verliezen van opgelost P uit de bodem naar grond- en oppervlaktewater. De uitdagingen van het gebruik van P in de landbouw dwingen ons ertoe om de productiviteit te verhogen en tegelijkertijd minerale P-meststoffen op een duurzame manier te gebruiken. Duurzame intensivering van de landbouw vereist een adequate input van P-meststoffen, het herstellen van het mondiale P evenwicht, en het vinden van manieren om de benutting van in de bodem aanwezig P te verbeteren. Dit laatste maakt het noodzakelijk om nieuwe manieren te vinden om de P-voorraden in de bodem beter beschikbaar te maken voor planten. Het onderzoeken van de rol die regenwormen spelen bij het beschikbaar maken van P voor planten kan daarom bijdragen aan het oplossen van de P vraagstukken waarmee we momenteel worden geconfronteerd. In dit proefschrift heb ik de rol van regenwormen in het verbeteren van de P-beschikbaarheid in de bodem onderzocht. Mijn onderzoek richtte zich met name op het effect van regenwormen in graslandssystemen in Nederland, aangezien graslanden belangrijk zijn voor de wereldwijde voedselproductie en daarnaast grote regenwormpopulaties kunnen herbergen.

Het hoofddoel van mijn proefschrift is het verhelderen van het effect van regenwormen op plant-beschikbaar P in de bodem en de P opname van gras, en het onderzoeken van de potentie van regenwormen voor een verduurzaming van de P-voeding van gras. De specifieke onderzoeksdoelstellingen zijn (1) het onderzoeken van de effecten van verschillende soorten regenwormen op plant-beschikbaar P en dit te relateren aan a) fysisch-chemische bodemeigenschappen en b) de ecologie van regenwormen; (2) het bepalen van de mechanismen die zorgen voor een verbeterde P-beschikbaarheid door regenwormen en het kwantificeren van het relatieve belang van alle bijdragende mechanismen; (3) het onderzoeken van het effect van diversiteit in regenwormensoorten voor plant-beschikbaar P; en (4) evalueren of het effect van regenwormen op plant-beschikbaar P resulteert in een verhoogde P opname en biomassa-productie van gras onder realistische veldomstandigheden. Deze doelstellingen komen aan de orde in vier studies (Hoofdstukken 2-5).

In Hoofdstuk 2 is aangetoond dat, hoewel alle onderzochte soorten regenwormen de totale hoeveelheid reversibel gebonden P verhoogden, er veel verschil is in het vermogen van regenwormsoorten om invloed te hebben op de mate waarin P aanwezig is in een direct voor de plant beschikbare vorm. Dit verschil was niet gerelateerd aan de

klassieke ecologische classificatie van regenwormen, en soorten uit dezelfde functionele groep lieten een grote variatie in de eigenschappen van wormenpoep zien, zoals de pH en hoeveelheid opgeloste koolstof gemeten in waterextracten. Deze variatie kon op zijn beurt worden gerelateerd aan de variatie in de oplosbaarheid van direct plant-beschikbaar P in dezelfde extracten. Op basis van deze gegevens kan worden geconcludeerd dat meerdere mechanismen bijdragen aan de door regenwormen verbeterde beschikbaarheid van P voor planten.

In Hoofdstuk 3 zijn deze mechanismen verder gekwantificeerd en werd hun relatieve belang vastgesteld. De meting en interpretatie van de binding van protonen (H^+) werd gebruikt om het reactieve oppervlak (RO) van een bodem te bepalen. Dit werd vervolgens gecombineerd met een geavanceerd model voor de binding van ionen aan het oppervlak van metaal-(hydr)oxiden in de bodem. Deze aanpak onthulde een tot nu toe onbekend mechanisme, namelijk de afname van het RO van metaal-(hydr)oxiden in wormenpoep door de groei van de metaal-(hydr)oxide deeltjes. Dit resulteert in een afname van het oppervlak dat beschikbaar is voor P-binding waardoor minder P gebonden wordt door de bodem en er dus meer P beschikbaar is voor opname door planten. De bijdrage van de mechanismen die zorgen voor een verbeterde P-beschikbaarheid door regenwormen in wormenpoep bestaat uit: a) een relatief klein effect van de verhoging van de pH in wormenpoep; b) een belangrijke bijdrage van de toevoeging van P door de toegenomen mineralisatie van organisch materiaal in de darmen van regenwormen; c) een potentieel belangrijk effect van een afname van het RO van wormenpoep door de groei van metaal-(hydr)oxide deeltjes; een proces dat wordt gekatalyseerd door de vorming van Fe^{2+} tijdens de reducerende omstandigheden in de worm die veroorzaakt worden door mineralisatie; en d) minder concurrentie tussen organische stof en P voor de bindingsplaatsen op metaal-(hydr)oxiden in wormenpoep in vergelijking met de bulkgrond. De verkleining van het RO werd alleen waargenomen in bodems die gedomineerd worden door Fe-(hydr)oxiden, en was afwezig of gering in Al-(hydr)oxide-gedomineerde bodems. Dit toonde aan dat de mineralogie van een bodem het effect van regenwormen op een verbeterde P-beschikbaarheid beïnvloedt, en het suggereert dat regenwormen het grootste potentieel hebben voor het verduurzamen van P-gebruik in door Fe-(hydr)oxide-gedomineerde bodems.

Alle mechanismen die zorgen voor een door regenwormen verbeterde P-beschikbaarheid zijn direct of indirect gerelateerd aan het vermogen van een regenworm om het organische materiaal dat hij opneemt te mineraliseren (d.w.z. het omzetten van organische verbindingen naar anorganische). Daarom is de mineralisatie van organisch P in meer detail onderzocht in Hoofdstuk 4. In dit hoofdstuk is aangetoond dat de potentiële activiteit van fosfatase-enzymen, die het mineralisatieproces van monoester P-verbindingen katalyseren, aanzienlijk door regenwormen wordt verhoogd in de volgorde wormenpoep > wormengangen > bulkgrond. De activiteit van fosfatase-enzymen varieerde tussen de verschillende soorten regenwormen en correleerde met het P-

gehalte van het gras. Dit laatste wijst op een direct effect van regenwormen op de P opname van gras door mineralisatie van organisch P.

Het mesocosm-velddexperiment van Hoofdstuk 5 onderzocht het effect van diversiteit in regenwormensoorten op de biomassaproductie en P opname van gras. Er werd geen effect waargenomen van een groter aantal soorten regenwormen op de P opname van het gras aan het einde van het experiment. Met behulp van een meervoudig lineair regressiemodel op de aanwezige wormengemeenschappen werden *A. longa* en *L. terrestris* echter wel geïdentificeerd als sleutelsoorten voor de verhoging van de P opname en biomassaproductie van gras. Verder toonde deze studie aan dat het effect van regenwormen op de P opname van gras niet alleen aanwezig is onder gecontroleerde kasomstandigheden, maar ook waarneembaar is in het veld onder meer realistische omstandigheden voor de praktijk.

In het laatste hoofdstuk van dit proefschrift (Hoofdstuk 6) heb ik mijn belangrijkste bevindingen samengevat, mijn resultaten in een breder perspectief geplaatst en de implicaties van mijn bevindingen voor de landbouwpraktijk besproken. Door regenwormen verbeterde P-beschikbaarheid kan in potentie bijdragen aan een duurzamere P-voeding van landbouwgraslanden in (toekomstig) Nederland. Echter, de uitdagingen van het gebruik van P in de landbouw zijn waarschijnlijk te complex voor een enkelvoudige benadering. Daarom heeft de landbouw een combinatie van verschillende methoden nodig om te voorzien in een adequate P-voeding van gras op bodems met een lage agronomische P-status. Mijn onderzoek laat zien dat het stimuleren van regenwormpopulaties om de benutting van P in de bodem te verbeteren één van die benaderingen zou kunnen zijn. Toekomstig onderzoek zou zich moeten richten op het bepalen van het effect van door regenwormen verbeterde P-beschikbaarheid op mondiale schaal, en op het vaststellen van de bijdrage van regenwormen aan de benutting van bodem P op de lange termijn. Tenslotte zou toekomstig onderzoek zich vooral moeten richten op het combineren van het gebruik van regenwormen met andere benaderingen die gericht zijn op het verhogen van de P opname uit bodems met een lage P-status

Resumen

El fósforo (P) es esencial para el crecimiento de las plantas, pero la mayor parte de P en el suelo no está disponible para ser absorbido por ellas, ya que se adhiere fuertemente a los componentes minerales. Además, el uso de P en la agricultura se ve desafiado tanto por el aumento actual de la población mundial como por el cambio de la dieta humana con tendencia hacia un mayor consumo de productos animales. Esto da como resultado una demanda creciente de alimentos y por consiguiente de P. Además, las reservas de roca fosfórica, como fuente de fertilizante de P mineral, están disminuyendo, mientras que las áreas que históricamente han recibido adiciones excesivas de P enfrentan riesgos ambientales relacionados con las pérdidas crónicas de P disuelto de los suelos a las aguas subterráneas y superficiales. Estos desafíos asociados con el uso agrícola de P nos obligan a aumentar la productividad mientras usamos los fertilizantes de P mineral en una manera más sostenible. La intensificación agrícola sostenible requerirá aportes adecuados de fertilizantes de P, superando los desequilibrios globales y encontrando formas de mejorar la utilización del P ya presente en el suelo. Esta última estrategia requiere encontrar nuevas formas de acceder a las reservas de P del suelo que están menos disponibles para las plantas. Por lo tanto, investigar la contribución de las lombrices de tierra en el mantenimiento de la adquisición óptima de P para las plantas puede contribuir a resolver los desafíos de P que actualmente enfrentamos. En este trabajo de grado, he investigado la contribución que tienen las lombrices al mejoramiento de la disponibilidad de P para la utilización del mismo en el suelo. Mi investigación se centró en el efecto de las lombrices sobre los sistemas de pasturas en los Países Bajos, ya que las pasturas son importantes para la producción mundial de alimentos y además tienen la ventaja de tolerar grandes poblaciones de lombrices.

El objetivo general de mi tesis fue elucidar el efecto de las lombrices de tierra sobre el P disponible en el suelo para las plantas y el consumo de P por el pasto, como también explorar su potencial para aumentar la sustentabilidad de la nutrición de P del pasto. Los objetivos específicos de mi investigación fueron (1) evaluar el efecto de diferentes especies de lombrices sobre el P disponible para las plantas y relacionarlo con a) las propiedades fisicoquímicas del suelo y b) la ecología de las lombrices; (2) determinar los mecanismos de control de la disponibilidad de P mejorada por las lombrices y cuantificar la importancia relativa de todos los mecanismos contribuyentes; (3) evaluar el efecto de la diversidad de lombrices en el P disponible para las plantas; y (4) evaluar si el efecto de las lombrices en el P disponible para las plantas da como resultado un aumento en el consumo de P por el pasto y la producción de biomasa en condiciones reales de campo. Estos objetivos están dirigidos en cuatro estudios (Capítulos 2-5).

El Capítulo 2 mostró que todas las especies de lombrices investigadas aumentaron la reserva total de P adsorbido de manera reversible, pero que existe una gran variación en la capacidad de las especies de alterar la medida en que el P está presente en formas fácilmente disponibles para las plantas. Esta variación no está relacionada con la

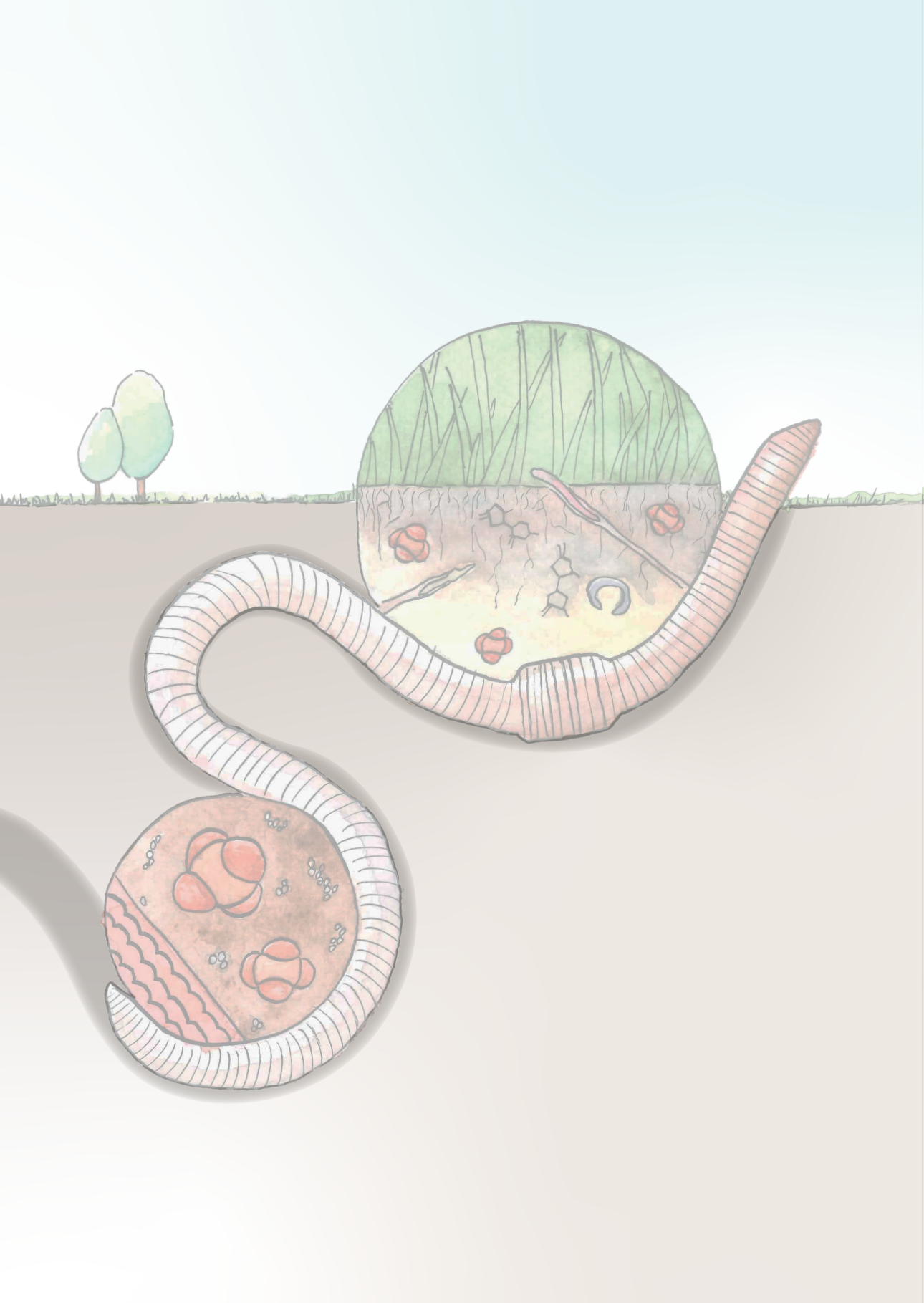
clasificación ecológica clásica de las lombrices, como también las especies del mismo grupo funcional mostraron una gran variación en las propiedades de sus excrementos, como el pH y el carbono orgánico disuelto medido en extractos de agua. A su vez, esta variación podría estar ligada a la variación que existe en la solubilidad de P directamente disponible para las plantas medida en los mismos extractos. Por lo tanto, se concluyó que múltiples mecanismos contribuyeron a la disponibilidad de P mejorada por las lombrices.

En el Capítulo 3, he cuantificado y evaluado estos múltiples mecanismos y su importancia relativa. La medición e interpretación de adsorción de protones (H^+), que deriva el área superficial reactiva (ASR) de un suelo, junto con un modelo avanzado de adsorción de iones a la superficie de los (hidr)óxidos de metal en el suelo, reveló un mecanismo que hasta ahora no había sido considerado: la disminución del ASR de (hidr)óxidos de metal en excrementos de lombrices causado por el crecimiento de partículas de (hidr)óxido de metal. Esto resulta en una disminución del área superficial que está disponible para la adsorción de P al suelo, entonces más P esta disponible para las plantas. La contribución de los mecanismos de control de la disponibilidad de P mejorada por las lombrices en sus excrementos consiste en: a) un efecto relativamente pequeño del aumento del pH en los excrementos; b) una mayor contribución de la adición de P a través de la mineralización de materia orgánica estimulada dentro del intestino de la lombriz; c) un efecto potencialmente mayor de una disminución en el ASR de los excrementos por crecimiento de partículas, que es catalizada por la formación de Fe^{2+} durante las condiciones reductoras causadas por la mineralización; y d) una disminución en la competencia entre la materia orgánica natural y el P por los sitios de unión de los (hidr)óxidos de metal en los excrementos de lombrices en comparación con el suelo. La reducción de la ASR solo se observó para suelos dominados por (hidr)óxido de hierro, mientras que estuvo ausente o fue menor en suelos dominados por (hidr)óxido de aluminio. Esto demostró que la mineralogía del suelo influye en la disponibilidad de P mejorada por las lombrices y sugiere que las lombrices tienen el mayor potencial para mejorar la sostenibilidad del uso de P en suelos dominados por (hidr)óxidos de hierro.

Todos los mecanismos de control de la disponibilidad de P mejorada por las lombrices están directa o indirectamente relacionados con la capacidad de una lombriz de mineralizar (es decir, convertir los compuestos orgánicos en compuestos inorgánicos) el material orgánico que ingiere. Por lo tanto, el mecanismo de mineralización del P orgánico se investigó con más detalle en el Capítulo 4. Esto mostró que la actividad potencial de las enzimas fosfatasa que catalizan el proceso de mineralización de los compuestos de mono éster P, aumentó considerablemente por efecto de las lombrices en el orden excrementos>madrigueras>suelo. La actividad de la fosfatasa varió entre las especies de lombrices y se correlacionó con el contenido de P del pasto, lo que sugiere un efecto directo inducido por las lombrices sobre el consumo de P por el pasto a través de la mineralización de P orgánico.

El experimento de campo sobre mesocosmos en el Capítulo 5 consideró el efecto de la diversidad de lombrices en la producción de biomasa y el consumo de P por el pasto. No se observó un efecto de mayor número de especies de lombrices sobre el consumo de P por el pasto al final del experimento. Sin embargo, utilizando un modelo de regresión lineal múltiple en las comunidades establecidas, se identificó a *A. longa* y *L. terrestris* como especies clave para aumentar el consumo de P por el pasto y la producción de biomasa. Además, este estudio mostró que el efecto de las lombrices en el consumo de P por el pasto no solo estaba presente en condiciones controladas en un invernadero, sino que también se puede observar el mismo efecto a escala mayor en condiciones reales de campo.

En el capítulo final de esta tesis (Capítulo 6), he resumido mis resultados más relevantes, los he ubicado en una perspectiva más amplia y he discutido las implicaciones de mis resultados en práctica. La disponibilidad de P mejorada por las lombrices tiene el potencial de contribuir con una nutrición de P más sostenible para las pasturas agrícolas en (el futuro de) los Países Bajos. Sin embargo, los desafíos asociados con el uso de P en la agricultura probablemente son demasiado grandes para ser resueltos con una sola estrategia. La agricultura necesita una combinación de varias estrategias para obtener una nutrición adecuada de P al pasto en suelos con bajo estado nutricional de P. Mi investigación muestra que estimular las poblaciones de lombrices de tierra para aumentar la utilización de P en el suelo podría ser una de esas estrategias. La investigación futura debe centrarse en evaluar el efecto de la disponibilidad de P mejorada por las lombrices a escala global y su contribución a la utilización del P del suelo durante tiempos más prolongados. Además, la investigación futura debería enfocarse particularmente en combinar lombrices con otras estrategias que tienen como objetivo aumentar la disponibilidad de P en suelos con un estado bajo del mismo.





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About the author

Publications

Affiliations of co-authors

PE&RC Training and Education Statement

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About the author

Margaretha Johanna (Hannah) Vos was born in Ede, the Netherlands, on the 16th of December 1988. She grew up in (what is in the Netherlands considered) a rural area among nature. After her secondary education she worked as a teacher in Guatemala where she discovered how much she liked to teach and explain things to others. After a year, her passion for science and research brought her back to the Netherlands to start the study Soil Water and Atmosphere at Wageningen University. Hannah specialised in soil chemistry and soil biology and during her MSc thesis she conducted a preliminary study to the effect of earthworms on phosphorus availability to grass. The results proved very interesting and led to a first publication and an idea for a research proposal. Meanwhile, the love for Latin America remained and Hannah conducted her internship at the Centro Internacional de Agricultura Tropical (CIAT) in Colombia. She graduated *cum laude* and worked as a researcher at Wageningen University and Universidade de São Paulo in Brazil. When the research proposal based on the results of her MSc thesis was granted, she started her PhD to explore the role of earthworms on enhancing phosphorus availability in soil in more detail. She greatly enjoyed the diversity of disciplines dealt with in this topic on the interphase of soil biology and soil chemistry (field work, laboratory experiments, chemical modelling), as well as the possibility to supervise students. She likes to pay attention to details and solve puzzles. Further, Hannah enjoys the freedom of a sailboat, hiking in nature and discovering the world.



Publications

Vos, H.M.J., Ros, M.B.H., Koopmans, G.F., van Groenigen, J.W., 2014. Do earthworms affect phosphorus availability to grass? A pot experiment. *Soil Biology & Biochemistry* 79, 34-42. doi: 10.1016/j.soilbio.2014.08.018

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Vos, H.M.J., Koopmans, G.F., Beezemer, L., de Goede, R.G.M., Hiemstra, T., Van Groenigen, J.W., 2019 Large variations in readily-available phosphorus in casts of eight earthworm species are linked to cast properties. *Soil Biology & Biochemistry*. 138, 107583. doi: 10.1016/j.soilbio.2019.107583

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Vos H.M.J., Koopmans G.F., Ferron, L.M.E., Oenema, O., Van Groenigen, J.W. 2022. Do earthworms increase grass biomass production and phosphorus uptake under field conditions? *Accepted for publication, subject to revisions, by Applied Soil Ecology*

Vos H.M.J., Hiemstra, T., Prado Lopez M., Van Groenigen, J.W., Voegelin, A., Mangold, S., Koopmans G.F. 2022. A new view on earthworm-enhanced solubility of soil phosphorus. *Accepted for publication, subject to revisions, by Geoderma*

Vos H.M.J., Zweig R., Koopmans G.F., Van Groenigen, J.W. Phosphatase activity in the drilosphere and its link to phosphorus uptake of grass. *In preparation*

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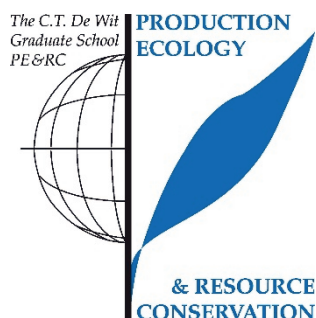
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PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)



Review of literature (4.5 ECTS)

- SUPPER: Stimulating Uptake of Phosphorus in Pastures through EaRthworms

Writing of project proposal (2 ECTS)

- Contribution to the NWO open programme proposal SUPPER: Stimulating Uptake of Phosphorus in Pastures through EaRthworms

Post-graduate courses (4.5 ECTS)

- Basic statistics; WUR (2016)
- The use of trait based approaches in community ecology and stress ecology; Instituto do Ambiente, Tecnologia e Vida; Coimbra, Portugal (2017)
- Soil ecology; Ecology & Evolution, PE&RC, SENSE (2019)

Invited review of (unpublished) journal manuscript (5 ECTS)

- Geoderma: influence of tropical millipedes on soil P (2016)
- Ecological Indicators: P availability in Nardus grasslands (2019)
- Soil Ecology Letters: effect of glyphosate on nutrient content of earthworm casts (2020)
- Applied Soil Ecology: effect of surrounding and ageing on properties of earthworm casts (2020)
- Geoderma: biochemical P transformations affected by earthworms (2021)

Deficiency, refresh, brush-up courses (6 ECTS)

- Ecological modelling and data analysis in R; WUR (2017)

Competence strengthening/skills courses (3.15 ECTS)

- Workshop the Choice; ESG (2016)
- Publish for impact; WGS (2017)
- Teaching and supervising thesis students; WGS (2017)
- Scientific integrity; WGS (2019)
- Career orientation; WGS (2021)
- Proposition writing; WGS (2021)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.55 ECTS)

- PE&RC First year weekend (2016)
- PE&RC Day (2016)
- PE&RC Midterm weekend (2019)
- PE&RC Last year online afternoon (2020)
- PE&RC Last year weekend (2021)

Discussion groups/local seminars or scientific meetings (6.95 ECTS)

- Plant-soil interactions discussion group (2016-2021)
- DISQ Thesis ring (2016-2021)
- Frontiers in ecology: the soil-plant interphase (2017)
- Festschrift symposium (2018)
- Wageningen Soil Conference; workshops on mycorrhiza characterization, fertilisation with chelates and spectral libraries (2019)
- Four seasons of soil (2019-2021)

International symposia, workshops and conferences (8.5 ECTS)

- NAEM; poster presentation; Lunteren, the Netherlands (2017)
- Annual meeting ASA, CSSA, SSSA; oral presentation; Tampa, Florida, USA (2017)
- 1st International Earthworm Congress (ISEE); oral presentation; Shanghai, China (2018)
- Symposium on Phosphorus in Soils and Plants (PSP6); oral presentation; Leuven, Belgium (2018)

Societally relevant exposure (1.55 ECTS)

- TV Episode of the NTR program: de kennis van nu (2017)
- Organisation of the Wageningen worm charming championship (2017, 2018, 2019)
- YouTube PhD pitch for website soil science cluster (2021)
- Earthworm activity at weekend van de wetenschap (2021)

Lecturing/supervision of practicals/tutorials (1.35 ECTS)

- Guest lecture biological interactions in soils (2016)
- Tutorial plant soil interactions (2017)
- Earthworm field lecture at lab skills course (2018, 2019, 2021)

BSc/MSc thesis supervision (15 ECTS)

- Variation in P related cast properties among earthworm species
- Phosphatase activity in earthworm cast
- Field experiment to earthworm effect on P uptake by grass; start
- Field experiment to earthworm effect on P uptake by grass; first growing season

- Field experiment to earthworm effect on P uptake by grass; second growing season
- Effect of earthworm community diversity on grass P uptake
- Earthworm activity effect on P uptake by grass
- Literature review on the effect of earthworms on nutrient availability in the field
- Field experiment to earthworm effect on P uptake by grass; end
- Effect of earthworm community diversity on grass P uptake and phosphatase activity

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