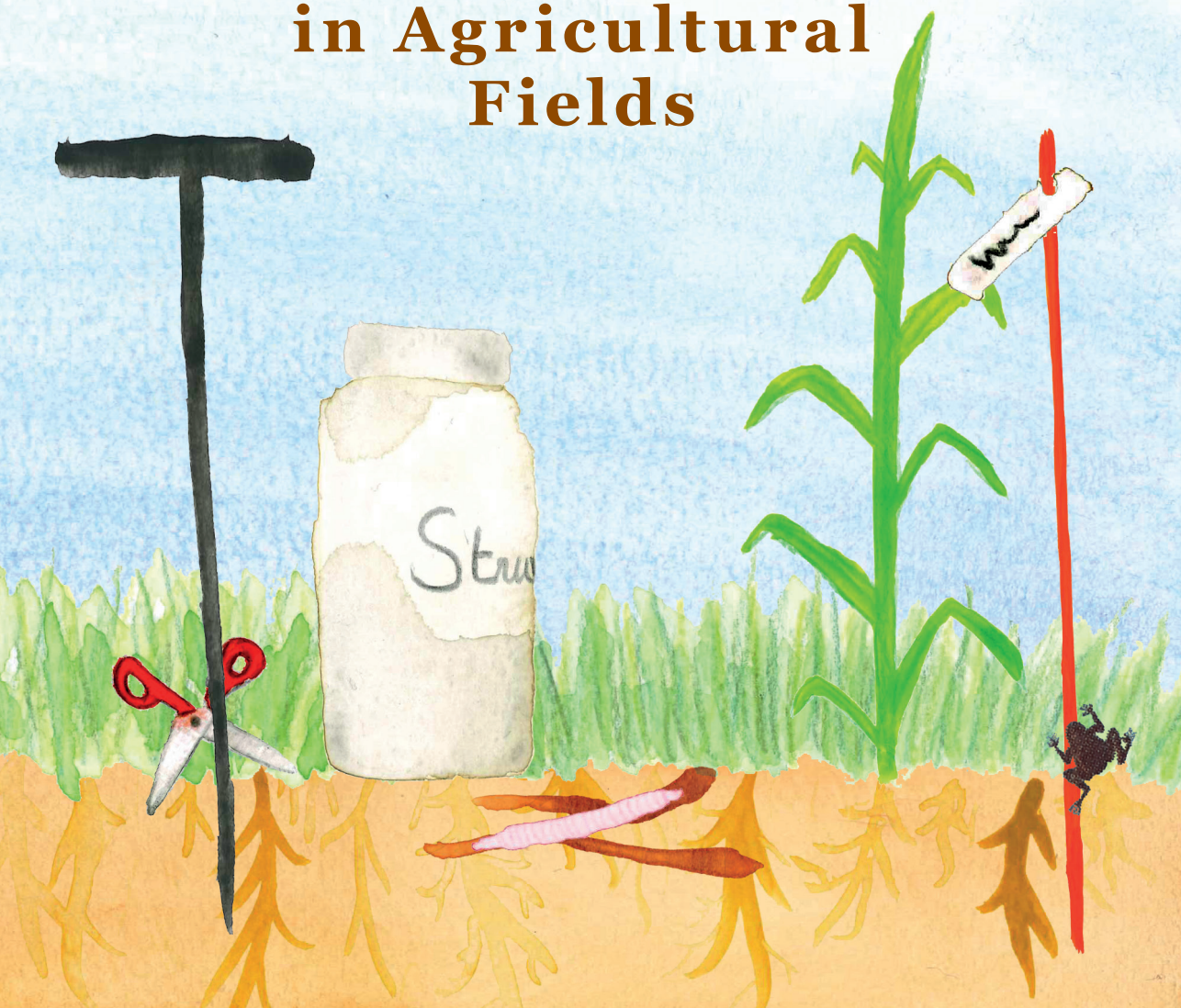
Struvite and Biodiversity to Improve Phosphorus Use in Agricultural Fields



Laura M.E. Ferron

Propositions

1. Struvite is a better phosphorus fertiliser than triple superphosphate. (this thesis)
2. The plant N:P ratio is an overrated tool to detect N or P limitations. (this thesis)
3. The detection of autism in utero will set back science.
4. That a PhD candidate cannot have a rich personal life is a self-fulfilling prophecy.
5. The invention of emails has increased the risk of burnout.
6. The higher rate of homebirth in the Netherlands leads to happier children.

Propositions belonging to the thesis, entitled

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Struvite and Biodiversity to Improve Phosphorus Use in Agricultural Fields

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Thesis

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Chapter 1

General Introduction

Laura M.E. Ferron

1 Agroecosystems in Crisis

1.1 Historical Reasons for Fertilisation Intensification

Humans have been growing their own food for thousands of years and the invention of agriculture is at the heart of the biological success of our species (Ladizinsky, 1998). After the domestication of crops and livestock to build our agroecosystems, the second most important factor of our agricultural success is the understanding and manipulation of biogeochemical cycles and the resulting need for fertilisation of crops (Scherer et al., 2009). For most of our agricultural history, these biogeochemical cycles have been constrained by what our planet could provide through natural mechanisms such as symbiotic nitrogen (N) fixation by rhizobacteria and weathering of naturally occurring phosphorus (P) minerals, and manure production from livestock (Allen, 2008; Emsley, 2002; Urbanski et al., 2022).

This has all changed during the last 150 years: there has been a parallel exponential growth of the global population and of the efficiency of growing food (Ritchie et al., 2023). This became possible, partly because of the mechanisation of agriculture, the innovation in plant breeding and protection and partly because of the intensification of fertilisation. According to Liebig's law of the minimum, the yield of a crop will be limited by the nutrient that is present in the relatively smallest amount with respect to plant needs (Ebelhar et al., 2008). In most environments, the limiting nutrient will either be N or P (Du et al., 2020). That is why during the second half of the 19th century, N and P-rich resources like guano, saltpetre and later phosphate rocks were transported around the globe to fertilise crops (Ashley et al., 2011; Mazoyer and Roudart, 2006). Mineral P such as single superphosphate and later triple superphosphate (TSP) started being produced first from bone meal, then later from phosphate rocks and introduced as a P fertiliser by Lawes and Gilbert at the turn of the century (Emsley, 2002). In 1910, the Haber-Bosh process was invented: it allowed the conversion from atmospheric dinitrogen (N2) to ammonia (NH3) (Barona et al., 2018). At first the NH3 produced served for the production of explosive and munition. Only after the Second World War and its hunger years, during the so-called Green Revolution initiated by European countries to ensure food self-sufficiency, N and P synthetic fertilisers started being used more systematically (Ashley et al., 2011; Dawson and Hilton, 2011; Emmerson et al., 2016). In addition to the popularisation of single and triple superphosphate (TSP) (Emsley, 2002), the Haber-Bosch process, which was first famous for its military uses, actually fed millions of people (Ritchie, 2017).

1.2 Associated Geopolitical and Environmental Issues

1.2.1 Crumbling Phosphate Rock Stocks and International Tensions

Although N is composing the majority of our atmosphere, the main concentrated resource of P accessible to humans for fertilisation is phosphate rocks. Beyond the environmental impact of mining an intrinsically finite resource, there are geopolitical tensions surrounding the use of phosphate rocks. Although the mineral is present in a number of countries, its distribution is very heterogenous: Morocco is claiming 70.4% of the global stocks, followed by China (4.5%), Egypt (3.9%) and Algeria (3.1%), while Europe had barely any reserves to speak of until the recent discovery of a large stock in Norway (Harper, 2023; U.S. Geological Survey, 2022). The important reserve claimed by Morocco is located in Western Sahara. The sovereignty of this territory has been disputed since the end of the colonisation by Spain in 1976 (Drury, 2013). However, the earlier claims that the world will reach "peak P" production within the next decades (Cordell et al., 2009) is increasingly debated because of both its calculation methods and the seemingly constant increase of the reserve size as new deposits are discovered or purity thresholds are modified. It is clear though, that this resource is causing geopolitical tensions and will remain to do so in the foreseen future (Cordell and White, 2013; Edixhoven et al., 2014; Rhodes, 2013; Walan et al., 2014). This is one of the reasons that the European Union has proclaimed P a "Critical Raw Material" (European Union Commission, 2020).

1.2.2 Leaking Biogeochemical Cycles and Eutrophication

Modern agriculture in developed countries has been remarkably successful in increasing both the soil N and P concentrations during the last century, either by direct mineral fertiliser application or by importing N and P-rich products in the form of animal feed (Demay et al., 2023; Nesme et al., 2018; Wang et al., 2022). This increase in N and P availably has a downside: agricultural practices increased the risk of soil erosion and leaching (Lehmann and Schroth, 2002; Montgomery, 2007), resulting in more N and P entering groundwater, ditches, lakes, waterwaysand eventually the sea (Lehmann and Schroth, 2002). The enrichment of surface waters with N and P can contribute to eutrophication when either of these nutrients limit primary production in freshwater ecosystems (Correll, 1998; Werner, 2009). The impacts of eutrophication include the excessive growth of algae, which can have serious consequences for human health and freshwater ecosystems (Akinnawo, 2023).

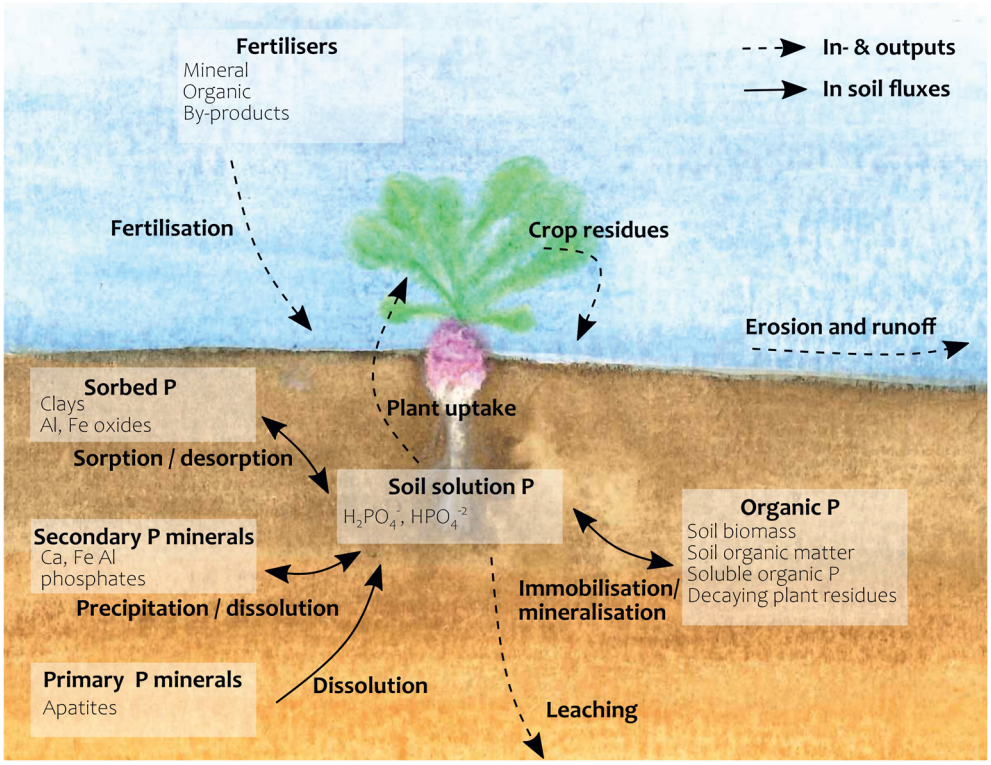


Figure 1.1 The soil P cycle in agroecosystems. Unlike N, P is typically only available in small quantities in the soil solution at with most soil P being present as inorganic P adsorbed to soil particles including Fe- and Al-(hydr)oxides and clay mineral edges or in calcium- or magnesium-phosphate minerals or as organic P when it is contained within organic matter (Gérard, 2016; Hesterberg, 2010; Weng et al., 2011). Figure adapted from Pierzynski et al (2005).

1.2.3 Laughing Gas with Serious Consequences

Besides problems with leaky N and P cycles, modern agriculture is also a crucial factor in climate change. The global loss of soil carbon (C), the deforestation, the use of fuel-consuming machinery and the fertiliser production are responsible for 739 Pg CO_2 emitted globally between 1851 and 2021, the equivalent of an average temperature increase of 0.33 °C (Jones et al., 2023). More than the consequence of an increased carbon dioxide (CO_2) concentration in the atmosphere, climate change is enhanced by emissions of trace gases such as nitrous oxide (N_2O) as it has a 273-fold higher global warming potential than CO_2 on a molecular basis (Intergovernmental Panel On Climate Change, 2023). Nitrous oxide emission is the result of a number of complex biochemical nitrogenous processes occurring at the interface of the soil and the atmosphere, chief among them nitrification and denitrification (Figure 1.2, van Groenigen et al., 2015). Although these processes

existed before the so-called Green Revolution, the application of N fertilisers in agriculture has exponentially increased the production rate of N_2O: it is estimated that between 1851 and 2021, land use (change) and forestry alone have resulted in 485 Tg N_2O globally, the equivalent of a 0.06 °C increase in average global temperature (Jones et al., 2023). In soils, N_2O emissions are impacted by (a-)biotic factors such as the climate, soil type (pH and texture) as well as the microbe biodiversity (Butterbach-Bahl et al., 2013; Cong Wang et al., 2021). While N_2O emissions are directly linked to N fertilisation practices, they are also impacted by the biogeochemical cycles of other elements such as C and P (Baral et al., 2017; Intergovernmental Panel On Climate Change, 2023; Li et al., 2021).

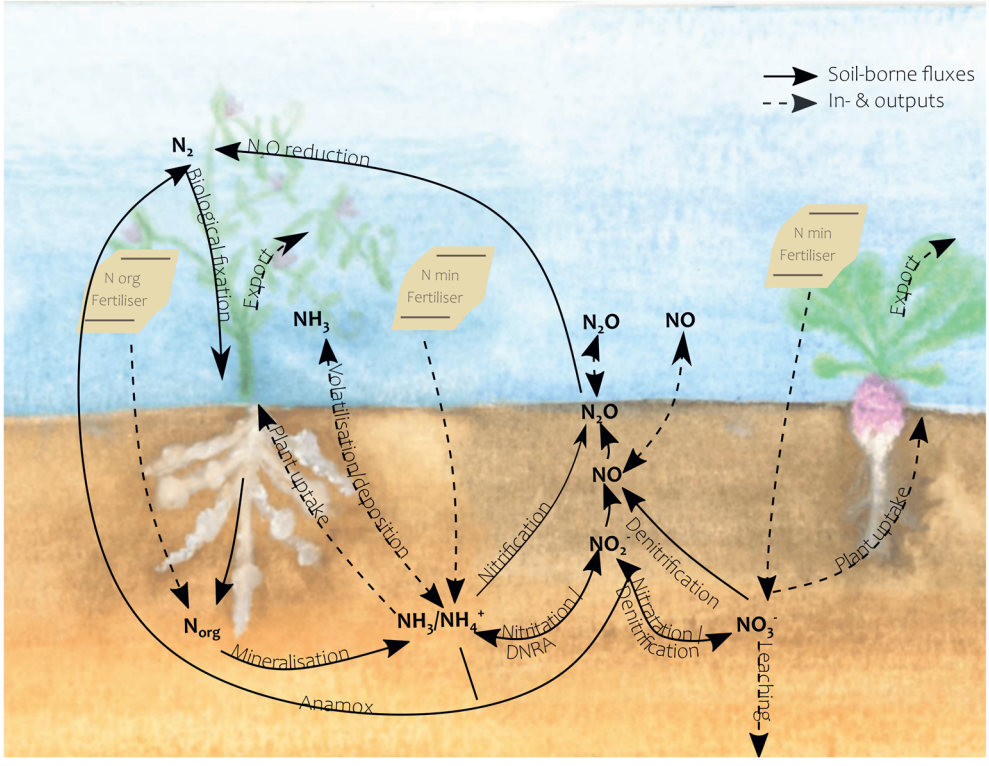


Figure 1.2 Soil N cycle in agroecosystems with a focus on nitrous oxide emissions. Fertilisers increase the pools of nitrate, ammonium and organic N respectively depending on their nature. Plants take up N in the form of nitrate or ammonium. Nitrous oxide emissions are the results of nitrification and denitrification mainly operated by soil micro-organisms. N_2: dinitrogen, NH_3: ammonia, NH_4^+ ammonium, NO_2^-: nitrite, NO_3^-: nitrate, NO: nitric oxide, N_2O: nitrous oxide, DNRA: Dissimilatory nitrate reduction to ammonium. Figure adapted from Pierzynski et al. (2005), Simon and Klotz (2013) and van Groenigen et al. (2015).

2 Towards More Sustainable Agroecosystems

Given the geopolitical tensions surrounding phosphate rocks and the environmental challenges related to the (over-)use of synthetic N and P fertilisers, agricultural practices need to be rethought. With respect to P, the various solutions fall into two categories: using circular P fertilisers and improving the P use efficiency (PUE) in the field (Cordell and White, 2013; Hinsinger and George, 2016). In this thesis, I focused on using struvite, a circular fertiliser, as well as making the most of biodiversity, both above- and belowground, to increase the PUE of agroecosystems.

2.1 Through a Novel Fertiliser

2.1.1 Struvite: a Slow-release, Circular Fertiliser

Struvite ($NH_4MgPO_4 \cdot 6\ H_2O$) is a mineral with equimolar amounts of N, P and magnesium. In the alkaline conditions of wastewater treatment plants, it precipitates spontaneously and thus has the potential to be recovered in a more controlled manner to be valorised as a fertiliser (Achilleos et al., 2022; Shih and Yan, 2016). The constant of solubility product (K_{sp}) of struvite is estimated to be between $4.37 \cdot 10^{-14}$ to $3.89 \cdot 10^{-10}$ (Bhuiyan et al., 2007) meaning that struvite will precipitate at relatively low ionic concentrations of magnesium, ammonium and phosphate with respect to wastewater. In return, this means that struvite has a much lower dissolution compared to other conventional P fertilisers (Degryse et al., 2017).

2.1.2 Struvite: a Performant Fertiliser?

Its use as a fertiliser has been tested in the last decade but results remain ambiguous. This is to a large extend related to the design of experimental trials used to test struvite as a fertiliser, which had a large impact on the obtained results (Anderson et al., 2020; Hertzberger et al., 2020; Huygens and Saveyn, 2018). Indeed, the duration of the experiment, the soil mass for pot trials and the soil P tests used all played a role in explaining the yield and P uptake under struvite fertilisation respective to conventional P fertilisers in a meta-analysis (Hertzberger et al., 2020). Struvite as often been tested in too small pots, with soils that do not call for P fertilisation and with very short experimental period (Hertzberger et al., 2020). There is a need for field-based trials that will better reflect the actual fertiliser potential of struvite.

Another parameter complexifying the assessment of struvite as a fertiliser is its low solubility: struvite dissolution varies depending on the soil pH, nutrient contents and the granular size of the fertiliser (Degryse et al., 2017; Talboys et al., 2016). Struvite dissolves better in slightly acid soils and in powdered form. Additionally, the low solubility of struvite makes the measurement of soil P tests challenging as struvite

Box 1 Dutch soil P tests

How to measure P in the soil is varying widely across countries because of soil types and historical differences (Jordan-Meille et al., 2012). In the Netherlands, a combination of two soil P tests are at the base of the classification of soils according to their P status determining the legal amount of P fertilisers farmers may apply (Commissie Bemesting Grasland en Voedergewassen, 2023).

The first test is the extraction of P in the soil solution with a 0.01 *M* $CaCl_2$ solution in a ratio of 1:10 (weight:volume) (Houba et al., 2000). This pool, referred to as P-$CaCl_2$, is in equilibrium with P adsorbed to poorly crystalline Fe- and Al-(hydr)-oxides and clay mineral edges in soil. As an intensity measurement, it is the reflection of the soil P buffering capacity (Nawara et al., 2017).

The second test is the extraction of P with a mixture of acetic acid and ammonium lactate (P-AL) at a 1:20 ratio (weight:volume) (Egnér et al., 1960). This pool is a quantity measurement and relates more directly to P loading of poorly crystalline Fe- and Al-(hydr)oxides as well as the amount of P adsorbed to these metal-(hydr)oxides (Schoumans and Groenendijk, 2000; van Doorn et al., 2023).

These two soil P test can be used to classify the agronomic soil P status, which subsequently can be used as a basis for P fertiliser recommendations (Commissie Bemesting Grasland en Voedergewassen, 2023).

An additional soil P test commonly used is the extraction of P with 0.2 *M* acid ammonium oxalate at a ratio of 1:20 (weight:volume) (Schwertmann, 1964). This pool refers to the amount of P reversibly adsorbed to Fe- and Al-(hydr)oxides. From this soil P test and the amounts of Fe- and Al-(hydr)oxides extracted simultaneously with the same extraction method, it is possible to calculate the P loading α of the soil (van der Zee and van Riemsdijk, 1988).

might be remaining in soils after harvest, potentially until the start of the next growing season and then only dissolve to some extend during soil tests, leading to an overestimation of soil P bioavailability (Gu et al., 2021). Soil P tests regularly used in the Netherlands (Box 1) have not yet been tested with soil containing struvite.

The poor dissolution of struvite could be an advantage as slow-release is a property sought after for fertilisers for some crops growing over winter like wheat or taking up P over long period of time, whereas some other crops such as maize need a timely availability of nutrients (Liu et al., 2014; Noordwijk et al., 1990; Smilde, 1972).

One general way to increase fertiliser efficiency is through placement near the plants in hotspots of band application. This is especially useful for plants that need a timely fertiliser input for less mobile nutrients such as P (Nkebiwe et al., 2016). Placed struvite has only been sporadically tested but could be advantageous for some plant species such as maize (Jama-Rodzeńska et al., 2023; Talboys et al., 2016).

Although until now most of the studies have focused on the capacity of struvite as a P fertiliser, it also contains a equimolar amount of N that should also be taken into

consideration as its slow release likely has environmental benefit with respect to the N biogeochemical cycle (Leon et al., 2024; Liu et al., 2011).

2.2 Through a More Biodiverse Agroecosystem

In recent years, in addition to modifying fertilisation practices, efforts have been made to apply ecological concepts to agrosystems in an attempt to increase their PUE and thus their sustainability. One such concept is the consideration of biodiversity at agricultural field level both for the cultivated species as well as for the inherent diversity of species the systems such as the earthworms (Wezel et al., 2020).

2.2.1 Cultivated Biodiversity

Increasing plant diversity has been shown to increase nutrient uptake (Chen and Chang, 2022). There are many ways to increase the diversity of cultivated plants at the field scale, for example using practices such as intercropping and crop rotation (Isbell et al., 2017). One approach is to convert ryegrass production grassland to a multi-species (other grasses as well as legumes and herbs) intensively managed grassland (Bullock et al., 2020). Plants use several mechanisms to take up nutrients from the soil and these can be classified into two categories (Marschener, 1998; Shen et al., 2011; Wen et al., 2019). First, *nutrient-mining* strategies (*sensu* Lambers et al., 2008) are relying on root exudation to enhance N and P availability in the soil surrounding living roots, the so-called rhizosphere (Hinsinger, 2001). Plants can for example modify the pH of the rhizosphere to increase the dissolution of secondary P minerals, secrete enzymes to help unlock nutrients to catalyse the mineralization of organic P. Exudates also serve as a C source to stimulate the activity and diversity of microorganisms in the rhizosphere, which in turn will make P more available through desorption, mineralisation and turnover effects (Richardson et al., 2009a). This first strategy is taping into the relative unavailability of P in a given soil volume. Second, *nutrient-scavenging* strategies (sensu Lambers et al., 2008) rely on soil exploration to take up more nutrients (Lynch and Brown, 2008; Richardson et al., 2009a). This is either done by elongation of the plant's own roots, resulting in thin, long roots with a high turnover, or by the extension of the plant's root systems through the hyphae of Arbuscular Mycorrhizal Fungi (AMF). This second strategy is taping into the immobility of P over different soil volumes. Although plants will use both of these strategies simultaneously to achieve adequate nutrient uptake, they are likely to favour one of them (Bergmann et al., 2020; Wen et al., 2019). Combining plants at the field-scale also enables combining their nutrient acquisition strategies, and this results in better overall nutrient uptake through effects of complementarity or competition (Oram et al., 2018). Although the effect of increased plant diversity on P cycling is well studied in natural grasslands (Hacker et al., 2015; Oelmann et

al., 2021, 2011), little work has been done on the implication of increased plant diversity in intensively managed grasslands. These systems are more fertilised, more often harvested and should meet nutrient requirement of livestock. The few studies that link biodiversity to the P biogeochemical cycle in intensively managed grasslands focused on the introduction of legumes (Rumpel et al., 2015) or the individual study of grass species (Ros et al., 2018). To my knowledge, the potential P benefits of combining several grass species has not been studied in the context of intensively managed grasslands.

2.2.2 Diversity of Soil Biota

An important soil organism in grassland ecosystems is earthworm. Earthworms are among the most abundant and important soil faunal groups in temperate regions, both in terms of density and diversity (Phillips et al., 2019). They have a positive effect on plant yield (van Groenigen et al., 2014). Earthworms benefit plant growth through their creation of hotspots of otherwise unavailable N and P through effect of concentration and enhance availability in their casts (Lang et al., 2023; van Groenigen et al., 2019, 2014; Vos, 2022). For P specifically, earthworms enhance the availability of both organic and inorganic P from the soil. They also impact the fate of some less available P fertilisers such as struvite, although the extend of this effect depends on earthworm species (Mackay et al., 1983; Vos, 2022). The impact of earthworms on struvite availability has not yet been studied, aside from the effect of their composted casts used as a bio-stimulant to increase struvite dissolution (Hernández Jiménez et al., 2020). A limitation of our knowledge on earthworms' impacts of the biogeochemical cycle is that it largely relies on pot experiment (Lang et al., 2023; van Groenigen et al., 2019; Vos, 2022).

Earthworm species are numerous with some 7000 species described worldwide, of which 30 can be found in the Netherlands (Krediet, 2019; Orgiazzi et al., 2016). The species are traditionally divided into three ecological groups. *Epigeic* earthworms live in the organic horizon of the soil and feed on the litter found there. *Endogeic* earthworms live deeper in the soil and feed on the soil organic matter. *Anecic* earthworms live in permanent burrows opening up at the soil surface where they forage for litter (Bouché, 1977; Örley, 1885). These three categories should be considered with nuance as few earthworms truly belong to one category but rather are characterised by their relationship to the three theoretical group mentioned above (Bottinelli et al., 2020). Moreover, the belonging of earthworms to one ecological group or another did not explain the variation of readily available P in their casts (Vos et al., 2019).

3 The Dutch Context

The Netherlands is known for its dairy production and this is reflected in its agricultural land use. In 2018, silage maize, which serves as feed for livestock covered 205574 ha or 10% of the agricultural land use (Centraal Bureau voor de Statistiek, 2024). The fertilisation of silage maize is advised to be done in band application and for both maize and grasslands, the recommended P fertilisation is based on the agronomic soil P status (Commissie Bemesting Grasland en Voedergewassen, 2023).

Grasslands are at the heart of the Dutch dairy production. In 2018, they covered 936000 ha of the country which represents 47% of the total agricultural land use (Centraal Bureau voor de Statistiek and Eurofins-Agro, 2018). English ryegrass (*Lolium perenne* L.) is the species of choice for grasslands (Remmelink et al., 2018). This species is intensively managed with four to six harvests and high N fertilisation rates, which could be as high as 382 kg $N \cdot ha^{-1}$ per year thanks to an European Union derogation that will stop to be effective in 2026 (Commissie Bemesting Grasland en Voedergewassen, 2023; Evers and de Haan, 2024).

Grasslands are largely localised on the country's sandy soils, with 44% of this land used allocated to sandy soils (Schils et al., 2002). These soils are naturally poorly fertile as they originally contain little nutrients and have a low cation exchange capacity due to the low amount of clay and soil organic matter. Moreover, they are prone to acidification, dryness and leaching (Huang and Hartemink, 2020). Dutch soils, however, have a history of P over-fertilisation and thus in 2013, 56% of Dutch grasslands had a soil P status considered as "high' (Amery and Schoumans, 2014). Recent restriction on the limit of P application, as well as the cancelling of the Eureopean Union derogation are likely reduce the overall P concentration of Dutch soils (Evers and de Haan, 2024; van Middelkoop et al., 2016).

4 Objectives and Outline

Because of environmental issues and geopolitical tensions, the agricultural P cycle is unsustainable and need to be rethought. This will guide my research objectives (RO).

One way improve the agricultural P cycle is to increase the PUE through using biodiversity in the fields. Although some agricultural systems as well as natural grasslands have been shown to further close the P cycle, an increase in cultivated species richness in intensively managed grasslands has not yet been demonstrated to increase the PUE. Furthermore, earthworms have an impact on the P cycle and may participate to increase the PUE in intensively managed grasslands. However,

our knowledge of their impact on soil P mostly come from controlled, greenhouse experiments and strong evidence that this is also true in the field is lacking. My first objective will be **(RO1) to assess the potential of cultivated and soil biodiversity to further increase the PUE in intensively managed grasslands**.

Another way to improve the agricultural P cycle could be to use struvite, a recycled, circular fertiliser that does not rely on the mining of a finite resource. Moreover, struvite may have environmental benefits in comparison to conventional P fertilisers because of its slow-release property. This slow release property is likely at the origin of the ambiguous nature of struvite as a fertiliser. To use struvite to its full potential, we first need **(RO2) to understand the plant and soil responses of agroecosystems fertilised with struvite**. Only after getting a better understanding of struvite's functioning as a fertiliser will we be able **(RO3) to determine the agronomic conditions for its successful use as a fertiliser**.

Within the Dutch context, the three research objectives led to the identification and realisation of four different studies, making up the respective chapters of this thesis.

In chapter 2, I investigate the benefit of increasing plant diversity in a field experiment mimicking an intensively managed grassland>. In order to understand the relations between plant diversity and soil P dynamics, I determine the plant P uptake, as well as the associated alteration of plant strategies in terms of roots, enzymes and microbial activity (RO1).

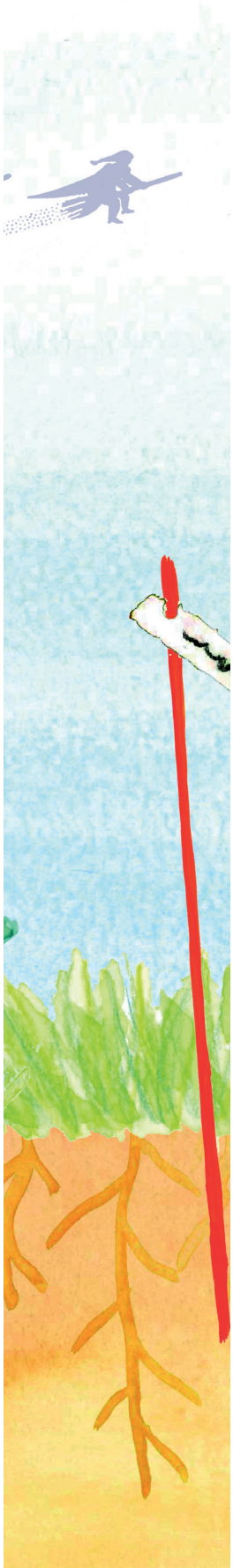
In chapter 3, I test struvite as a placed P fertiliser for maize in a greenhouse pot experiment with a noncalcareous sandy soil and a loamy soil (RO2) and look at the P of plants and soils to get a better understanding of the mechanisms affecting effectiveness of struvite fertilisation. Furthermore, I test the validity of two soil P tests (i.e., P-$CaCl_2$ and P-AL) for the two soils amended with struvite; as these tests

are routinely used for determining the agronomic soil P status within the context of the Dutch fertiliser recommendation system (RO3).

In chapter 4, I investigate an understudied but important aspect of struvite fertilisation: its contribution to N cycling, in particular its impact on N_2O emissions under two soils with a contrasting agronomic soil P status in a greenhouse pot experiment (RO3). I also compare differences between two different forms of struvite (granules and powder) as a source of N for plant growth (RO2).

In chapter 5, I combine both the circular fertilisation and biodiversity aspects, studying the effectiveness of struvite in field conditions (RO3) under various rates of earthworm diversity in relation to root traits (RO1 and 3).

Finally, in chapter 6, the results of the four individual studies are brought together and discussed within the framework of the three research objectives. Additionally, methodological challenges encountered are reflected upon.



Chapter 2

Combination of *Lolium perenne* L. and *Festuca arundinacea* Schreb. Improve Yields Under Low Phosphorus Availability

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Abstract

Phosphorus (P) is one of the main nutrients for all plants, including grasses. However, sources of P fertiliser are not renewable, are not evenly distributed and over-fertilisation can lead to serious environmental degradation. Smart combinations of grasses may be able to more efficiently take up P from soils through complementarity. In a two-year field mesocosm experiment, we compared the performance of *Lolium perenne* L. and *Festuca arundinacea* Schreb. in monocultures and in combination, as well as a mixture of both species with a tetraploid variety of *L. perenne* and *Phleum pratense* L. Plants were grown in an unfertilised low P soil and in P fertilised soil for two growing seasons. We measured biomass production, root traits, nutrient uptake, microbial biomass and enzymatic activities. In the unfertilised plots, the combination of *L. perenne* and *F. arundinacea* generated the highest cumulative yields (25951 ± 4059 $kg \cdot ha^{-1}$), relative total yield (>1) and P nutrition index (0.79). We related this to the complementarity found in root traits and lower intraspecific competition of *F. arundinacea* and *L. perenne* diploid. *F. arundinacea* produced higher root biomass than *L. perenne* diploid at deeper soil layers (98 *vs* 44 $g \cdot m^{-2}$; $p < 0.05$). On the other hand, *L. perenne* diploid had significantly finer roots than *F. arundinacea* both at topsoil and bottom layers (0.19 *vs* 0.22 mm and 0.19 *vs* 0.23 mm at top and bottom layers respectively). The 4 species combination did not result in higher yields. Our results show that, in low P soils, combinations of grass species with contrasting root traits could lead to significantly higher yields than monocultures.

Keywords

Phosphorus, grassland, root morphology, microorganisms, complementarity

1 Introduction

Phosphorus (P) is one of the main nutrients for plants and therefore essential for grassland production (Aydin and Uzun, 2005). P has also been included in the list of critical raw materials by the European Union because of its uneven distribution across the globe (Bertrand et al., 2016). At the same time, over-fertilisation with P in the past has turned almost 60% of European grassland soils unresponsive to P fertilisation (Recena et al., 2022) and has caused eutrophication and degradation of natural ecosystems (Yuan et al., 2018). It is therefore urgent to reduce the environmental impacts of P fertilisation by reducing the overall demand for P fertilisers in agriculture without compromising agricultural production (Garske et al., 2020).

Seeding mixtures of plants can be an option to reduce P dependency while increasing plant yields and nutrient uptake in agricultural systems (Postma and Lynch, 2012; Xue et al., 2016). In situations of P limitation, complementary root traits and delayed resource access by different plants could result in higher yields in mixtures than in monocultures (Bakker et al., 2018; Oram et al., 2018). Moreover, some grass species can facilitate the uptake of P of the other species they are grown in combination. This can be achieved through a change in soil chemical properties, such as pH, due to the release of root exudates, or by the promotion of soil microbial activity that could ultimately lead to a higher P availability (Giles et al., 2017; Khan et al., 2007; Xue et al., 2016). Research on such mechanisms has focused mostly in natural and permanent grasslands, yet complementary use of P in intensively managed grasslands remains poorly investigated.

In Europe, perennial or English ryegrass (*Lolium perenne L.)* is one of the main cultivated grass species (Becker et al., 2020; Rogers et al., 2019). This grass species is well adapted to a temperate climate and it provides high yields and feeding values (Becker et al., 2020). In recent years, the combination of *L. perenne* with tall fescue (*Festuca arundinacea* Schreb.) has gained interest because of *F. arundinacea*'s higher tolerance to drought (Cougnon et al., 2013), providing better access to water under drought events. This quite common combination of *L. perenne* and *F. arundinacea*, however, has received little attention as a potential way to increase P access to grasslands. This combination could be of special interest in areas of the world where the inputs of P fertiliser are expected to decline such as north-western Europe. There are a few challenges related to *F. arundinacea* that could inhibit its widespread cultivation in temperate regions. One of its major drawbacks is its low palatability compared to *L. perenne* (Cougnon et al., 2013). Moreover, the high silicon content of the leaves of *F. arundinacea* results in low digestibility (Hartley et

al., 2015). To mitigate these disadvantages, one strategy is to combine *F. arundinacea* in grass mixtures with species with higher palatability.

Here we quantified the P uptake and yield benefits of introducing *F. arundinacea* in combination with *L. perenne*, or in association with other palatable grass species (timothy (*Phleum pratense L.*) and a tetraploid variety of *L. perenne*). We compared performance with and without P fertilisation, and investigated the relationships between yields and root traits, soil microbial activity or soil properties. We tested the general hypothesis that *F. arundinacea*, with its deeper rooting system, would facilitate access by the grassland mixtures to P in the soil and improve yields in low P conditions, and that this could be related to a complementarity effect in root traits as well as an increased P solubilization by the microbial community.

2 Materials and Methods

2.1 Experimental Setup

We conducted a two-year (2019 – 2020) field mesocosm experiment on the campus of Wageningen University, The Netherlands (51.989° N, 5.657' E). The climate at the field location is temperate maritime (Cfb according to Köppen-Geiger classification). Further detailed climatic information during the two seasons can be found in the supplementary material (Figure 2.S1). The mesocosms consisted of wooden boxes (0.75 wide x 0.75 tall m and 0.40 m deep) that were installed in the field (Figure 2.S2). The boxes were then divided in half, so each mesocosm consisted of two plots with a surface of 0.28 m^2 each. The mesocosms had a weed control fabric on the bottom side that allowed water to leach out but prevented roots from exploring the soil outside the wood box, both downwards and sideways. Two years before the start of the experiment, the boxes were filled with a pre-homogenised low P sandy soil from Achterberg, The Netherlands (51.593° N, 5.352° E). This soil is classified as a plaggic podzol (IUSS Working Group WRB, 2015) and was collected from the top 0 – 0.25 m from an extensively managed grassland that had not been fertilised with P for more than 25 years. This soil had a low $CaCl_2$ extractable P (0.5 mg $P \cdot kg^{-1}$) fraction, acidic pH (5.59) and a cation exchange capacity of 7 $cmol \cdot kg^{-1}$. More information about this soil is available in Supplementary material (Table 2.S1).

L. perenne diploid (Lp2) and *F. arundinacea* (Fa) were planted in monocultures and in a 50/50 combination (Lp2Fa). We used the seeding rate recommended by the seed producer (Barenbrug BV, The Netherlands) which was 4 and 6 g $seeds \cdot m^{-2}$ for Lp2 and Fa respectively. For the Lp2Fa combination, we used 50% of the recommended rate for each grass species, resulting in 2 and 3 $seeds \cdot m^{-2}$ respectively. In addition, to improve the palatability of the Lp2Fa grass combination, we studied a four-species

combination (4sp) with timothy (*P. pratense*) (Pp) and a tetraploid variety of *L. perenne* L. (Lp4). Both Pp and Lp4 are considered highly palatable (Balocchi and López, 2009; Ogle et al., 2010). The seeding rate for each species in the 4sp treatment was 25% of the recommended seeding rate for each species, resulting in 1, 1.5, 0.4 and 1.5 seeds·m^{-2} for Lp2, Lp4, Pp and Fa respectively. We also grew Pp and Lp4 in monocultures to calculate the relative yield total for the 4sp treatment. This resulted in a randomized block design with six plant mixtures replicated four times, leading to 24 boxes each split in two plots (with and without P fertilisation on the northern and southern sides respectively, not randomized) for a total of 48 plots distributed over four blocks. Nine harvests were performed over two growing seasons (S1 and S2) for the above-ground plant biomass, and roots and soils were sampled at the last harvest at the end of the experiment. Specific dates of the harvests can be found in the Supplementary material (Table 2.S2).

All plots were fertilised with nitrogen (N) and potassium (K). N fertiliser was added in the form of calcium ammonium nitrate (CAN), which was added before sowing the grasses as well as after each grass cut, leading to a total application 350 kg N·ha^{-1} for S1 and 311 kg N·ha^{-1} for S2. Potassium fertiliser was added in the form of potassium sulphate, once at the beginning of each growing season, at a rate of 30 kg K·ha^{-1} for S1 and 36 kg K·ha^{-1} for S2. In addition, P was applied in the form of triple super phosphate ($Ca(H_2PO_4)_2 \cdot H_2O$) on the northern half of each box (P+), at a rate of 22 kg P·ha^{-1} for S1 and 50 kg of P·ha^{-1} for S2. We increased the P application in S2 to ensure the alleviation of any P limitation in the P+ plots. The southern side of each box did not receive any P fertiliser (P-). Rates were based on the Dutch fertiliser guidelines for grasslands (Commissie Bemesting Grasland en Voedergewassen, 2017). Detailed information on the amount of fertilisation treatments and sowing can be found in the Supplementary material (Table 2.S2). All fertilisers used were broadcasted uniformly by hand over the soil surface in solid granular form as provided by the commercial suppliers.

2.2 Plant Analyses

Aboveground biomass samples were taken at each harvest (Table 2.S2) by cutting grass shoots 0.05 m above the soil surface. A metal frame (0.2 x 0.2 m) was used as a guide to cut the grass at the same location at each harvest. The grass outside the metal frame was also clipped at each harvest and discarded. After harvesting, samples were sorted by grass species and dried for 48 h at 70 °C.

We calculated the relative yields (RY) of the grass species grown in the mixtures and the relative yield total (RYT) of the full mixture [1] (de Wit, 1960).

[1] $RYT = \sum Yield\ mixture_i / Mean\ yield\ monoculture_i$

Where "i" represents the grass species. RY is calculated similarly but only for one of the species.

Root samples were taken at two different depths (0 – 0.15 and 0.15 – 0.40 m) at the end of the experiment using a soil core with a diameter of 30 mm. Three cores were taken from each plot and pooled. Roots were rinsed with water and separated from soil using a 1 mm mesh sieve. For analyses of root traits, a subsample of fresh roots was scanned after being stained with a neutral red solution (0.5 $g \cdot L^{-1}$). A resolution of 600 dpi on a 0.15 x 0.25 m tray was used for scanning. Root length (m) and diameter (mm) were calculated using the software WinRHIZO Pro (Regent Instruments). Specific root length (SRL) was calculated by dividing root length by root biomass ($m \cdot g^{-1}$). The remaining roots were dried at 70 °C for 48 h.

N and P contents in shoots were measured colourimetrically in a segmented flow analyser (SFA, Skalar, SAN^{++}, Breda, the Netherlands) after digestion with H_2SO_4/Se (Novozamsky et al., 1983). In short, shoot samples were placed in digestion tubes with a mixture of salicylic acid, sulphuric acid and selenium to prevent loss of N. The sample was incubated for 2 h at room temperature followed by heating at 100 °C for 2 h. After the tubes were allowed to cool down, hydrogen peroxide was added and heated at 330 °C. This step was repeated twice. Finally, the samples were allowed to stand overnight before homogenization and measurement. Measurements were done separately for each of the species in the grass mixtures treatments (Lp2Fa and 4sp). The N and P contents at the treatment level was calculated as the weighted average of the N and P contents present in a given treatment according to the relative biomass contribution of each species for that treatment.

We calculated the phosphorus nutrition index (PNI) [2] (Duru and Ducrocq, 1996) and the nitrogen nutrition index (NNI) [3] (Lemaire and Gastal, 1997) in the aboveground biomass as follows:

[2] $PNI = P\ (\%) / 0.15 + 0.065 \times N\ (\%)$

[3] $NNI = N\ (\%) / 4.8 \times Dry\ matter^{-0.32}$

Values of PNI or NNI > 1 indicate no nutrient limitation and < 0.8 show plant growth limitation. These indexes were calculated on pastures at field level and are designed to assess the direct and indirect effects of P and N fertilisation on grass growth and nutritional status.

2.3 Soil Analyses

Soil samples were taken at the end of the experiment from a depth of 0 – 0.1 m. Representative samples were taken using a Grass Plot Sampler (Eijkelkamp, Giesbeek, the Netherlands) with a diameter of 23 mm. The soil cores were sampled randomly over the soil surface of the plot and pooled after sampling *c.a.* 500 g of fresh soil in each plot. Soil samples were stored at –80 °C for biological analyses or dried at 40 °C for 48 h for soil chemical analyses.

Soluble P, dissolved organic nitrogen (DON), ammonium (NH_4), nitrate (NO_3), dissolved organic carbon (DOC) and pH were determined on dried samples. For the determination of pH, soil was shaken with a 0.01 *M* $CaCl_2$ solution for 2 h and measured with a combined glass electrode. The solution was then also used for the colorimetric determination of the beforementioned C, N and P parameters using a SFA (Skalar, SAN^{++}, Breda, the Netherlands) (Houba et al., 2000).

Microbial C, N and P, and enzymatic activities were measured on fresh or frozen (-80 °C) samples. Due to technical constraints, we were not able to measure biological variables in the Pp and Lp4 monoculture treatments. Microbial C and N were measured with the chloroform fumigation/extraction method in fresh samples (Jenkinson and Powlson, 1976). Briefly, 30 g of soil was fumigated under vacuum conditions for 24 h. Subsequently, C and N were extracted with 100 mL of a 0.05 *M* K_2SO_4 solution for 1 h. The measurements of C and N were performed in a TOC analyser (Shimadzu SSM-5000A/TOC-VCSH Carbon, Shimadzu, Kyoto, Japan). In parallel, unfumigated samples were also extracted as the controls. Microbial C and N were then calculated by subtracting the values of C and N in the control unfumigated samples from the values in the fumigated samples. Microbial P was also estimated using the chloroform fumigation/extraction method (Brookes et al., 1982). In short, 5 g of fresh soil was fumigated under vacuum conditions for 24 h. P was then extracted with 100 mL of a 0.5 *M* sodium bicarbonate solution at pH 8.5 for 30 min. Similarly, in parallel, P was extracted from unfumigated samples as controls. P content was measured colourimetrically by the molybdate blue method (Murphy and Riley, 1962). Microbial P was calculated by subtracting the values of P in the control samples from the values in the fumigated samples. Microbial C, N and P are expressed in mg of C, N or P·kg^{-1}. No correction factors were applied for microbial C, N or P.

Potential soil enzymatic activities were measured on frozen soil colourimetrically using a 96-well microplate technique (ISO 20130:2018) (Cheviron et al., 2022). Enzymes linked to the C, N and P cycles were analysed. The enzymes measured were

β-glucosidase (BGLU; EC: 3.2.1.21), N-acetyl-glucosaminidase (NAG; EC: 3.2.1.30), arylamidase (ARYLN; EC: 3.5.1.5), acid phosphatase (PAC; EC: 3.1.3.1) and alkaline phosphatase (PAK; EC: 3.1.3.2). In short, 4 g of fresh soil was mixed with H_2O for BGLU and NAG during 10 min. For ARYLN, PAC and PAK, a solution of Trizma buffer (50 mM) at pH 7.5, 5.5 and 11 respectively was used. Soil solutions were incubated with 4-nitrophenyl β-D-glucopyranoside 0.05 *M* for BGLU, 4-N-acetyl-β-D-glucosaminide 0.01 *M* for NAG, L-leucine β-naphthylamide hydrochloride 0.008 *M* for ARYLN and 4-nitrophenylphosphate disodium salt hexahydrate 0.05 *M* for PAC and PAK. After incubation, the reaction was stopped and the colouration revealed by the addition of 0.5 *M* $CaCl_2$ and 0.1 *M* Tris at pH 12 for BGLU, NAG, PAC and PAK activities. For ARYLN, ethanol and p-dimethylaminocinnamaldehyde were added (DMCA). The absorbance was measured on a Varioskan Flash-Thermo microplate reader (Thermo Fisher Scientific, Waltham, United States) at 405 nm for BGLU, NAG, PAC and PAK and at 540 for ARYLN. Enzymatic activities were expressed in nmol PNP (paranitrophenol)·min^{-1}·g^{-1} of dry soil for BGLU, NAG, PAC and PAK activities and in nmol β-naphthylamine·min^{-1}·g^{-1} of dry soil for ARYLN activities.

2.4 Statistical Analyses

Statistical analyses and figures were done using the software RStudio v1.4.1717 (R Core Team, 2022). Figures were made using the R package *ggplot2* (Wickham, 2016).

The effect of plant species and time on yields, nutrient content in the leaf, PNI and NNI were analysed by conducting a linear mixed effect model, with the plot's ID nested within blocks as a random factor using the R package *lme4* separately on P- and P+ plots (Bates et al., 2015). When the effect of the plant species was significant ($p < 0.05$) we conducted Tukey's post-hoc test using the R package *emmeans* (Lenth et al., 2024). When the interaction of the model is significant, it is not possible to interpret the effect of the plant species, as it depends on the time of observation. In this situation, we performed Tukey's posthoc test on single main effects after estimating marginal means using the R package *emmeans*. This allows to test the effect of factor accounting for the interaction effect.

The effects of plant species on root morphology, soil chemical and biological variables were analysed using one-way analyses of variance (ANOVA) separately on P- and P+ plots including block as a random effect using the package *lme4* separately on P+ and P- plots. When the ANOVA showed statistical differences, $p < 0.05$, we conducted Tukey's HSD posthoc test to reveal pairwise differences between plant

treatments using the R package *emmeans*. We also performed one-way ANOVAs at a plant species level within the mixtures to understand which of the grass species within the mixture was driving P uptake or increased yields at a given harvest. One-way ANOVA model residuals were examined for normality and homoscedasticity assumptions.

Over- or under-yielding of the grass species grown in mixtures was tested by performing a one-tailed t-test on the RY of each species. When the RY of Lp2 or Fa grown in the Lp2Fa mixture were significantly ($p < 0.05$) higher or lower than 0.5 overyielding or underperformance respectively is assumed. For the grasses grown in the 4sp mixture, over- or under-yielding was assumed when Fa or Pp's RY was significantly higher or lower than 0.25. The distinction between Lp2 and Lp4 in the 4sp mixture is not possible, and for this reason, overyielding or underperformance was assumed when Lp's RY was significantly higher or lower than 0.5.

Raw data is available at ZENODO online repository (Velasco-Sánchez et al., 2023).

3 Results

3.1 Aboveground Biomass and Yields

The results of the mixed linear model showed a significant effect of grass species on cumulative yields ($p = 0.03$) and a significant interaction of grass species and harvest date ($p < 0.01$) in the plots that received no P fertilisation (P-). After examination of single main effects, we observed that Lp2Fa mixture showed significantly higher cumulative yield than any other treatment, including Lp2 (*L. perenne*) and Fa (*F. arundinacea*) in monoculture ($p < 0.05$) at months 14, 16 and 18 after sowing (Figure 2.1). On average, Lp2Fa produced 25950 ± 8117 $kg \cdot ha^{-1}$ (mean ± standard error, $n = 4$) of dry biomass over two growing seasons, a 69% increase compared to the mean of all other treatments combined (15333 ± 924, $n = 20$). No statistical differences in cumulative yield were found between plant treatments in the P+ plots (Figure 2.1). The cumulative yields were also, on average, the highest in the Lp2Fa mixture in the P- plots at the end of season 1 (10720 ± 1242 $kg \cdot ha^{-1}$) and season 2 (15231 ± 2882 $kg \cdot ha^{-1}$) yet the differences were not significant.

The RYT was, generally, higher than 1 in all the harvests of the P- plots for the Lp2Fa treatment but not always for the 4sp treatment (Figure 2.2). In the P+ plots, neither the Lp2Fa nor the 4sp mixture resulted in consistent RYT values above 1. The highly variable RYT values in the P+ and P- plots in June of season 1 (first harvest) are associated with the low yields at the first cut of the experiment.

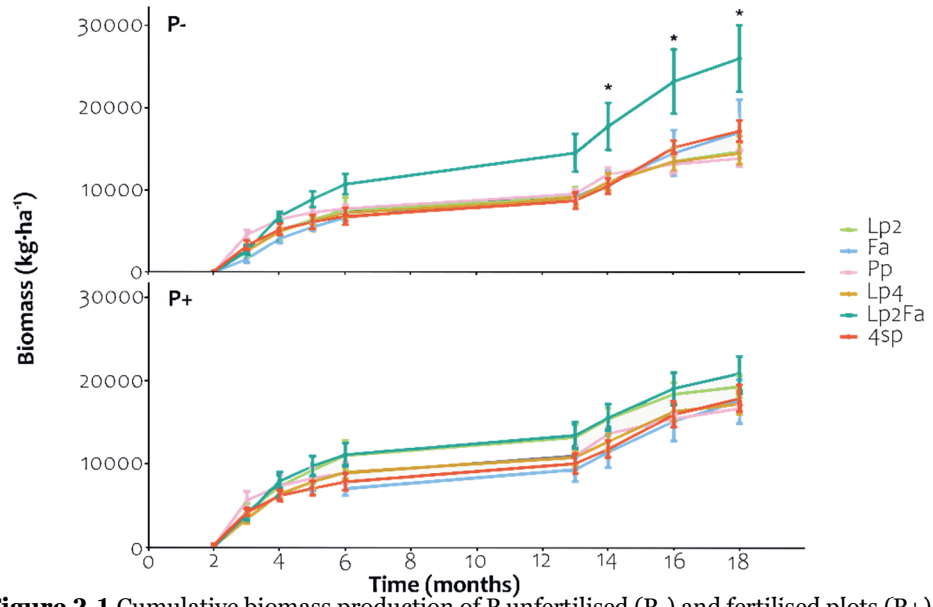

Figure 2.1 Cumulative biomass production of P unfertilised (P-) and fertilised plots (P+) for the different grass species treatments. Lp2 = *L. perenne* diploid; Fa = *F. arundinacea;* Pp = *P. pratense;* Lp4 = *L. perenne* tetraploid; Lp2Fa = Lp2 and Fa combination; 4sp = Lp2, Lp4, Fa and Pp combination. Bars indicate standard errors of the mean, n = 4. Asterisks indicate Tukey's significant differences of single main effects. Months indicate months after sowing.

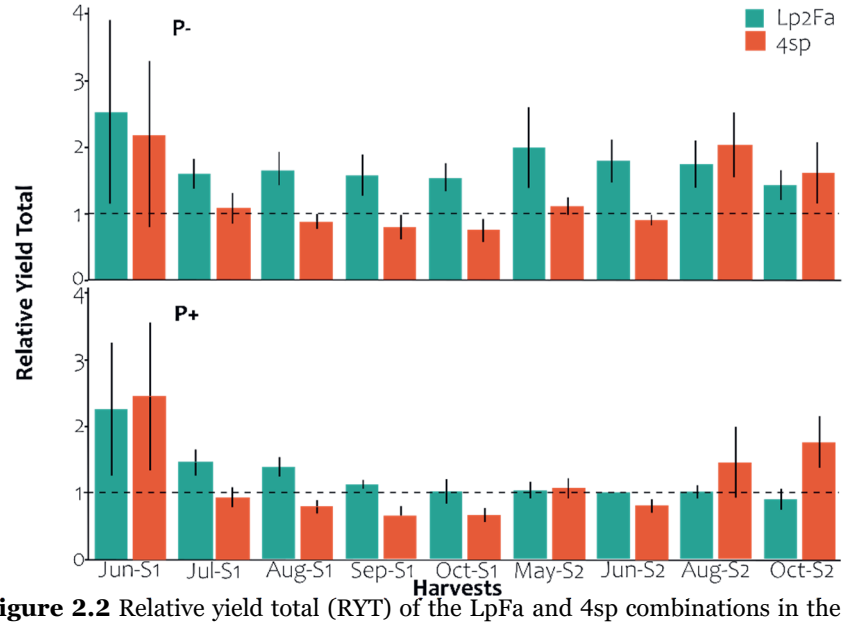

Figure 2.2 Relative yield total (RYT) of the LpFa and 4sp combinations in the P fertilised (P+) and P unfertilised (P-) plots at each harvest. S1 = First season, S2 = Second season, Lp2Fa = *L. perenne* diploid and *F. arundinacea* combination and 4sp = *L. perenne* diploid, *L. perenne* tetraploid, *F. arundinacea* and *P. pratense* combination. Bars indicate standard errors, n = 4. Values of RYT > 1 indicate higher yields than monoculture.

In P- plots, Lp2 dominated the harvests of season 1 and Fa dominated the ones of season 2 (Table 2.1). *L. perenne* produced significantly ($p < 0.05$) higher relative yields (RY > 0.5, over-yielding) in the Lp2Fa mixture than in monoculture throughout the grass harvests of the first season (July, August, September and October). Conversely, Fa over-yielded significantly throughout the second season (June, August and October). In the 4sp mixture, Lp over-yielded in the harvest of May, June and August of season 2. *F. arundinacea* over-yielded in the first cut of season 1, yet it significantly under-yielded (RY < 0.25) in the harvest of August, September and October in season 1 and in May in season 2. *P. pratense* also significantly under-yielded in the 4sp mixture in the last cut of season 2.

In the P+ plots, the trends were more erratic. Over-yielding was found for Lp2 in the Lp2Fa mixture only in the cut of August in season 1 and under-yielded in the cut of October in season 2 (Table 2.1). *F. arundinacea* grown in the Lp2Fa mixture over-yielded in the harvests of July of season 1 and in the harvests of May and October of season 2. Grass species generally under-yielded in the 4sp mixture grown in the P+ plots, Fa under-yielded throughout the first season (July, August, September and October) and also in the cut of June in season 2. Similarly, Pp under-yielded in the cut of October in season 2. Only Lp (not possible to differentiate Lp2 from Lp4 in the 4sp mixture) over-yielded (RY > 0.5) in the cut of October in season 2.

Results of the mixed linear model showed a significant effect of grass species on P nutrition (N:P and PNI) and a significant interaction between grass species and season (S1 *vs* S2) ($p < 0.01$) in the P- plots (Figure 2.3). No differences were observed between grass species at the end of S1 in P- plots, the average N:P value was 13.49 ± 0.36, n = 4 and the PNI values at the end of S1 were close to the 0.8 threshold for P limitation (Figure 2.3A and B). At the end of S2, in the P- plots, the N:P ratio increased significantly ($p < 0.05$) in the Lp2, Lp4 and 4sp treatments compared to S1. Likewise, PNI decreased significantly at the end of S2 compared to S1 for Lp2 and the 4sp mixture in the P- plots. The N:P ratios were significantly higher in the Lp2, Lp4 and 4sp treatments, followed by Pp, Lp2Fa and Fa in the P- plots (Figure 2.3A) and the PNI values indicated near adequate P levels (0.8) in the Lp2Fa and Fa treatments as well as a strong P limitation in the Lp2, Lp4 and 4sp combinations (Figure 2.3B) at the end of S2. In P+ plots, no statistical difference was found among grass species on N:P or PNI and these values did not change across seasons (Figure 2.3C and D). The PNI values in the P+ plots were close to 0.8 in both S1 and S2, suggesting adequate P nutrition (Figure 2.3D).

Table 2.1 Relative yield (RY) of each grass species grown in mixtures in P fertilised (P+) and unfertilised plots (P-). Values significantly higher or lower than 0.5 in the Lp2Fa mixture indicate over-yielding or under-yielding compared to the monoculture. In the 4sp mixture, values of RY significantly higher or lower than 0.25 for the Fa and Pp species denote over-yielding or under-yielding. In the 4sp mixture, the distinction between Lp2 and Lp4 is not possible (Lp), RY values significantly higher or lower than 0.5 denote over-yielding or under-yielding. S1 = First season; S2 = Second season; Lp2 = *L. perenne* diploid; Fa = *F. arundinacea*; Pp = *P. pratense*; Lp4 = *L. perenne* tetraploid; Lp2Fa = Lp2 and Fa combination; 4sp = Lp2, Lp4, Fa and Pp combination; + = significant over-yielding; - = significant under-yielding. Values indicate mean ± standard error, n = 4. Bold values indicate significantly higher or lower than the threshold (one-tailed t-test).

		Jun-S1	Jul-S1	Aug-S1	Sep-S1	Oct-S1	May-S2	Jun-S2	Aug-S2	Oct-S2
P-										
Lp2Fa	Lp	0.53 ± 0.30	**0.71 ± 0.06 (+)**	**0.90 ± 0.11 (+)**	**0.92 ± 0.14 (+)**	**0.78 ± 0.10 (+)**	1.09 ± 0.45	0.74 ± 0.23	0.78 ± 0.30	0.60 ± 0.33
	Fa	1.99 ± 1.11	0.89 ± 0.20	0.75 ± 0.19	0.66 ± 0.20	0.75 ± 0.16	0.90 ± 0.22	**1.05 ± 0.18 (+)**	**0.96 ± 0.11 (+)**	**0.83 ± 0.11 (+)**
4sp	Lp	1.25 ± 0.74	0.65 ± 0.17	0.45 ± 0.10	0.44 ± 0.15	0.46 ± 0.15	**0.75 ± 0.08 (+)**	**0.68 ± 0.04 (+)**	**1.21 ± 0.25 (+)**	1.34 ± 0.50
	Fa	**0.56 ± 0.12 (+)**	0.14 ± 0.05	**0.06 ± 0.02 (-)**	**0.07 ± 0.02 (-)**	**0.07 ± 0.03 (-)**	**0.10 ± 0.03 (-)**	0.13 ± 0.06	0.42 ± 0.12	0.19 ± 0.11
	Pp	0.35 ± 0.27	0.29 ± 0.03	0.37 ± 0.08	0.29 ± 0.08	0.23 ± 0.08	0.26 ± 0.18	0.10 ± 0.08	0.20 ± 0.17	**0.07 ± 0.05 (-)**
P+										
Lp2Fa	Lp	0.62 ± 0.30	0.66 ± 0.08	**0.79 ± 0.06 (+)**	0.55 ± 0.05	0.48 ± 0.13	0.38 ± 0.14	0.37 ± 0.09	0.30 ± 0.13	**0.20 ± 0.13 (-)**
	Fa	1.63 ± 0.73	**0.81 ± 0.11 (+)**	0.60 ± 0.10	0.57 ± 0.06	0.54 ± 0.08	**0.66 ± 0.06 (+)**	0.62 ± 0.08	0.71 ± 0.11	**0.69 ± 0.07 (+)**
4sp	Lp	1.44 ± 0.72	0.51 ± 0.20	0.40 ± 0.14	0.36 ± 0.15	0.39 ± 0.15	0.62 ± 0.23	0.57 ± 0.05	0.82 ± 0.21	**1.48 ± 0.48 (+)**
	Fa	0.52 ± 0.31	**0.07 ± 0.02 (-)**	**0.06 ± 0.02 (-)**	**0.08 ± 0.04 (-)**	**0.10 ± 0.04 (-)**	0.22 ± 0.10	**0.12 ± 0.05 (-)**	0.31 ± 0.08	0.24 ± 0.15
	Pp	0.49 ± 0.39	0.36 ± 0.08	0.34 ± 0.09	0.21 ± 0.06	0.18 ± 0.07	0.24 ± 0.17	0.12 ± 0.09	0.32 ± 0.29	**0.04 ± 0.03 (-)**

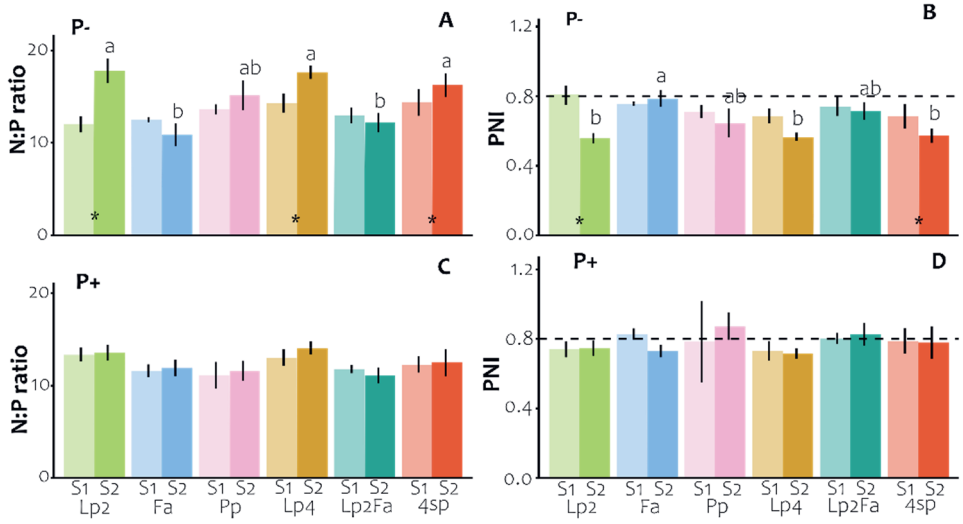

Figure 2.3 Averaged nutrient uptake in P unfertilised (P-) (A and B) and P fertilised (P+) (C and D) plots at last harvest of season 1 (S1) and season 2 (S2). PNI = Phosphorus nutrition index; Lp2 = *L. perenne* diploid; Fa = *F. arundinacea;* Pp = *P. pratense;* Lp4 = *L. perenne* tetraploid; Lp2Fa = combination of Lp2 and F*a;* 4sp = combination of Lp2, Lp4, Fa and Pp. Letters indicate significant differences among grass species after single main effect evaluation (Tukey test). Asterisks indicate significant differences between S1 and S2. Horizontal line at 0.8 in panels B and D shows threshold for adequate PNI. Bars show standard errors, n = 4.

When examining the differences in P nutrition at a species level within the mixtures we observed no significant differences in N:P ratios of PNI by the end of S1 and S2 in the P- and P+ plots (Table 2.S3). In the P+ plots, the PNI values were close to the 0.8 threshold, indicating no strong P limitation (Table 2.S3).

We did not find strong N limitation in either P- and P+ plots as shown by the high nitrogen nutrition index (NNI) values, which were on average higher than 0.8 (Table 2.S4). We observed that the 4sp was the mixture with the lowest NNI values both in the P+ and P- plots at S1 and S2 (Table 2.S4). Detailed information on N and P nutrition as well as NNI and PNI indexes can be found in Table 2.S4.

3.2 Root Morphology

Root biomass showed no statistical differences between plant treatments in the P- and P+ plots in the top soil layer (0 – 0.15 m) (Figure 2.4A and D). Specific root length was found significantly lowest for Fa in both P- and P+ plots (1.41 ± 0.08 and 1.48 ± 0.15 $m \cdot g^{-1}$ for P- and P+ respectively). Root diameter was found the highest for Fa in both P- and P + with an average diameter of 0.23 ± 0.01 and 0.22 ± 0.01 mm respectively (Figure 2.4C and F).

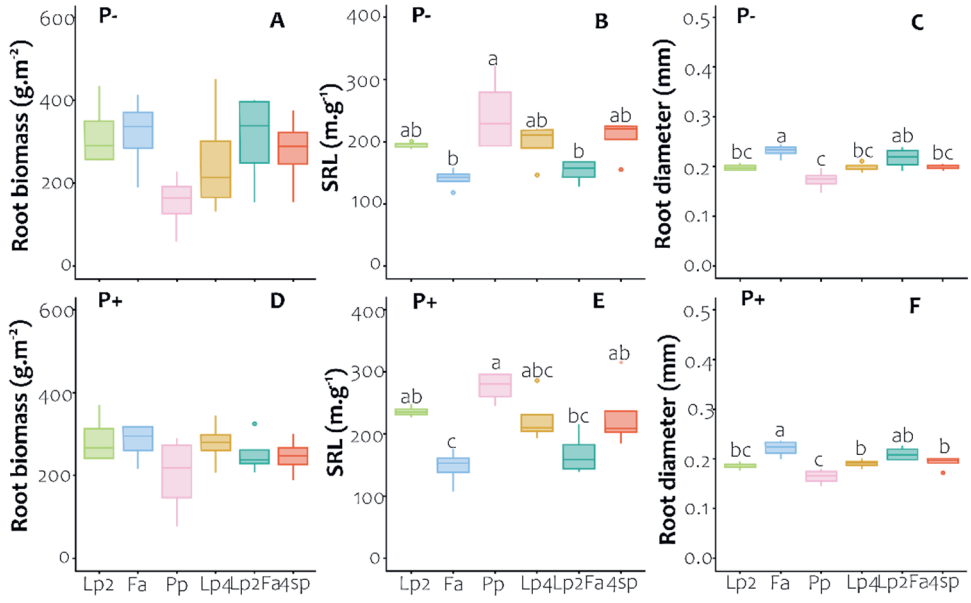

Figure 2.4 Root traits in P unfertilised (P-) (A, B and C) and P fertilised (P+) (D, E and F) plots at last harvest in the *topsoil* (0 – 0.15 m). Lp2 = *L. perenne* diploid; Fa = *F. arundinacea;* Pp = *P. pratense;* Lp4 = *L. perenne* tetraploid; Lp2Fa = combination of Lp2 and Fa; 4sp = combination of Lp2, Lp4, Fa and Pp; SRL = Specific root length. Letters are displayed when ANOVA is significant and indicate the results of Tukey's HSD test.

In the deeper soil layers (0.15 – 0.40 m) we observed significantly ($p < 0.05$) higher root biomass and diameter for Fa (Figure 2.5A, C, D and F). The average root biomass of Fa at the deeper soil layers was 98.14 ± 18.61 $g \cdot m^{-2}$ for the P- plots and 93.9 ± 24.69 for the P+ plots $g \cdot m^{-2}$, n = 4 (Figure 2.5A and D). Root diameter was highest in the Fa monoculture and lowest in the Pp monoculture in both P- and P+ plots (Figure 2.5C and F). The SRL was also significantly lower in the Fa compared to the rest of the plant treatments in the P+ plots. In the P- plots, no statistically significant differences between species were found, yet Fa also showed, on average, the lowest SRL (Figure 2.5B and E).

Root traits were also significantly correlated with P nutrition values (Table 2.2). In P- plots, we observed that PNI and P uptake were positively correlated with top root diameter, deep root biomass and deep root diameter. PNI was also negatively correlated with top and deep SRL. Shoot P concentration was not correlated with any of the root traits in the P- plots. In the P+ plots, P concentration and PNI were positively correlated with top and deep SRL. Moreover, shoot P concentration was negatively correlated with top and deep root diameters and total root biomass.

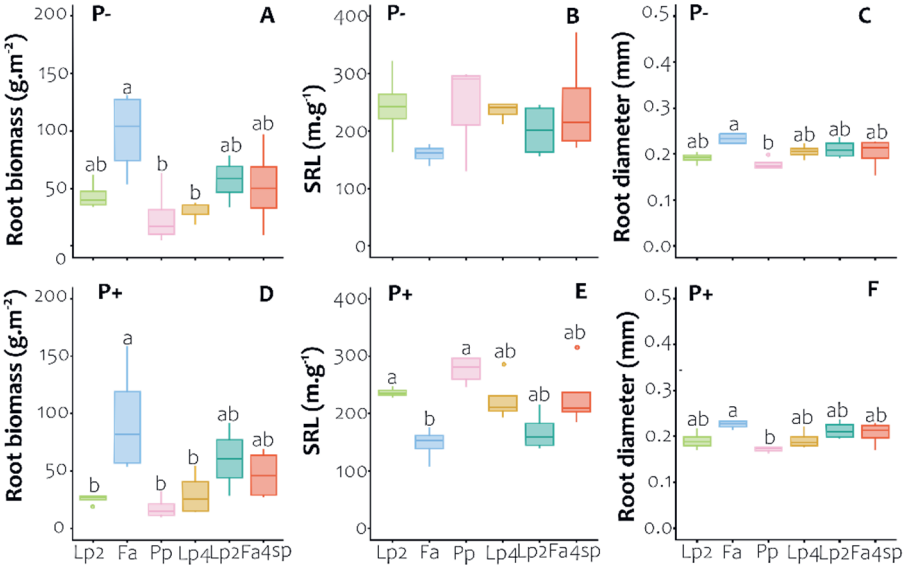

Figure 2.5 Root traits in P unfertilised (P-) (A, B and C) and P fertilised (P+) (D, E and F) plots at last harvest in the deeper layers of the soil (0.15 – 0.40 m). Lp2 = *L. perenne* diploid; Fa = *F. arundinacea;* Pp = *P. pratense;* Lp4 = *L. perenne* tetraploid; Lp2Fa = combination of Lp2 and Fa; 4sp = combination of Lp2, Lp4, Fa and Pp; SRL = Specific root length. Letters are displayed when ANOVA is significant and indicate the results of Tukey's HSD test.

Table 2.2 Correlations between P nutrition in grasses and root morphology. Top root: 0 - 0.15 m, deep root: 0.15 - 0.40 m, P uptake in kg P·ha^{-1}.PNI: Phosphorus nutrition index, SRL: Specific Root Length. Values indicate Pearson's correlation coefficients, highlighted in bold are statistically significant correlations.

	Top root biomass (g·m^{-2})	Top SRL (m·g^{-1})	Top root diameter (mm)	Deep root biomass (g·m^{-2})	Deep SRL (m·g^{-1})	Deep root diameter (mm)	Total root biomass (g·m^{-2})
P-							
P (%)	- 0.11	- 0.07	0.14	0.32	- 0.27	0.13	-0.01
P uptake	0.21	- 0.40	**0.56**	**0.57**	- 0.29	**0.42**	0.34
PNI	0.07	**- 0.47**	**0.49**	**0.58**	**- 0.52**	**0.44**	0.21
P+							
P (%)	- 0.35	**0.48**	**- 0.53**	- 0.32	**0.50**	**- 0.52**	**- 0.40**
P uptake	- 0.36	- 0.12	0.19	**0.41**	- 0.24	0.16	- 0.10
PNI	-0.29	**0.46**	**- 0.53**	- 0.24	**0.46**	**- 0.50**	- 0.32

3.3 Soil Chemical and Biological Differences

No statistical differences were found between the plant treatments for most of the soil chemical variables analysed (readily available P, DOC, DON, NH_4, NO_3 and pH) (Table 2.S5). Fertilisation with P roughly doubled the levels of readily available P ($CaCl_2$ extractable P), on average soluble P was 0.02 ± 0.01 mg·kg^{-1} of dry soil for the P- plots and 0.05 ± 0.01 mg·kg^{-1} for the P+ plots. Plant available P (Olsen P) was also increased in the fertilised plots, 9.76 ± 0.42 *vs* 20.63 ± 0.75 mg P·kg^{-1}. The pH of the soil was unaffected by the plant treatments, the pH of the P- plots was 5.23 ± 0.03 and 5.26 ± 0.04 in the P+ plots, n = 24. On average, the 4sp treatment had the highest DOC values (6.43 ± 0.16 mg C·kg^{-1}, n = 24) in the P- plots, yet the differences were found non-significant (p = 0.096).

Similarly to soil chemical variables, the effect of the different plant treatments was non-significant in most of the soil biological variables measured (microbial C, microbial N, microbial P, N-acetyl-glucosaminidase (NAG), arylamidase (ARYLN), acid phosphatase (PAC) and alkaline phosphatase (PAK)) with the exception of β-glucosidase (BGLU) (Table 2.3). BGLU potential activity in the P- plots was the highest for the 4sp treatment (18.92 ± 1.86 PNP·min^{-1}·g^{-1} of dry soil, n = 4). The 4sp treatment also showed the highest average of microbial C, N and P biomass and highest NAG, ARYLN, PAC and PAK, yet these differences were found non-significant.

4 Discussion

4.1 Root Morphology Changes and Reduced Intraspecific Competition Improve P Nutrition

The aim of this experiment was to evaluate the introduction of Fa in grass mixtures with Lp alone or with other grass species to improve P acquisition and therefore dependency on P fertilisers. We hypothesised that Fa, because of its deeper root system could potentially explore and make use of more soil P compared to other grass species. Our results confirm our initial hypothesis showing a significantly higher cumulative biomass production in the Lp2Fa treatment compared to the Lp2 or Fa monocultures (Figure 2.1) and a RYT consistently greater than 1 in the plots with no P fertilisation (P-), indicating over-yielding (Figure 2.2 and Table 2.1).

The increased cumulative yields and RYT of the Lp2Fa combination can be explained by the complementary use of resources over time and space as well as a lower intraspecific competition. During the first season Lp2 grown in the Lp2Fa mixture

Table 2.3 Average values of soil biological variables measured at the end of the experiment in P (P+) fertilised and P (P-) unfertilised plots. BGLU = β-glucosidase, NAG = N-acetyl-glucosaminidase, ARYLN = arylamidase, PAC = Acid phosphatase and PAK = Alkaline phosphatase. Microbial biomass expressed in $mg \cdot kg^{-1}$ of dry soil, BGLU, NAG, PAC and PAK are expressed in $PNP \cdot min^{-1} \cdot g^{-1}$ of dry soil and ARYLN is expressed in β-naphthylamine $\cdot min^{-1} \cdot g^{-1}$ of dry. In bold, significant effect of the plant treatment (ANOVA) and different letters indicate the results of Tukey's HSD test. Values indicate mean ± standard error, n = 4. Lp2 = *L. perenne* diploid; Fa = *F. arundinacea*; Lp2Fa = combination of Lp2 and Fa; 4sp = combination of Lp2, Fa, *P. pratense* and *L. perenne* tetraploid.

	Microbial C	Microbial N	Microbial P	BGLU	NAG	ARYLN	PAC	PAK
P-								
ANOVA	p = 0.454	p = 0.146	p = 0.120	**p = 0.004**	p = 0.279	p = 0.166	p = 0.358	p = 0.199
Lp2	254.26 ± 16.1	6.56 ± 0.63	0.42 ± 0.30	12.31 ± 0.87 **b**	6.15 ± 0.77	0.48 ± 0.14	64.28 ± 3.92	13.11 ± 1.44
Fa	279.04 ± 16.41	8.88 ± 1.56	0.94 ± 0.23	13.70 ± 0.99 **b**	6.21 ± 0.48	0.68 ± 0.11	61.69 ± 3.15	15.33 ± 2.14
Lp2Fa	281.19 ± 15.55	9.56 ± 1.21	0.74 ± 0.17	14.09 ± 0.82 **b**	5.22 ± 0.87	0.72 ± 0.07	52.49 ± 5.06	15.34 ± 0.66
4sp	287.07 ± 11.25	9.63 ± 1.45	1.22 ± 0.16	18.92 ± 1.86 **a**	6.25 ± 0.48	0.80 ± 0.15	68.84 ± 10.70	18.04 ± 1.46
P+								
ANOVA	p = 0.881	p = 0.972	p = 0.529	p = 0.515	p = 0.497	p = 0.144	p = 0.325	p = 0.952
Lp2	280.04 ± 15.56	8.68 ± 3.03	0.77 ± 0.72	13.92 ± 0.99	6.09 ± 1.05	0.46 ± 0.08	56.85 ± 3.30	14.66 ± 3.05
Fa	281.57 ± 6.25	9.34 ± 1.66	0.42 ± 0.31	13.49 ± 1.11	5.43 ± 0.46	0.65 ± 0.06	56.95 ± 2.83	13.74 ± 0.97
Lp2Fa	284.16 ± 12.14	10.02 ± 1.12	1.53 ± 0.72	15.44 ± 0.52	6.56 ± 0.71	0.67 ± 0.03	64.36 ± 3.21	15.48 ± 0.63
4sp	271.39 ± 16.86	9.85 ± 2.48	0.48 ± 0.48	15.11 ± 1.37	5.17 ± 0.29	0.65 ± 0.09	63.83 ± 4.99	14.19 ± 3.05

significantly over-yielded compared to the monoculture, whereas, in the second season, Fa was the species that over-yielded (Table 2.1). During the first season, Lp2, a faster-growing grass (Finn et al., 2013), possibly benefited from more available P, as shown by the ideal PNI values (Figure 2.3B and D). It also could have benefited from a lower intraspecific competition, as the seeding rate was reduced in the Lp2Fa compared to the monoculture (2 *vs* 4 $g \cdot m^{-2}$). Conversely, at the end of the second season, Fa, a slower-growing grass (Gastal et al., 2010) up and started to benefit from its distinct root traits and associated increased access to more nutrients (Cougnon et al., 2013) (Table 2.2). Likewise, Fa could also benefited from a lower intraspecific competition, as the seeding rate in the mixture was also reduced compared to the monoculture (3 *vs* 6 $g \cdot m^{-2}$).

We argue that the Lp2Fa mixture benefited from the niche complementarity of Lp2 and Fa, which ultimately also facilitated P nutrition. By the end of the experiment, irrespective of the P fertilisation regime, Lp2 in monoculture had consistently and significantly finer roots than Fa (Figures 2.4C and F, 2.5C and F) and Fa had significantly more root biomass at deeper soil layers (Figure 2.5A and D). Finer roots, because of an associated higher specific root length (Eissenstat, 1992; Tshewang et al., 2022) and deeper root biomass, because of increased access to nutrient pools (Ros et al., 2018), have been suggested as relevant for P uptake in grass mixtures. Our results are in line with previous research in which Fa was shown to have higher root biomass at deeper soil layers (Cougnon et al., 2017, 2013; Ros et al., 2018) and relatively thick roots (Cougnon et al., 2017). The higher root biomass in the deeper soil layers potentially allowed Fa to keep up P uptake under limiting conditions both in monoculture and when grown in the Lp2Fa mixture (Figure 2.3 and Table 2.2). This was not the case for Lp2 in monoculture, which was strongly P-limited as shown by the PNI and N:P values at the end of season 2 (Figure 2.3A and B). In this case, the higher intraspecific competition in the monoculture could have resulted in a decrease in P acquisition, as also shown in global meta-analyses (Adler et al., 2018; Zhu et al., 2023).

We also hypothesized that an increased complementarity between roots would result in a higher P uptake and yields under P limitation in the mixture with 4 species (Bi et al., 2019). However, when we introduced Fa in the more palatable 4sp mixture, we did not observe any increased yield or RYT compared to the monocultures (Figures 2.1 and 2.2). In fact, we observed under-yielding for some of the species in the mixture at many of the harvests, particularly for Fa (Table 2.1). Other studies have also demonstrated that increasing the number of species in a grass mixture does not necessarily result in increased yields in semi-natural grasslands (Mangan et al.,

2011; Roscher et al., 2011) and that high interspecific competition could result in lower P uptake in grasslands (Guiz et al., 2018).

Concerns of Fa palatability should be taken into consideration when examining the higher yields of the Lp2Fa mixture under P limitation. Nevertheless, newer varieties of Fa are considerably improved in palatability (Becker et al., 2020; Kindiger, 2022). Moreover, post-harvest techniques, such as silage, are known to increase the nutritional value of harvested grass (Peratoner et al., 2011). In this sense, Lp2Fa could be an interesting grass mixture in situations of P limitation. Lastly, the adaptation of Fa in different types of soils should be considered. In our mesocosms, we used an homogeneous sandy soil with a low P content. It is unknown how different grasses would react in soils with a finer texture or different P availability distribution, as these variables are important for root traits and plant growth (Table 2.2) (Becker et al., 2020).

4.2 Contribution of Soil Microorganisms Towards P Nutrition

Soil microorganisms are able to solubilise poorly soluble forms of P and eventually increase the plant available pool of P (Khan et al., 2007; Richardson and Simpson, 2011). As such, we hypothesised that soil microorganisms could improve the uptake of P by grasses in situations of P limitation. However, we did not observe any relationship between microbial activities (enzyme activities) or microbial biomass with P uptake in both P+ and P- plots. Our results indicate that none of the species studied contributed to significantly change the microbial community of the soil in a way that resulted in higher yield or P mobilisation.

We did observe that, in the P- plots, the 4sp treatment resulted in significantly higher BGLU activities. There was also a trend for increased microbial C, N and P biomass and increased ARYLN, NAG, PAC and PAK activities compared to Lp2, albeit non-significant. This might be related to an increased interspecific competition between grass species in the 4sp treatment (Bybee-Finley et al., 2022). For example, Pp was well established in the 4sp mix early in the experiment, with an initial RY of 0.49, indicating neither over- nor under-yielding. At the end of the second season, however, at the time of soil sampling, Pp had almost entirely been out-competed and had therefore likely provided additional biomass available for microbial mineralisation. So, higher turnover of organic material, because of a higher competition among species, could have caused the increased microbial activities and biomass (Schofield et al., 2019; Sun et al., 2021). Moreover, a higher species diversity could have resulted in a higher exudation of organic compounds that might have stimulated microbial activities (Steinauer et al., 2016).

Measuring soil microbial activities and biomass throughout the growing season might have yielded different results as the activities of soil microorganisms could be time-dependent (Gao et al., 2021). Other P-related enzymes, such as phytase, could have also dominated the P release from poorly soluble P forms (Rizwanuddin et al., 2023) and their effects might have been different depending on the most abundant poorly soluble P species in the soil (Velasco-Sánchez et al., 2024). Activities could also have been markedly different at deeper soil layers, where the root biomass was dominated by Fa (Fairbanks et al., 2020). Similarly, measuring arbuscular mycorrhiza fungi (AMF) colonization could have also explained the differences between grass species, yet preliminary measurements during the first season showed no statistical differences.

5 Conclusion

We tested grass mixture combinations containing *F. arundinacea* as a way to reduce the inputs of P fertiliser in intensively managed grasslands. We hypothesised that *F. arundinacea*, because of its deeper root system, would be able to access more P than other grass species. We also postulated that, in a situation of P limitation, soil microorganisms would be of importance to achieve high yields. Our results showed that the combination of *L. perenne* and *F. arundinacea* resulted in significantly higher yields than the monocultures, possibly because of the contrasting root systems of *L. perenne* and *F. arundinacea* and the ability of *F. arundinacea* to withstand P limitation. Additionally, we did not observe any contribution of the soil microbial community towards higher yields or P mobilisation.

More research is needed on the combination of *L. perenne* and *F. arundinacea* to allow its widespread use as a P limitation-tolerant grass mixture. In particular, further experiments should aim to better understand the palatability issues of *F. arundinacea*, potentially including grazing animals in their experimental design. Moreover, experiments conducted on different soils must be performed that account for vertical heterogeneity of soil properties. Lastly, further experiments should also focus on exploring the role of soil microorganisms on P cycling at the deeper soil layers where *F. arundinacea* was predominant. We conclude that a combination of *L. perenne* and *F. arundinacea* species in intensively managed grassland could lead to the alleviation of P limitation and higher yields in situations of low P availability.

Acknowledgments

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Supplementary Information

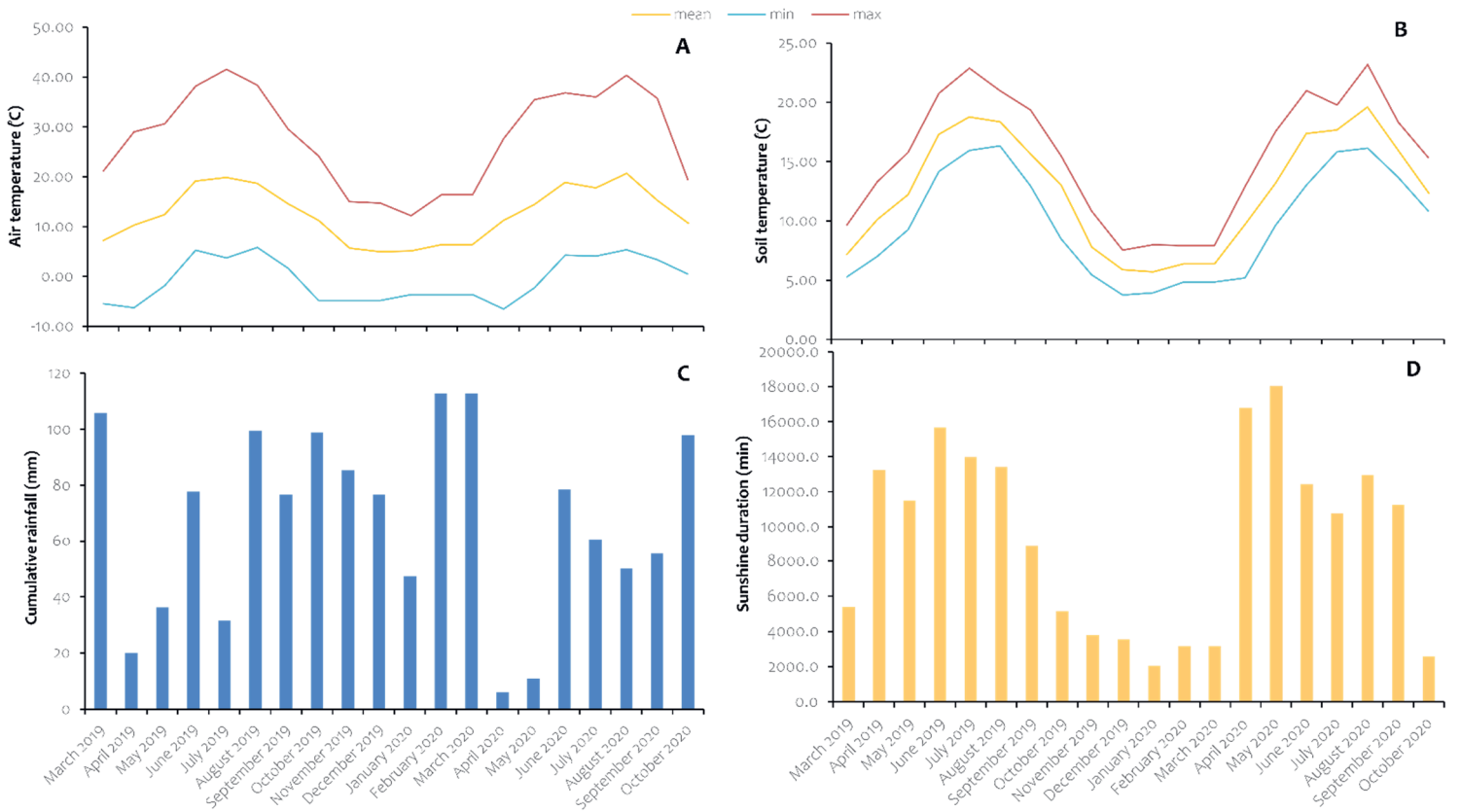


Figure 2.S1 Detailed climatic information from Veenkampen weather station (Wageningen, The Netherlands) during the 2019 and 2020 growing seasons. Shielded above 0.1 m air temperature (A), soil temperature at 0.1 m depth under grassland (B), cumulative monthly precipitation (C) and cumulative monthly sunshine duration (D).

Figure 2.S2 Mesocosm field experiment at Wageningen University, The Netherlands.

Table 2.S1 Initial soil analysis.

Variable	Value
Sand (%) [a]	87.7
Silt (%) [a]	6
Clay (%) [a]	1.6
Organic matter (%) [b]	3.8
pH [c]	5.59
CEC (cmol(+)·kg^{-1}) [d]	7
DOC (mg·kg^{-1}) [c]	60
N-NH_4 (mg·kg^{-1}) [c]	2.0
N-(NO_3+NO_2) (mg·kg^{-1}) [c]	3.9
Nts (mg·kg^{-1}) [c]	11
P-$CaCl_2$ (mg·kg^{-1}) [c]	0.5
P-AL (mg·kg^{-1})[e]	116
P-ox (mg·kg^{-1}) [f]	268
K (mg·kg^{-1}) [c]	26
S (mg·kg^{-1}) [c]	1.8
Mg (mg·kg^{-1}) [c]	88.9
Na (mg·kg^{-1}) [c]	6
Al (mg·kg^{-1}) [f]	1553
Fe (mg·kg^{-1}) [f]	656

CEC = Cation exchange capacity, [a] = measured by laser diffraction, [b] = loss on ignition, 105-550°C, [c] = measured in 0.01 *M* $CaCl_2$, SFA (except for pH), [d] = Not buffered, ICP-AES, [e] = measured in ammonium lactate, SFA, [f] = measured in ammonium oxalate – oxalic acid, ICP-AES.

Table 2.S2 Timeline of experiment (2019 - 2020).

Activity	Season 1 (2019)										
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Harvests				Sowing		H1	H2	H3	H4	H5		
N				82		109	64	56	39			
K				30								
P				22								
	Season 2 (2020)											
Harvests					H6	H7		H8		H9		
N			82		109	64		56				
K			36									
P			50									

The plots were fertilised with CAN = Calcium ammonium nitrate, TSP = Triple super phosphate and K_2SO_4 = potassium sulphate. P fertiliser was only added in the P+ plots. Fertilisers were applied after each grass cut. H = harvest.

Table 2.S4 Nutrient (P and N) concentrations, uptakes and nutritional indicators (PNI, NNI and N:P) at the end of season 1 and season 2 in shoots. PNI = Phosphorus nutrition index, NNI = Nitrogen nutrition index, Lp2 = *L. perenne* diploid, Fa = *F. arundinacea*, Lp2Fa = combination of Lp2 and Fa and 4sp = combination of Lp2, *L. perenne* tetraploid, Fa and *P. pratense*, S1 = end of season 1, S2 = end of season 2. Values show mean ± standard error, n = 4. Significant effects are highlighted in bold. Differences among groups are shown based on Tukey's significant differences. In case of significant interactions, Tukey test is performed on single main effects.

		P (%)	P uptake (kg P ha^{-1})	PNI	N (%)	N uptake (kg N ha^{-1})	NNI	N:P
P -								
S1	Lp2	0.32 ± 0.02	3.91 ± 0.39 **ab**	0.81 ± 0.05	3.87 ± 0.27	47.67 ± 7.42 **ab**	1.34 ± 0.13 **a**	12.02 ± 0.87
	Fa	0.30 ± 0.01	3.46 ± 0.61 **a**	0.76 ± 0.01	3.73 ± 0.18	43.33 ± 7.75 **a**	1.26 ± 0.10 **a**	12.52 ± 0.28
	Pp	0.29 ± 0.02	1.39 ± 0.38 **b**	0.71 ± 0.04	3.89 ± 0.20	18.52 ± 4.52 **ab**	0.98 ± 0.11 **ab**	13.65 ± 0.48
	Lp4	0.28 ± 0.01	2.69 ± 0.57 **ab**	0.68 ± 0.04	3.92 ± 0.13	37.40 ± 6.29 **ab**	1.24 ± 0.07 **a**	14.32 ± 0.98
	Lp2Fa	0.29 ± 0.02	2.64 ± 0.35 **ab**	0.74 ± 0.04	3.69 ± 0.09	33.26 ± 3.71 **ab**	1.15 ± 0.04 **ab**	12.96 ± 0.58
	4sp	0.27 ± 0.02	0.70 ± 0.23 **b**	0.68 ± 0.04	3.75 ± 0.16	9.36 ± 3.08 **b**	0.70 ± 0.08 **b**	14.40 ± 0.88
S2	Lp2	0.23 ± 0.01	2.66 ± 0.67 **ab**	0.56 ± 0.03 **b**	4.12 ± 0.19 **a**	46.71 ± 11.39 **ab**	1.37 ± 0.10 **a**	17.86 ± 1.28 **a**
	Fa	0.26 ± 0.01	6.40 ± 2.86 **a**	0.79 ± 0.05 **a**	2.85 ± 0.35 **b**	61.32 ± 24.59 **a**	1.04 ± 0.15 **a**	10.89 ± 1.23 **b**
	Pp	0.26 ± 0.04	1.79 ± 0.77 **b**	0.65 ± 0.08 **ab**	3.69 ± 0.35 **ab**	24.42 ± 8.20 **ab**	1.01 ± 0.11 **ab**	15.14 ± 1.61 **ab**
	Lp4	0.24 ± 0.02	2.34 ± 0.45 **ab**	0.57 ± 0.03 **b**	4.21 ± 0.20 **a**	42.17 ± 9.77 **ab**	1.34 ± 0.10 **a**	17.64 ± 0.72 **a**
	Lp2Fa	0.24 ± 0.01	3.19 ± 0.77 **ab**	0.71 ± 0.03 **ab**	2.88 ± 0.09 **b**	39.10 ± 9.76 **ab**	0.95 ± 0.10 **ab**	12.18 ± 0.73 **b**
	4sp	0.21 ± 0.01	1.52 ± 0.55 **b**	0.57 ± 0.03 **b**	3.43 ± 0.11 **ab**	25.46 ± 10.12 **b**	0.84 ± 0.13 **b**	16.30 ± 0.73 **a**
Species		p = 0.582	**p = 0.028**	p = 0.076	**p = 0.018**	**p = 0.002**	**p < 0.001**	**p = 0.013**
Season		**p < 0.001**	p = 0.265	**p < 0.001**	**p = 0.006**	p = 0.176	p = 0.804	**p < 0.001**
Species x Season		p = 0.664	p = 0.291	**p = 0.043**	**p = 0.003**	p = 0.928	p = 0.433	**p < 0.001**

P +								
S1	Lp2	0.30 ± 0.03	5.26 ± 0.70 **a**	0.74 ± 0.04	4.01 ± 0.27	68.60 ± 5.60 **a**	1.54 ± 0.07 **a**	13.30 ± 0.76
	Fa	0.32 ± 0.01	3.21 ± 0.63 **ab**	0.83 ± 0.03	3.75 ± 0.15	37.04 ± 6.97 **ab**	1.20 ± 0.09 **ab**	11.58 ± 0.66
	Pp	0.32 ± 0.11	1.85 ± 0.59 **ab**	0.79 ± 0.23	3.22 ± 0.97	19.29 ± 6.14 **b**	0.88 ± 0.27 **abc**	11.09 ± 1.48
	Lp4	0.28 ± 0.03	3.28 ± 0.50 **ab**	0.73 ± 0.06	3.64 ± 0.23	41.62 ± 4.90 **ab**	1.23 ± 0.07 **ab**	13.00 ± 0.92
	Lp2Fa	0.31 ± 0.01	2.15 ± 0.45 **b**	0.80 ± 0.02	3.62 ± 0.15	24.73 ± 4.84 **b**	1.02 ± 0.07 **b**	11.76 ± 0.35
	4sp	0.31 ± 0.02	0.84 ± 0.31 **b**	0.79 ± 0.04	3.70 ± 0.15	9.91 ± 3.69 **b**	0.69 ± 0.09 **c**	12.25 ± 0.53
S2	Lp2	0.32 ± 0.02	2.75 ± 0.20 **ab**	0.75 ± 0.04	4.24 ± 0.07 **a**	36.72 ± 2.18 **ab**	1.31 ± 0.03 **a**	13.49 ± 0.89
	Fa	0.25 ± 0.01	5.83 ± 0.76 **a**	0.73 ± 0.04	2.91 ± 0.16 **b**	68.06 ± 6.82 **a**	1.24 ± 0.06 **ab**	11.89 ± 0.93
	Pp	0.36 ± 0.03	4.33 ± 1.29 **ab**	0.87 ± 0.08	4.07 ± 0.12 **ab**	50.34 ± 14.86 **ab**	1.37 ± 0.13 **abc**	11.53 ± 1.10
	Lp4	0.31 ± 0.01	2.89 ± 0.35 **ab**	0.72 ± 0.03	4.31 ± 0.22 **a**	40.09 ± 4.29 **ab**	1.36 ± 0.06 **ab**	14.04 ± 0.77
	Lp2Fa	0.30 ± 0.02	2.36 ± 0.75 **b**	0.83 ± 0.05	3.27 ± 0.17 **ab**	26.59 ± 8.21 **b**	0.84 ± 0.11 **b**	11.06 ± 0.62
	4sp	0.29 ± 0.02	1.82 ± 0.60 **b**	0.78 ± 0.06	3.52 ± 0.13 **ab**	25.52 ± 8.89 **b**	0.83 ± 0.15 **c**	12.49 ± 0.86
Species		p = 0.836	**p < 0.001**	p = 0.822	p = 0.176	**p < 0.001**	**p < 0.001**	p = 0.067
Season		p = 0.805	p = 0.165	p = 0.999	p = 0.683	p = 0.120	p = 0.439	p = 0.630
Species x Season		p = 0.547	**p = 0.014**	p = 0.889	**p = 0.029**	**p = 0.009**	p = 0.185	p = 0.944

Table 2.S3 N:P ratios and PNI values of grass species grown in mixtures at the end of season 1 (S1) and season 2 (S2) in P fertilised plots (P+) and unfertilised plots (P-). On the left of each variable, the p-value from the ANOVA analysis is displayed. Letters indicate differences between groups. PNI = Phosphorus nutrition index, Lp2 = *L. perenne* diploid, Fa = *F. arundinacea*, Lp2Fa = combination of Lp2 and Fa and 4sp = combination of Lp2, *L. perenne* tetraploid, Fa and *P. pratense*, S1 = end of season 1, S2 = end of season 2. Values show mean ± standard error, n = 4.

Treatment	Grass	N:P – S1		N:P – S2		PNI – S1		PNI – S2
P-								
Lp2Fa	Lp2	p = 0.969	12.94 ± 0.69	p = 0.905	12.08 ± 0.52	p = 0.784	0.73 ± 0.04	p = 0.781	0.73 ± 0.03
	Fa		12.99 ± 1.06		12.28 ± 1.50		0.75 ± 0.08		0.70 ± 0.07
4sp	Lp	p = 0.105	15.10 ± 1.84	p = 0.053	15.60 ± 1.09	p = 0.162	0.64 ± 0.08	p = 0.059	0.61 ± 0.04
	Fa		14.43 ± 0.65		17.71 ± 0.65		0.68 ± 0.03		0.51 ± 0.03
	Pp		13.69 ± 1.75		15.34 ± 1.80		0.72 ± 0.10		0.61 ± 0.06
P+								
Lp2Fa	Lp2	p = 0.518	11.51 ± 0.56	p = 0.218	10.26 ± 0.66	p = 0.358	0.83 ± 0.03	p = 0.066	0.92 ± 0.05
	Fa		12.01 ± 0.46		11.86 ± 0.96		0.78 ± 0.04		0.74 ± 0.05
4sp	Lp	p = 0.480	12.92 ± 0.75	p = 0.078	13.08 ± 0.97	p = 0.494	0.74 ± 0.06	p = 0.123	0.74 ± 0.06
	Fa		12.52 ± 1.38		14.12 ± 1.46		0.77 ± 0.12		0.68 ± 0.07
	Pp		11.31 ± 0.41		9.53 ± 0.87		0.86 ± 0.03		0.96 ± 0.12

Table 2.S5 Average values of soil chemical variables measured at the end of the experiment in P fertilised (P+) and P unfertilised (P-) plots at the end of the experiment. Lp2 = *L. perenne* diploid; Fa = *F. arundinacea*; Lp2Fa = combination of Lp2 and Fa; 4sp = combination of Lp2, *L. perenne* tetraploid (Lp4), Fa and *P. pratense* (Pp); DOC = dissolved organic carbon; DON = dissolved organic nitrogen. All results with the exception of pH are expressed in $mg \cdot kg^{-1}$ of dry soil. All variables were measured in a 0.01 *M* $CaCl_2$ extract. Values indicate mean ± standard error, n = 4.

	P	DOC	DON	NH_4	NO_3	pH
P-						
ANOVA	p = 0.761	p = 0.096	p = 0.436	p = 0.876	p = 0.536	p = 0.165
Lp2	0.023 ± 0.002	5.50 ± 0.34	0.51 ± 0.23	0.32 ± 0.06	0.18 ± 0.02	5.18 ± 0.06
Fa	0.021 ± 0.002	5.58 ± 0.27	0.68 ± 0.36	0.27 ± 0.04	0.14 ± 0.03	5.28 ± 0.10
Pp	0.024 ± 0.002	5.88 ± 0.29	0.27 ± 0.08	0.40 ± 0.20	0.35 ± 0.19	5.07 ± 0.08
Lp4	0.024 ± 0.001	5.65 ± 0.41	0.26 ± 0.04	0.36 ± 0.08	0.24 ± 0.03	5.28 ± 0.08
Lp2Fa	0.023 ± 0.001	5.72 ± 0.26	0.32 ± 0.03	0.30 ± 0.03	0.21 ± 0.06	5.36 ± 0.07
4sp	0.024 ± 0.001	6.43 ± 0.16	0.26 ± 0.03	0.28 ± 0.03	0.24 ± 0.04	5.19 ± 0.06
P+						
ANOVA	p = 0.767	p = 0.137	p = 0.757	p = 0.773	p = 0.417	p = 0.073
Lp2	0.049 ± 0.008	5.78 ± 0.39	0.56 ± 0.33	0.40 ± 0.10	0.21 ± 0.06	5.13 ± 0.07
Fa	0.048 ± 0.008	5.89 ± 0.47	0.31 ± 0.07	0.39 ± 0.04	0.26 ± 0.02	5.49 ± 0.02
Pp	0.045 ± 0.005	5.91 ± 0.48	0.27 ± 0.04	0.62 ± 0.37	0.17 ± 0.02	5.13 ± 0.16
Lp4	0.062 ± 0.008	6.46 ± 0.33	0.35 ± 0.07	0.53 ± 0.14	0.25 ± 0.02	5.32 ± 0.02
Lp2Fa	0.052 ± 0.005	6.78 ± 0.37	0.38 ± 0.10	0.84 ± 0.48	0.20 ± 0.05	5.24 ± 0.07
4sp	0.058 ± 0.016	6.20 ± 0.22	0.31 ± 0.02	0.39 ± 0.05	0.52 ± 0.29	5.27 ± 0.09

Annexe to Chapter 2: Additional Species Mixtures

The experiment presented in Chapter 2 also contained all the other possible two-species mixtures from *L. perenne* diploid (Lp2), tetraploid (Lp4), *F. arundinacea* (Fa) and *P. pratense* (Pp), although only the combination of Lp2 and Fa were presented in Chapter 2. The reason for this is the technical constraints that prevented me from analysing the microbial biomass and enzymatic activity on these plots. The data regarding the plants, however, were collected on all the plant treatments and are presented below.

A.1. Plant Mixture Performance

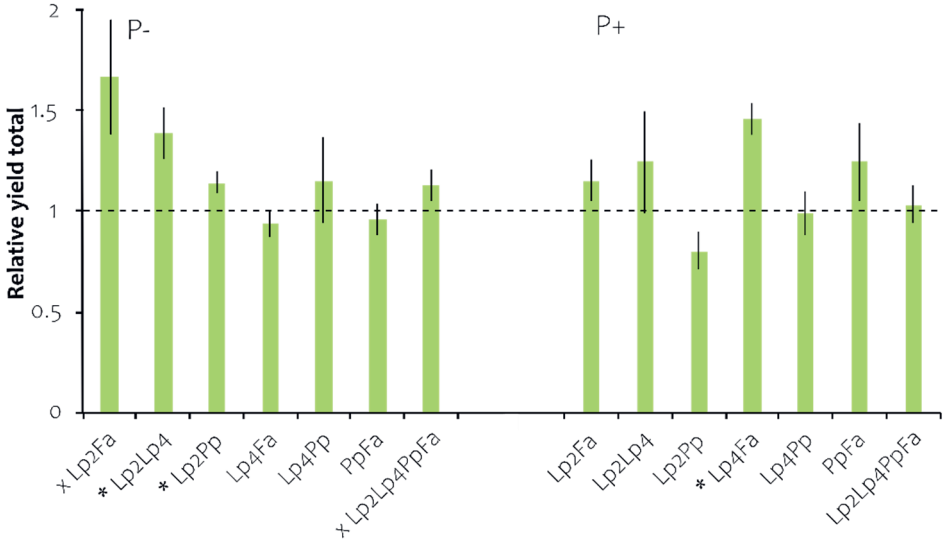


Figure 2.A1 Relative yield total (RYT) of all the grass combinations in the P fertilised (P+) and P unfertilised (P-) plots over the two growing seasons. The contributions of Lp2 and Lp4 to the yield when both species were present are assumed to be equal. Lp2 = *L. perenne* diploid, Lp4 = *L. perenne* tetraploid, Fa = *F. arundinacea*, Pp = *P. pratense*. Bars indicate standard errors, n = 4. Values of RYT > 1 indicate higher yields than monoculture. Results of one-tailed t-test: x = p-value < 0.10, * = p-value < 0.05

Combining the yield of the two seasons, the relative yield totals (RYT) of the grass mixtures were not always higher than one. Only Lp2Lp4 and Lp2Pp in the P unfertilised plots as well as Lp4Fa in the P fertilised plot showed a RYT significantly higher than one, although the P-Lp2Fa treatment was very close to being significant (p-value = 0.0503).

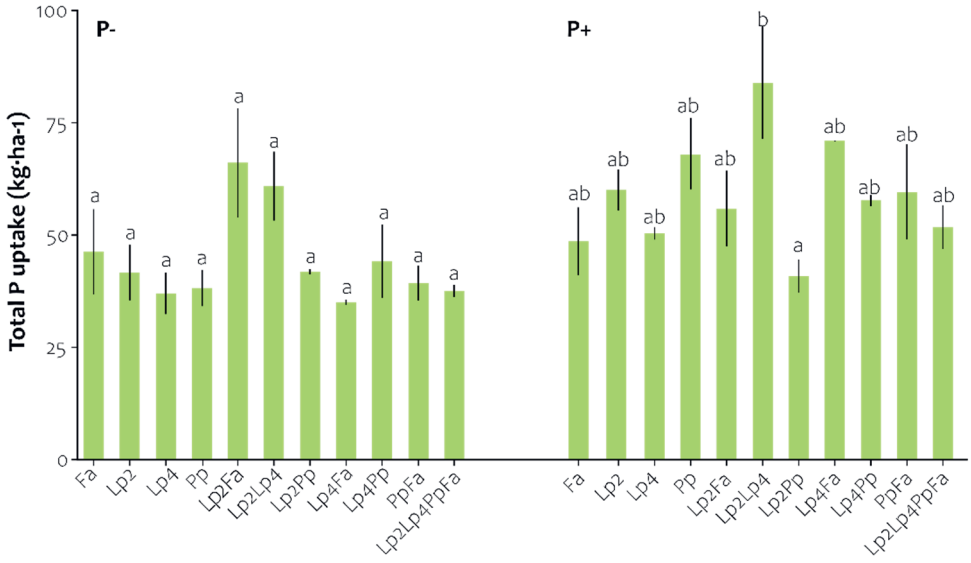


Figure 2.A2 P uptake of all the species combinations in the P fertilised (P+) and P unfertilised (P-) plots for the two growing seasons. The P uptake is calculated assuming that the P content at the end of the growing season is representative of the P content for the harvests of that season. Lp2 = *L. perenne* diploid, Lp4 = *L. perenne* tetraploid, Fa = *F. arundinacea*, Pp = *P. pratense*. Bars indicate standard errors, n = 4. Letters indicate significant differences among grass treatments (Tukey test done separately on the P- and P+ after significant ANOVAs).

The total P uptake for the two seasons is presented in Figure 2.A2. Although the ANOVA reported a significant difference among the different P uptakes of plant treatments for the P unfertilised conditions, the Tukey test was not able to pick up different groups. Yet, visually, we can see a pattern in the data, with Lp2Fa and Lp2Lp4 having a higher P uptake than most other grass treatments. In the P-fertilised conditions, Lp2Pp led to significantly less P uptake than Lp2Lp4. In the P-fertilised conditions, Lp2Fa did not lead to the maximum P uptake.

A.2. Root Traits

At the end of the first growing season, the N and P contents of the roots were measured. Having the N concentration in the roots, the SRL, the average diameter and the root tissue density (RTD) of the plant treatment, I could visualise the root traits of the first season using the root economics space (RES) concept (Bergmann et al., 2020). To this mean, I performed a PCA using the data of these four variables, for the P+ and P- plots separately.

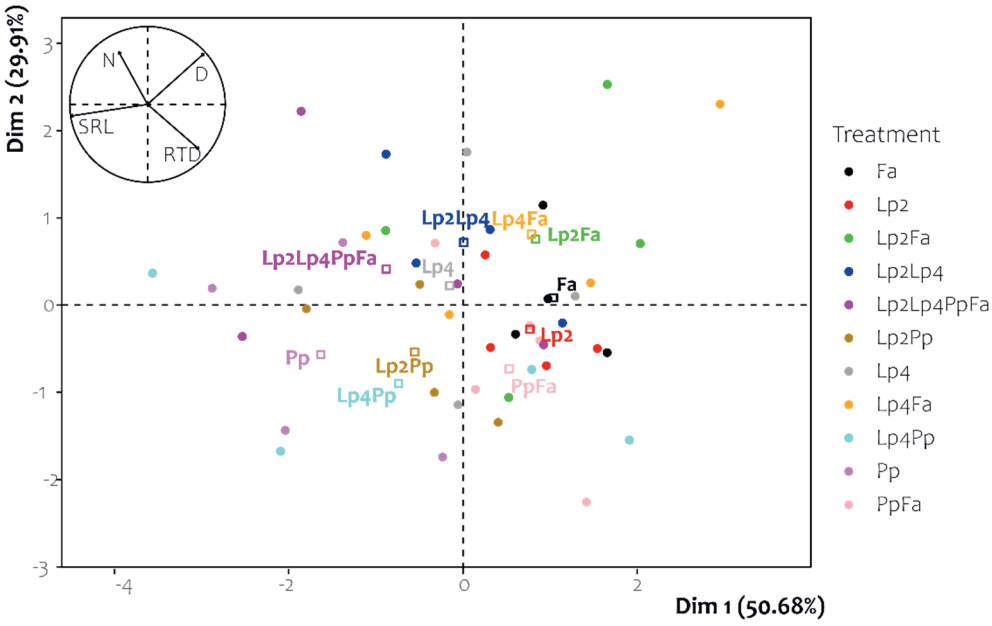


Figure 2.A3 Root economics space of all the plant treatments in P- conditions at the end of the first season for the 0 - 15cm soil layer. Lp2 = *L. perenne* diploid, Lp4 = *L. perenne* tetraploid, Fa = *F. arundinacea*, Pp = *P. pratense*. Bars indicate standard errors, n = 4.

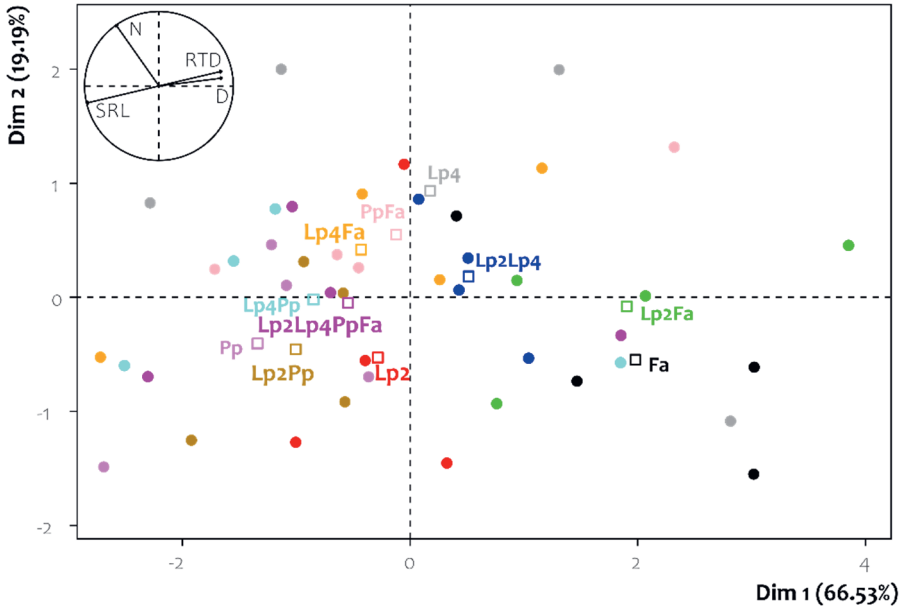
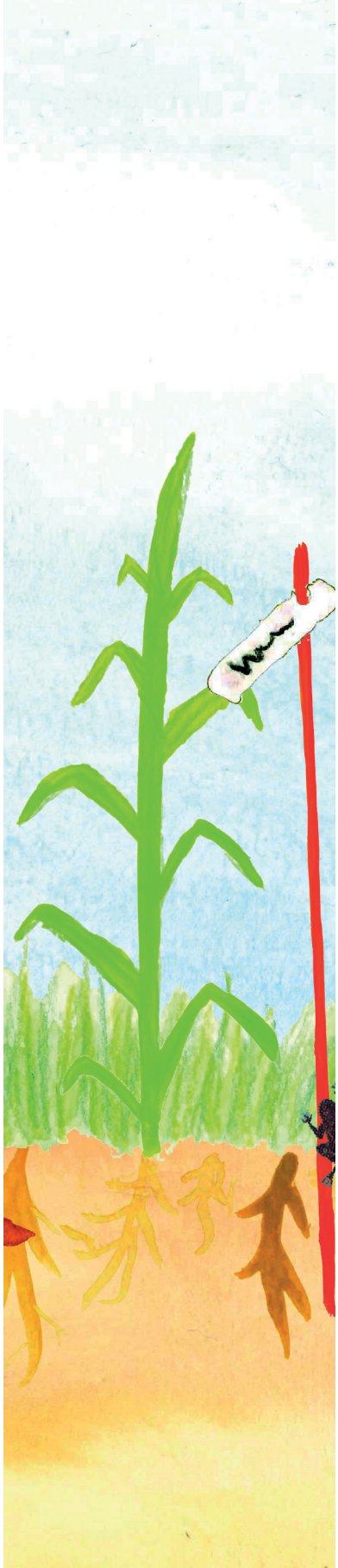


Figure 2.A4 Root economics space of all the plant treatments in P+ conditions at the end of the first season for the 0 - 15cm soil layer. Lp2 = *L. perenne* diploid, Lp4 = *L. perenne* tetraploid, Fa = *F. arundinacea*, Pp = *P. pratense*. Bars indicate standard errors, n = 4. For legend, see Figure 2.A3.

In P unfertilised conditions, the RES was as described by Bergmann et al. (2020), with the collaboration gradient (average diameter and SRL) being perpendicular to the conservation gradient (N content of the roots and RTD) (Figure 2.A3). In the P fertilised conditions, however, the gradients were not maintained, as the average diameter was positively correlated to the RTD (Figure 2.A4). On both PCA, Pp was opposed to Fa along the collaboration gradient, with Pp closer to the "do-it-yourself" pole and Fa closer to the "outsourcing" pole. The conservation gradient separated between the two Lp, with Lp4 closer to the "fast" pole and Lp2 closer to the "slow" pole. Some species mixtures placed themselves between the position of their respective monoculture (for example Lp2Pp), while others did not (for example Lp2Fa).

These results will be further discussed in Chapter 6.



Chapter 3

A Better Understanding of the Effectiveness of Placed Phosphorus Fertilisation with Struvite for Maize: A Pot Experiment

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Abstract

Struvite is emerging as a circular, slow-release phosphorus (P) fertiliser. However, its effectiveness for crops and its impact on soil P tests still remain largely unclear. We analysed the effectiveness of struvite as a row fertiliser in a pot experiment with maize in a sandy soil and a loamy soil, both exhibiting a low agronomic soil P status. Maize was seeded in both soils, amended with either granular struvite or water-soluble di-ammonium phosphate (DAP) as row fertilisers. A positive control received an excess of sodium phosphate, mixed homogeneously through both soils; a negative control did not receive additional P. We measured the agronomic maize performance and assessed the suitability of two established agronomic soil P tests (*i.e.* P-$CaCl_2$ and P-AL) to predict plant-available P in the soils amended with struvite. Furthermore, we performed an additional batch experiment to better understand the dissolution dynamics of struvite during a prolonged period of extraction of soil in 0.01 *M* $CaCl_2$. Placed struvite application led to a lower performance of maize in comparison to DAP in terms of biomass production as well as P uptake (53 and 71% lower, respectively). Yet the agronomic performance of struvite in general was higher than the performance of the negative control, confirming the potential of struvite as an emerging P fertiliser. Surprisingly, both soils fertilised with struvite showed significantly higher soil P availability than any other P treatment in our pot experiment. This was an artefact due to the dissolution of residual struvite granules during the soil extraction procedures. These results call for a reconsideration of how to interpret P-$CaCl_2$ and P-AL as a basis for P fertiliser recommendations for soils receiving struvite as a P fertiliser.

Keywords

Struvite, soil P tests, P fertiliser effectiveness, maize

1 Introduction

Phosphorus (P) is one of the main nutrients limiting plant growth globally, both on natural and agricultural lands (Du et al., 2020; Hou et al., 2020). Although soils generally do contain large amounts of P, it is usually largely unavailable to plants if it is part of primary minerals, or bound to metal (hydr)oxides and clay mineral edges, or present as organic P (Hesterberg, 2010; Yang et al., 2013). To remediate the resulting P-limited plant growth conditions, P fertilisers are applied to agricultural land. The most commonly used mineral P fertilisers, such as triple super phosphate (TSP), come from phosphate rock, which is a non-renewable, limited resource. In addition to being concentrated in the soil of only a few countries, this source will become depleted somewhere in the coming centuries (Cordell et al., 2009; Koppelaar and Weikard, 2013; Walan et al., 2014). This necessitates a rethinking of the origin of the P fertiliser we apply in agriculture. In particular, recycling P from waste streams seems a promising alternative to phosphate rock-based fertilisers (Koppelaar and Weikard, 2013; van Dijk et al., 2016; Vollaro et al., 2017).

Perhaps the most promising recycled P fertiliser is struvite ($Mg(NH_4)PO_4{\cdot}(6H_2O)_{(s)}$). This salt can be recovered from a wide range of wastes such as wastewater, urine, and digestate (Ahmed et al., 2018; Kataki and Baruah, 2018). Although mainly considered a P fertiliser, struvite contains equimolar amounts of nitrogen (N) in the form of ammonium. Mineral fertilisers containing both ammonium and phosphate such as diammonium phosphate (DAP) are already being used and are soluble in water (Kongshaug et al., 2014). Struvite, however, has a lower solubility than presently used mineral P fertilisers (Degryse et al., 2017). Depending on the experimental conditions and the crops, the slow dissolution of struvite can lead to various effects on agronomic crop performance (Ahmed et al., 2018; Hertzberger et al., 2020). Maize is a crop for which adequate P fertilisation in the early growth stage is critical (Bender and Heijden, 2015). Struvite fertilisation trials on maize have led to inconclusive results: some studies report a higher performance of maize when fertilized with struvite as compared to a conventional fertiliser counterpart (Robles-Aguilar et al., 2020; Szymańska et al., 2019), while others report a similar performance (Gell et al., 2011; Muys et al., 2021; Nongqwenga et al., 2017; Uysal et al., 2014). Some studies report a lower maize performance with struvite as compared to a conventional counterpart (Hertzberger et al., 2021; Liu et al., 2011). Although there are various qualities of recovered struvite, a selection of seven struvite samples out of the tested eight resulted in the same performance of maize seedlings in a greenhouse trial (Muys et al., 2021). Thus, the variation in study outcomes with respect to maize performance is likely a result of the different experimental setups and the growing conditions of the plant, especially the struvite dose, the soil P

characteristics, and the length of the experiment (Hertzberger et al., 2020). Furthermore, all these studies focused on broadcast application or homogeneous spreading of struvite while maize usually benefits from row application of fertilisers (Alam et al., 2018). Moreover, the fertilization effect of struvite seems to differ per soil (Nongqwenga et al., 2017).

In addition to measuring the agronomic performance of crops after fertiliser application, the use of agronomic soil P tests to extract P from soil provides a more complete insight into the effectiveness of a P fertiliser to raise plant-available P. In the Netherlands, P-$CaCl_2$ and P-AL are used as a combined indicator to classify the agronomic soil P status of arable land and grassland as a basis for P fertiliser recommendation (Commissie Bemesting Grasland en Voedergewassen, 2023). With the introduction of P-$CaCl_2$ as an intensity method and P-AL as a quantity method (Nawara et al., 2017), it became possible to bring the role of soil P buffering into the Dutch P fertiliser recommendation system as the ratio of P-AL over P-$CaCl_2$ provides a more mechanistic insight into the availability of P in soil for plant uptake (Reijneveld et al., 2022; van Rotterdam et al., 2012). For determining P-$CaCl_2$, the multi-nutrient 0.01 *M* $CaCl_2$ extraction method based on a soil-to-solution ratio (SSR) of 1:10 (w:v) can be used, which mimics soil solution conditions (Houba et al., 2000; McDowell and Sharpley, 2001). The size of the P-$CaCl_2$ pool, which is commonly interpreted as a measure of the so-called readily plant-available P (Houba et al., 1996; Nawara et al., 2017; Reijneveld et al., 2022; van Doorn et al., 2023), is determined by P desorption being a function of the P loading of poorly crystalline Fe- and Al-(hydr)oxides in soil and soil properties like pH and soil organic matter (SOM) (Koopmans et al., 2004a; Weng et al., 2011). Poorly crystalline Fe- and Al-(hydr)oxides ([Fe+Al]-ox) and P reversibly adsorbed to these (hydr)oxides (P-ox) can be extracted from soil with 0.2 *M* acid ammonium oxalate (Schwertmann, 1964). The P loading of Fe- and Al-(hydr)oxides (α) in soil can be calculated as the molar ratio between P-ox and [Fe+Al]-ox (van der Zee and van Riemsdijk, 1988). With P-AL, soil is extracted with a mixture of acetic acid and ammonium lactate (Egnér et al., 1960). The size of the P-AL pool largely depends on the P loading α as well as the amount of P reversibly adsorbed to poorly crystalline Fe- and Al-(hydr)oxides (Schoumans and Groenendijk, 2000; van Doorn et al., 2023). Although P-$CaCl_2$ and P-AL are established agronomic soil P tests (Nawara et al., 2017; Reijneveld et al., 2022; van Doorn et al., 2023), their reliability has not been tested yet for soils to which struvite has been applied, while other agronomic soil P tests (Mehlich-3, Bray-1, and Olsen P) have been shown to give unreliable results for struvite-amended soils (Gu et al., 2021).

The objective of this study was twofold: first, we aimed to further the understanding of the effectiveness of struvite as a placed P fertiliser for maize and second, we wanted to assess the suitability of P-$CaCl_2$ and P-AL as routinely employed agronomic soil P tests for determining plant-available P in soils amended with struvite. To this end, we performed a pot experiment with maize, using two soils with contrasting soil properties but a low agronomic soil P status for comparing the performance of maize with placed fertilisation of struvite versus placed DAP fertilisation. Row P fertilisation is often used as an application technique for maize cropping in the Netherlands (van Schooten et al., 2019). Soil samples taken at the end of the pot experiment were used to determine P-$CaCl_2$ and P-AL so as to assess how plant-available P was related to aboveground biomass production and P uptake by maize. Furthermore, the 0.2 *M* acid ammonium oxalate extraction method was used to quantify how P-$CaCl_2$ in the struvite- and DAP-amended soils was related to the P loading α. Lastly, a batch experiment was done to quantify struvite dissolution during soil extraction with 0.01 *M* $CaCl_2$ using a shaking time varying from 1 to 24 h instead of the prescribed 2 h (Houba et al., 2000)

2 Materials and Methods

2.1 Soils

The pot experiment was conducted with a sandy soil and a loamy soil. The sandy soil, a Plaggic Podzol (IUSS Working Group WRB, 2015), was sampled from an extensively managed grassland (Achterberg, the Netherlands), whereas the loamy soil, a Gleyic Fluvisol (IUSS Working Group WRB, 2015), was taken from a floodplain along the river Meuse (Lottum, the Netherlands). Both soils were collected from the top layer (0-25 cm). For the pot experiment, soils were air-dried and sieved over 5 mm. Prior to chemical analysis, a subsample from both soils was oven-dried (40˚C) and passed through a 2 mm-sieve. Soil texture was determined via pipette and sieve after removal of $CaCO_3$ and organic matter (Houba et al., 1997). Near-infra-red spectroscopy was used to determine $CaCO_3$ in the loamy soil (Eurofins Agro, Wageningen, the Netherlands). The loss-on-ignition method (550˚C) (Hoogsteen et al., 2015) was used to determine SOM. An extraction with 0.01 *M* $CaCl_2$ was used to measure the pH as well as N-NO_3, N-NH_4, and P-PO_4 (Houba et al., 2000). Moreover, P-AL (Egnér et al., 1960) was measured and an extraction with 0.2 *M* acid ammonium oxalate (Schwertmann, 1964) was used to determine P-ox and [Fe+Al]-ox and to calculate the P loading α (van der Zee and van Riemsdijk, 1988). Analytical details of the aforementioned extraction methods are given in Section 2.4.

2.2 Experimental Design

We set up a pot experiment with the two soils and four P fertiliser treatments with five replicates each, following a random block design. In order to simulate row fertilisation in our pot experiment, DAP and struvite granules were not mixed homogeneously through the soil but placed 2 cm below the soil surface at the centre of the pot (Figure S1). The zone of 5.5 cm diameter around this centre was named the "fertiliser zone", i.e. the zone with DAP or struvite, whereas the bulk soil of these treatments did not receive any P fertiliser. The amount of P applied with struvite and DAP was the same, i.e. 17 kg $P \cdot ha^{-1}$. This P dosage was calculated as the average P dose recommended for both soils as a row fertiliser for maize, based on the initial P-$CaCl_2$ and P-AL of both soils (Table 3.1) (Commissie Bemesting Grasland en Voedergewassen, 2023). DAP and struvite were obtained from a commercial supplier (Triferto Fertilizers, Doetinchem, the Netherlands) and a sewage treatment plant for industrial and domestic waste water (Land van Cuijk, Cuijk, the Netherlands), respectively. The required DAP and struvite dosages were calculated using the claimed P contents of these fertilisers. The actual chemical composition of both fertilisers was measured afterwards. To this end, subsamples of DAP and struvite were digested with a mixture of H_2SO_4, salicylic acid, H_2O_2, and selenium (Novozamsky et al., 1983). The N and P concentrations in the digests were measured with a segmented flow analyser (SFA). Furthermore, a subsample of the struvite was digested with Aqua Regia (Houba et al., 1997) to measure the contents of magnesium (Mg) and other elements using an inductively coupled plasma - optical emission spectrometer (ICP-OES). The results are reported in Table 3.S1. The measured N, P, and Mg contents in struvite and DAP deviated by a maximum of 5% from the claimed contents. The remaining two P fertiliser treatments of the pot experiment are controls: a negative control (control[-]) without P fertilisation and a positive control (control[+]) receiving P in solution ($NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot H_2O$ mixed in a 1:1 molar ratio) to homogeneously increase soil P availability in the fertiliser zone and the bulk soil alike. For the control[+] treatment, we raised the agronomic P status of both soils from the category "low" to the category "sufficient" (Ministerie van Binnenlandse Zaken en Koninkrijksrelaties, 2023). Since the loamy soil had a lower P-$CaCl_2$ and P-AL than the sandy soil (Table 3.1), the former received a higher P dosage (64 kg $P \cdot ha^{-1}$) than the latter (47 kg $P \cdot ha^{-1}$). Pots from all treatments received N, Mg, potassium (K), sulphur (S), copper (Cu), manganese (Mn), and boron (B), applied as solutions and mixed homogeneously through the soil, both in the bulk soil and the fertiliser zone. The amounts of N and Mg added with DAP or struvite were subtracted from the intended N and Mg applications (Table 3.S2). A combination of solutions prepared from NH_4NO_3, $Mg(NO_3)_2 \cdot 6H_2O$, KCl, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot H_2O$,

$Na_2SO_4 \cdot 10H_2O$, and H_3BO_3 was used to reach a final fertilisation level of 1.20 g N·pot^{-1} for the control[-], control[+], and DAP treatments and 1.23 g N·pot^{-1} for the struvite treatment, 0.28 g Mg·pot^{-1}, 1.33 g K·pot^{-1}, 0.20 g S·pot^{-1}, 0.06 g Cu·pot^{-1}, 0.06 g Mn·pot^{-1}, and 4 mg B·pot^{-1}. The conversion of the recommended fertilisation doses expressed per hectare into doses at the pot scale was done based on the number of maize plants in the field, using a typical maize plant density of 100000 plants·ha^{-1} (van Schooten et al., 2019), while we had one maize plant in each pot at the start of our pot experiment.

Each pot (volume = 4 L and ø = 20 cm) was filled with 4.2 kg of sandy soil or 3.8 kg of loamy soil including the combination of solutions with the above-mentioned fertilisers and additional demi-water to reach 60% of the maximum soil water holding capacity. Four maize seedlings (pregerminated in the dark at 25˚C for 72 h) of the variety Movanna (Zaadhandel Neutkens, Vessem, the Netherlands) were planted in a circle, 2.7 cm from the centre of each pot. Seven days after planting, the best-performing plant was selected and the remaining three plants were removed to ensure the presence of one viable maize plant in each pot (Figure 3.S1). Each pot was watered every other day with demi-water. Each week, the five blocks were rotated clockwise to minimize possible effects of local differences in plant growth conditions in the greenhouse. Plant height was weekly measured. The greenhouse temperature was kept at 25˚C and relative air humidity was 65%. Artificial light was supplied for one hour before and after sunset. On day 40 at which some of the maize plants had reached the end of the vegetative growth stage, the experiment was terminated: maize shoots were harvested and roots and soil were sampled, as described hereafter.

2.3 Plant Analyses

After harvest, maize shoots were oven-dried (70˚C), followed by measuring their biomass and grinding of the material. Total N and P in the ground biomass were measured by digesting a subsample according to Novozamsky et al. (1983), followed by measurement of the N and P concentrations with a SFA. The uptake of N and P by maize was calculated by multiplying the harvested dry biomass with the N and P contents. The P use efficiency (PUE) was calculated as follows:

$$PUE\ (\%) = \frac{F - P_0}{P_1} \times 100$$

with F being the amount of P fertiliser added per pot, P_0 the P uptake of the control[-] treatment, and P1 the P uptake of the fertilised pot.

For all treatments, roots were sampled at two locations in the pot: the fertiliser zone and the bulk soil (Figure 3.S1). To do so, we extracted two soil cylinders (ø = 5.5 cm and height = 15 cm) at equidistance from the maize stubble. The soil sampled with

the cylinders was sieved (2 mm) to separate the roots from the soil. Next, the soil taken with the cylinder from the bulk soil was mixed with the rest of the bulk soil to create one composite bulk soil sample, whereas the soil taken with the cylinder from the fertiliser zone was used as it was. The collected roots were further cleaned by washing over a 0.5 mm-sieve and collected carefully with tweezers. Roots were dried (70˚C) and their biomass was measured. We calculated the ratio of root biomass in the fertiliser zone and the bulk soil as a measure for the maize root distribution in the potted soil.

2.4 Soil Analyses

The soil samples of the first three blocks out of the in total five blocks were oven-dried (40˚C) and 2 mm-sieved prior to chemical analysis. The soil samples were extracted with 0.01 *M* $CaCl_2$ at a SSR of 1:10 (w:v) with horizontal shaking for two hours (160 strokes·min^{-1}) and centrifugation (1800 x g for 10 min) following Houba et al. (2000). One part of the supernatant was used to measure the pH and the other part was 0.45 µm-filtered (Aqua 30/0.45 CA Whatman) and acidified by adding 25 µL 5 *M* HCl for sample conservation before chemical analysis. We determined phosphate (P-PO_4) (hereafter referred as P-$CaCl_2$), dissolved organic carbon (DOC), N-NO_3, N-NH_4, and total dissolved N (Nts) with an SFA and Al, Fe, K, Mg, and total dissolved P (TDP) with an ICP-OES. The results of the pH and TDP, DOC, N-NH_4, N-NO_3, and Nts measurements are shown in Table 3.S3. Determination of P-AL was done by extracting soil with a mixture of acetic acid and ammonium lactate (pH = 3.75) at a SSR of 1:20 (w:v) with horizontal shaking for four hours (160 strokes·min^{-1}), centrifugation (1800 x g for 10 min), and 0.45 µm-filtration (Egnér et al., 1960). In the resulting extract, we measured P-PO_4 using the SFA to obtain P-AL. To determine P-ox and [Fe+Al]-ox, soil was extracted with 0.2 *M* acid ammonium oxalate following Schwertmann (1964), using a SSR of 1:20 (w:v) with horizontal shaking for two hours (160 strokes·min^{-1}) in the dark and centrifugation (3000 x g for 10 min). We then measured P, Al and Fe in the resulting extract with an ICP-OES. Knowing P-ox and [Fe+Al]-ox, the P loading α was calculated (van der Zee and van Riemsdijk, 1988). The results of the 0.2 *M* acid ammonium oxalate extraction are shown in Table 3.S4. To assess the possibility of struvite dissolving during 0.01 *M* $CaCl_2$ extraction after the standard shaking time of 2 h as prescribed by Houba et al. (2000) had ended, we performed an additional batch experiment using a shaking time of up to 24 h. This experiment is described in detail in Section S1.

2.5 Statistics

Statistical analyses were performed with R (version 3.6.0, "Planting of a Tree") and the *stats*, *agricolae* and *dplyr* packages. The normality and homoscedasticity of the data were checked with the Shapiro and Bartlett tests, respectively. We performed analyses of variances (ANOVA) after checking on whether the residuals followed normality. When the normality assumption was not satisfied, we log-transformed the data. This was the case for the results on plant P uptake, P-ox, and P loading α. In the case of heteroscedasticity, we used the varIdent variance structure (*nlme* package) by treatment to correct it (Zuur et al., 2009). When the ANOVA yielded a significant difference among treatments, we proceeded with Tukey's honestly significant difference (HSD) test. For the root distribution ratio, we excluded the control[-] from the statistical analysis, because the variation of the root distribution ratio for this treatment was higher than for the other treatments but the actual root biomasses in the control[-] were very low. On average per pot, sampled root biomass were 0.021, 0.123, 0.192, and 0.091 g for the control[-], control[+], DAP, and struvite treatments, respectively. Furthermore, we excluded the control[-] for P-$CaCl_2$ of both soils as it was the only way to meet the assumptions for the ANOVA, while P-$CaCl_2$ was low for the control[-] of both soils.

3 Results and Discussion

3.1 Initial Soil Properties

The sandy soil and the loamy soil differed in texture and $CaCO_3$ content (Table 3.1). The sandy soil was noncalcareous with only 2% clay, while the loamy soil contained 17% clay as well as 0.6% $CaCO_3$. The soils had a different pH, with the sandy soil being acidic (pH = 5.5), whereas the loamy soil had a near-neutral pH of 6.5. The loamy soil had a higher SOM content (6.0%) than the sandy soil (4.2%). Furthermore, the loamy soil had a higher [Fe+Al]-ox content than the sandy soil, whereas the amount of reversibly adsorbed P-ox was slightly lower. Consequently, the P loading α for the loamy soil was lower (α = 0.09) than for the sandy soil (α = 0.14). This is in line with the higher P-$CaCl_2$ of the sandy soil compared to the loamy soil. Yet, the P loading α is rather low for both soils when compared to a large set of representative Dutch agricultural topsoils (Koopmans et al., 2006). The soil P status of both soils was classified as "poor", based on P-$CaCl_2$ and P-AL (Ministerie van Binnenlandse Zaken en Koninkrijksrelaties, 2023).

Table 3.1. Properties of the two soils used in this study.

Property	Unit	Sandy soil	Loamy soil
Clay	%	2	17
Silt	%	8	46
Sand	%	90	37
SOM[1]	%	4.2	6.0
$CaCO_3$	%	nd[2]	0.6
pH-$CaCl_2$	-	5.5	6.5
N-NO_3 ($CaCl_2$)	mg N·kg^{-1}	9.8	5.0
N-NH_4 ($CaCl_2$)	mg N·kg^{-1}	2.8	0.2
P-$CaCl_2$[3]	mg P·kg^{-1}	0.37	0.16
P-AL	mg P·kg^{-1}	116	48
P-ox	mg P·kg^{-1}	301	278
[Fe+Al]-ox	mmol·kg^{-1}	71	102
α[4]	-	0.14	0.09

[1]Soil organic matter.
[2]Not determined.
[3]P-$CaCl_2$ refers to P-PO_4 as measured by SFA in a 0.01 *M* $CaCl_2$ soil extract (Houba et al., 2000).
[4]P loading of poorly crystalline Fe- and Al-(hydr)oxides (van der Zee and van Riemsdijk, 1988).

3.2 Aboveground Biomass Production and Nutrient Uptake

For the control[+], DAP, and struvite treatments, maize reached a significantly higher aboveground biomass production in the loamy soil compared to the sandy soil (Figure 3.1A). Overall, the biomass was highest for the control[+] and DAP treatments, followed by the struvite treatment, and then the control[-] treatment. For the loamy soil, the differences in biomass between the four P treatments were in line with the overall effect of P fertilisation. For the sandy soil, however, this was not the case. For the latter, the biomass of the struvite treatment did not differ significantly from the results of the control[-] and control[+] treatments. The biomass results correspond closely to those of the maize plant height (Figure 3.S2).

The results of the nutrient uptake by maize (Figure 3.2A and B) are reasonably in line with those of the aboveground biomass production. For the control[+] and DAP treatments, N and P uptake were significantly higher for the loamy soil than for the sandy soil, whereas no difference was found between both soils for the struvite treatment. Overall, N and P uptake was lowest for the control[-] treatment. For the

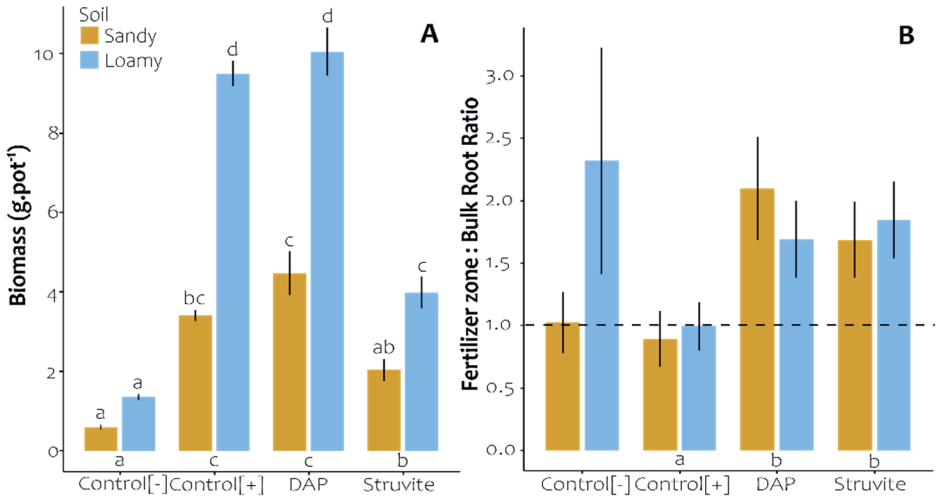

Figure 3.1. Aboveground biomass production of maize (A) and root biomass ratio (B) for the four P treatments of the sandy soil and the loamy soil (n = 5). The dashed line in Figure 3.2B at a root ratio = 1 indicates an equal root biomass in the fertiliser zone and in the bulk soil sample. Because of a very low root biomass of the control[-] treatment for both soils, the latter was excluded from statistical analyses of the root ratio. The error bars represent the standard error of the mean and letters indicate the results of the Tukey test.

struvite treatment, N and P uptake was significantly higher than for the control[-] treatment, but significantly lower than for the control[+] and DAP treatments. For both soils, the differences in N uptake between the four P treatments aligns with the overall effect of P fertilisation, whereas this was not the case for P uptake. For the sandy soil, P uptake of the DAP treatment was significantly higher than for the three other P treatments. However, P uptake of the struvite treatment did not differ significantly from the results of the control[-] and control[+] treatments. The control[-] treatment had a significantly lower P uptake than the control[+] treatment. For the loamy soil, P uptake was significantly different for all four P treatments, with the control[+] treatment having the highest P uptake, followed by the DAP treatment, the struvite treatment, and then the control[-] treatment. Compared to the control[-] treatment, the higher N and P uptake of the DAP and struvite treatments of both soils (with the exception of P uptake from the struvite treatment of the sandy soil) is most likely facilitated by proliferation of maize roots in the fertiliser zone (Figure 3.1B). This will be further discussed in Section 3.3.

The stoichiometric ratio of N and P in the aboveground biomass has widely been used to investigate the nature of nutrient limitation for the growth of natural vegetation as well as several crops including maize (Bélanger et al., 2012; Koerselman and Meuleman, 1996; Sadras, 2006; Ziadi et al., 2007). Overall, P fertilisation helped to alleviate P limitation for both soils, as the N:P ratio was highest

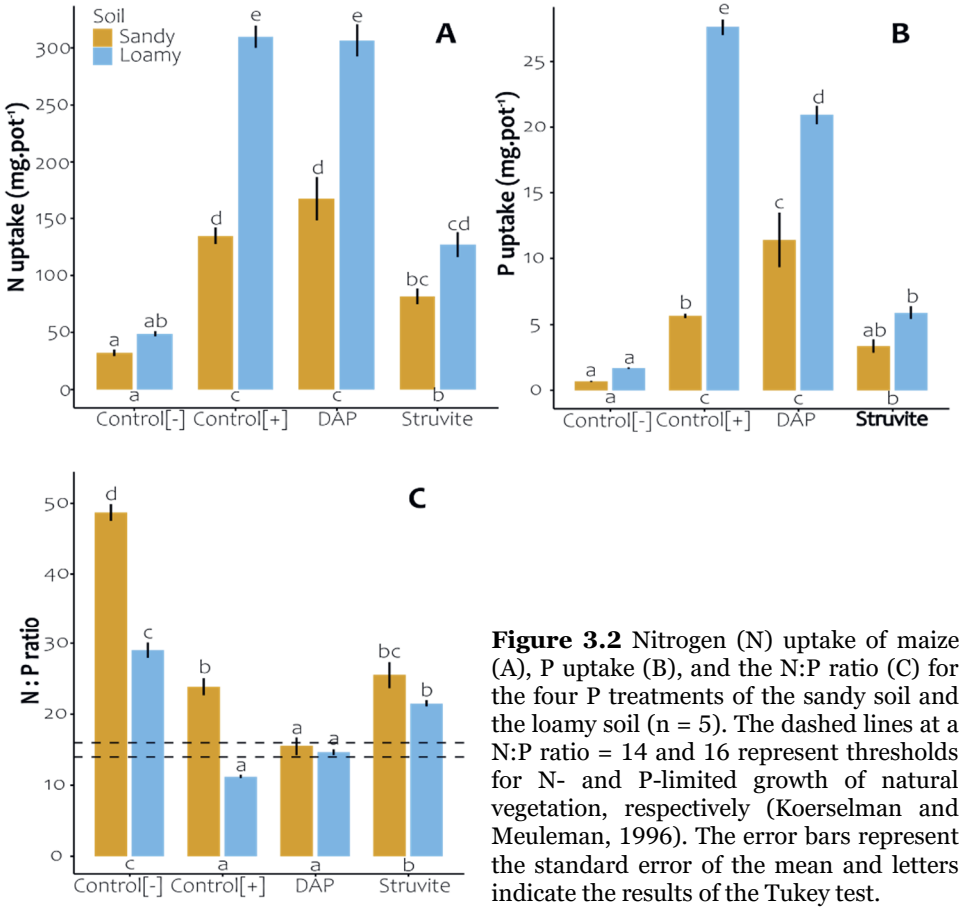

Figure 3.2 Nitrogen (N) uptake of maize (A), P uptake (B), and the N:P ratio (C) for the four P treatments of the sandy soil and the loamy soil (n = 5). The dashed lines at a N:P ratio = 14 and 16 represent thresholds for N- and P-limited growth of natural vegetation, respectively (Koerselman and Meuleman, 1996). The error bars represent the standard error of the mean and letters indicate the results of the Tukey test.

for the control[-] treatment, followed by the struvite treatment, and then the control[+] and DAP treatments (Figure 3.2C). These results were in line with those of the overall effect of P fertilisation on the aboveground biomass production of maize (Figure 3.2A). For the control[-] treatment of both soils, the N:P ratio was far above 16, which is used as a threshold to indicate P limitation for natural vegetation (Koerselman and Meuleman, 1996). This is in accordance with the classification of the agronomic soil P status of both soils as "poor", based on the initial P-$CaCl_2$ and P-AL (Table 3.1). However, the sandy soil of the control[-] treatment had a higher N:P ratio (i.e. 49.3) than the loamy soil (i.e. 30.0), although the sandy soil had both a higher initial P-$CaCl_2$ and P-AL (Table 3.1). Furthermore, P application in the control[+] treatment lowered the N:P ratio to below 14, which would indicate N limitation (Koerselman and Meuleman, 1996). However, this was not the case for the sandy soil, as the N:P ratio of this treatment was significantly higher and well

above 16. For the DAP and struvite treatment, no significant differences in N:P ratio were found between the sandy soil and the loamy soil. The N:P ratio was well above 16 for both soils when struvite was applied. In case of DAP addition, the N:P ratio was between 14 and 16. Hence, DAP was more effective in alleviating P limitation than struvite, although the amounts of P applied with both fertilisers were the same (Table 3.S2). This may be attributed to the poor solubility of struvite, while DAP has a higher solubility and quickly dissolves in soil (Talboys et al., 2016).

3.3 Placement Effect of P Fertiliser

For both soils, DAP was equally effective in increasing the aboveground biomass production of maize as the P fertiliser applied in the control[+] treatment (Figure 3.1A). Likewise, struvite had the same effectiveness as the control[+] treatment for the sandy soil. This is the effect of placed DAP and struvite application in the fertiliser zone (Figure 3.S1). In soils with a low agronomic P status such as used here (Table 3.1), P placement in a small volume of soil leads to a locally larger increase in plant-available P than the same amount of P mixed homogeneously through a larger soil volume, thereby enhancing both P uptake and crop yield (Grant et al., 2001; Lu and Miller, 1993). According to a meta-analysis of Nkebiwe et al. (2016), P fertiliser placement led to a 14.3% higher crop yield than broadcast P application for equal amounts of P applied. Remarkably, the amounts of P applied with DAP and struvite to the fertiliser zone of both soils (i.e. 17 kg P·ha^{-1}) in our pot experiment were even 2.8 and 3.8 times lower than the amounts of P mixed through the entire volume of potted soil for the control[+] treatment of the sandy soil (i.e. 47 kg P ha^{-1}) and the loamy soil (i.e. 64 kg P·ha^{-1}), respectively. Plants can respond to P limitation as in the control[-] treatment of the sandy soil and the loamy soil (Figure 3.2C) by various adaptations including root proliferation in regions of the soil with an elevated level of plant-available P (Grant et al., 2001). For this reason, we sampled roots in equal volumes of soil in the fertiliser zone and in the bulk soil of the four P treatments of both soils (Figure 3.S1). The ratio of the root biomass in the fertiliser zone to the root biomass in the bulk soil was close to one for the control[-] treatment of the sandy soil (Figure 3.1B). Hence, no root proliferation took place in the fertiliser zone, which is understandable as no P was applied at all in this treatment. For the control[-] treatment of the loamy soil, however, the ratio was unexpectedly above one. Since the root biomass in the control[-] treatment of both soils was very low (see Section 2.5), these ratios were sensitive to variation as any root fragment collected in either the fertiliser zone or the bulk soil may have had a large impact on the resulting ratios. For this reason, the ratios of the control[-] treatments of both soils were not included in further statistical analyses. The ratio for the control[+] treatment of both soils did not significantly differ from one, meaning there was no preferential root growth in

one zone or the other. This was to be expected since the P fertiliser was mixed through the entire volume of potted soil. For the DAP and struvite treatments of both soils, the ratios were higher than the ratios of the control[+] treatment and significantly higher than one. Hence, root proliferation took place in the fertiliser zone, probably enabling the maize plant to directly tap P from the locally elevated pool of plant-available P resulting from placed DAP and struvite application. In the next section, the response of P-$CaCl_2$ and P-AL to P fertilisation will be discussed.

3.4 Response of Soil P Tests to P Fertilisation

In Figure 3.3, the results of P-$CaCl_2$ and P-AL measured in the bulk and fertiliser zone samples from the four P treatments of the sandy soil and the loamy soil are presented. For both soils, the bulk soil samples of all P treatments except those from the control[+] treatment had the same P-$CaCl_2$ and P-AL. The P-$CaCl_2$ and P-AL in the bulk soil samples of the control[+] treatment were higher as P was mixed through the entire volume of potted soil, whereas struvite and DAP were placed in the fertiliser zone (Figure 3.S1). For the fertiliser zone of the control[+] treatment, P application caused P-$CaCl_2$ to increase to 1.77 ± 0.06 mg·kg^{-1} for the sandy soil and

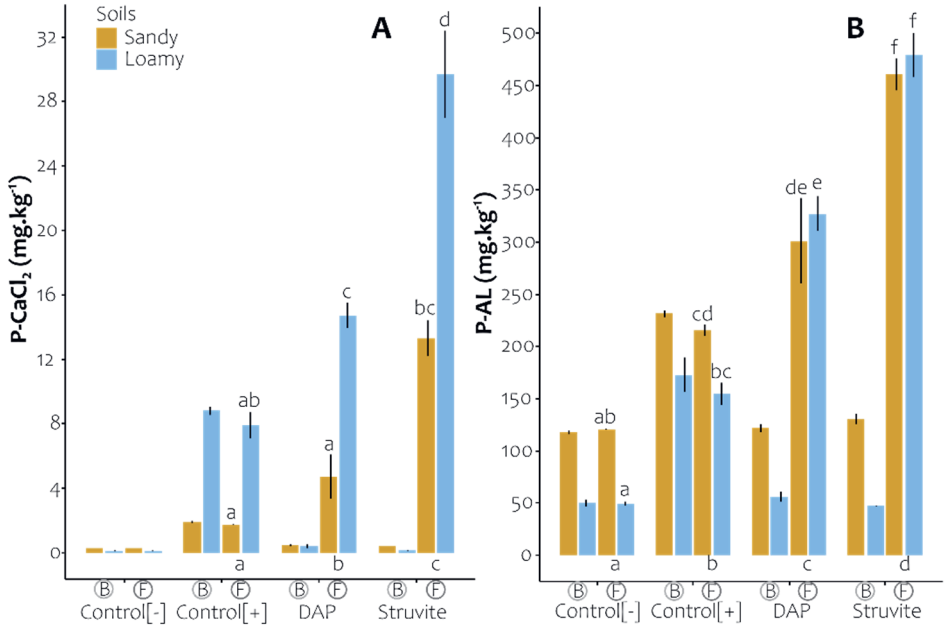

Figure 3.3 The amounts of P-$CaCl_2$ (A) and P-AL (B) extracted from the soil samples taken from the bulk soil (circled B) and the samples from fertiliser zone (circled F) of the four P treatments of the sandy soil and the loamy soil (n = 3). The error bars represent the standard error of the mean and letters indicate the results of the Tukey test. Bulk soil samples violated normality assumption so ANOVA was not performed for this part of the data.

7.90 ± 1.40 mg·kg^{-1} for the loamy soil. Although the loamy soil of this treatment had a higher P-ox than the sandy soil (Table 3.S4), yet its P loading α (i.e. 0.14) was lower than the α of the sandy soil (i.e. 0.19) resulting from the higher [Fe+Al]-ox content of the loamy soil (Table 3.1). Hence, P application in the control[+] treatment was more effective in raising P-$CaCl_2$ for the loamy soil than for the sandy soil, despite the lower P loading α of the loamy soil. For the treatments with DAP and struvite, the same effect was observed: P-$CaCl_2$ in the fertiliser zone of the sandy soil increased to 4.73 ± 2.38 mg·kg^{-1} for the DAP treatment and 13.3 ± 1.9 mg·kg^{-1} for the struvite treatment, whereas P-$CaCl_2$ in the fertiliser zone sample of the loamy soil increased to 14.7 ± 1.4 mg·kg^{-1} and 29.7 ± 4.7 mg·kg^{-1} for the DAP and struvite treatments, respectively. Yet again, the P loading α values of the DAP- and struvite-amended loamy soil were lower than those of the sandy soil (Table 3.S4). The difference in the effectiveness of P application between both soils to raise P-$CaCl_2$ could be the result of a different P buffering capacity (Ehlert et al., 2003). This is illustrated by plotting the P loading α against P-$CaCl_2$ for the soil samples from all P treatments of the sandy soil and the loamy soil in Figure 3.4, revealing the P adsorption isotherm of both soils (Koopmans et al., 2004b). Since the adsorption isotherm of the loamy soil has a lower slope at any given P-$CaCl_2$, this soil has a lower P buffering capacity than the sandy soil (Ehlert et al., 2003). This may be due to the higher pH and SOM content of the loamy soil (Table 3.1). Adsorption of P to Fe- and Al-(hydr)oxides decreases when the pH increases (Antelo et al., 2010; Weng et al., 2012). Furthermore, P may compete with organic matter for binding to poorly crystalline Fe- and Al-(hydr)oxides as both P and organic matter bind to metal-(hydr)oxides, which lowers P adsorption (Antelo et al., 2007; Hiemstra et al., 2010; Regelink et al., 2015; Weng et al., 2012).

There is one aspect which complicates the interpretation of the adsorption isotherms in Figure 3.4. Remnants of struvite granules were clearly visible when the fertiliser zone samples were taken from the sandy soil and the loamy soil at the end of the pot experiment. There were no discernible DAP granules. This explains why the levels of TDP, N-NH_4, and Mg in the 0.01 *M* $CaCl_2$ soil extracts of the fertiliser zone samples from the struvite treatment of both soils were significantly higher than those of the other P treatments (Table 3.S3). In an additional batch experiment, we extracted one fertiliser zone soil sample from each P treatment with 0.01 *M* $CaCl_2$, using a shaking time varying from 1 to 24 h. In the standard procedure of the 0.01 *M* $CaCl_2$ extraction method of Houba et al. (2000), a shaking time of 2 h is prescribed. This shaking time is commonly interpreted as the time required to reach equilibrium for the desorption of multiple nutrients including N-NH_4 and P-PO_4 (Koopmans et al., 2004b, 2004a; van Erp et al., 1998). The results of this batch experiment are discussed in detail in Section S1 of the Supplementary Information. In brief, clear indications for the

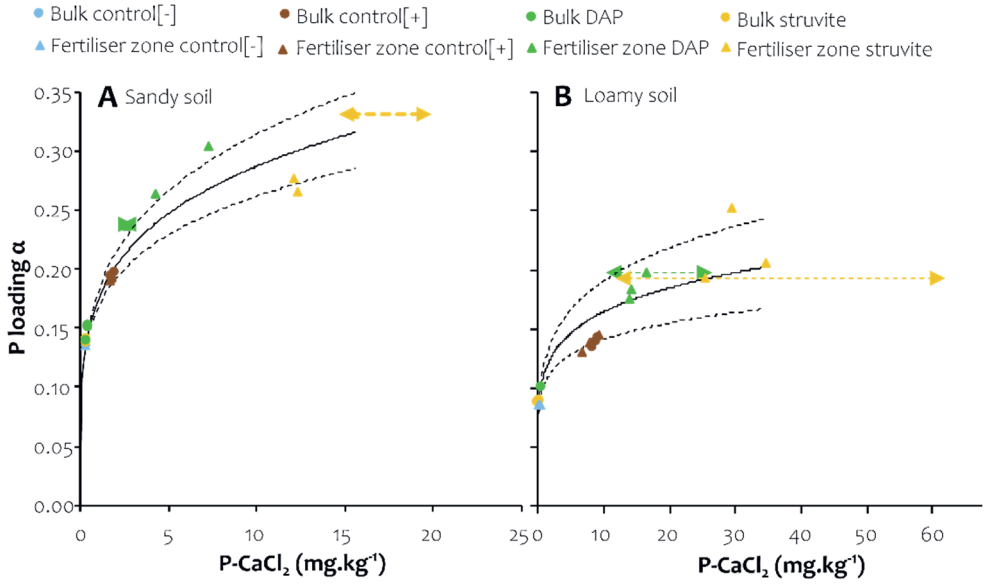

Figure 3.4. The P loading α calculated as the molar ratio between P-ox and $[Al+Fe]_{ox}$ plotted against P-$CaCl_2$ extracted from the bulk and fertiliser zone samples from the four P treatments of the sandy soil (A) and the loamy soil (B) (n = 3). The P-$CaCl_2$ is based on the prescribed shaking time of 2 h (Houba et al., 2000). To guide the eye, a Freundlich equation has been fitted to the results of both soils, with K_F = 0.17 and n = 0.22 for the sandy soil and K_F = 0.11 and n = 0.17 for the loamy soil. The dotted lines on both sides of the Freundlich equation represent the 95%-confidence interval. The arrows with dotted lines for one of the three fertiliser zone soil samples from the DAP and struvite treatments of both soils represent the results of the additional batch experiment in which the fertiliser zone samples were extracted with 0.01 *M* $CaCl_2$ using a shaking time varying from 1 to 24 h. Since the effect of shaking time on the extraction of P-PO_4 from the DAP-amended sandy soil by 0.01 *M* $CaCl_2$ in the batch experiment was limited, the arrow with the dotted line cannot be seen in the figure.

dissolution of residual struvite were found, as significant quantities of N-NH_4 and P-PO_4 were released from the struvite-amended sandy soil and loamy soil in 0.01 *M* $CaCl_2$ when the shaking time increased from 1 to 24 h (Figure 3.S3). For the DAP-amended loamy soil, some residual DAP dissolution was observed, whereas this was hardly the case for the sandy soil. The results are consistent with the well-known difference in dissolution behaviour between both P fertilisers as struvite has a lower solubility than DAP, leading to slow struvite dissolution with time (Talboys et al., 2016). Following the approach of Chapter 4, we used the increase in the amount of N-NH_4 extracted by 0.01 *M* $CaCl_2$ from a shaking time of 2 to 24 h as a proxy to quantify residual struvite and DAP dissolution. For the sandy soil, 13% of the initially added amount of struvite dissolved during the batch experiment, whereas this was 12% for the loamy soil. This is likely an underestimation for both soils, because residual struvite dissolution still seemed to continue at a shaking time of 24 h in the

batch experiment. Since $N\text{-}NH_4$ mainly binds to clay mineral particles (Chunying Wang et al., 2021; Wang and Alva, 2000), struvite dissolution is further underestimated for the loamy soil. Because of the lower P buffering capacity of the loamy soil, the increase in $P\text{-}PO_4$ in 0.01 *M* $CaCl_2$ from a shaking time of 1 to 24 h was especially prominent for this soil (i.e. 50.2 $mg \cdot kg^{-1}$), as indicated by the arrow in Figure 3.4. The same applies to the increase in $P\text{-}PO_4$ extracted with 0.01 *M* $CaCl_2$ from the DAP-amended loamy soil (i.e. 16.1 $mg \cdot kg^{-1}$). For the struvite-amended sandy soil, the increase in $P\text{-}PO_4$ was much less (i.e. 5.3 $mg \cdot kg^{-1}$), as this soil has a higher P buffering capacity. The adsorption isotherms in Figure 3.4 were based on $P\text{-}CaCl_2$, determined according to the standard protocol of Houba et al. (2000) including the prescribed shaking time of 2 h. However, dissolution of remnants of struvite in both soils and DAP in the loamy soil may have led to an accumulation of $P\text{-}PO_4$ in 0.01 *M* $CaCl_2$ already within 2 h of shaking, as revealed by the results of our additional batch experiment. As such, $P\text{-}CaCl_2$ does not reflect the actual equilibrium for $P\text{-}PO_4$ desorption from these soils. To this the possible uncertainty in $P\text{-}CaCl_2$ must be added, because any variation in the standard shaking time of 2 h or the time taken to centrifuge and filter the 0.01 *M* $CaCl_2$ soil suspensions after shaking will have had an impact on the extracted amount of $P\text{-}PO_4$, as is clearly demonstrated by the arrows for the struvite-amended soils and the DAP-amended loamy soil in Figure 3.4. The presence of remnants of struvite in both soils and DAP in the loamy soil may have played a role as well when determining P-AL in the fertiliser zone samples of the DAP and struvite treatments for both soils (Figure 3.3). Application of P in the control[+] treatment led to a higher P-AL for the sandy soil and the loamy soil, as P-AL increases with P-loading α and P-ox (Table 3.S4) (Schoumans and Groenendijk, 2000; van Doorn et al., 2023). Likewise, P-AL increased even further in the fertiliser zone samples of the DAP and struvite treatments of both soils, which can, in part, again be explained by the increase in P-loading α and P-ox (Table 3.S4). Since the pH of the extraction solution of P-AL is low (i.e. pH = 3.75) (Egnér et al., 1960), residual struvite and DAP will probably have dissolved in the mixture of acetic acid and ammonium lactate, leading to an overestimation of P-AL. The same is likely true for the 0.2 *M* acid ammonium oxalate extraction method, due to the low pH of the extraction solution (i.e. pH = 3.0) (Schwertmann, 1964). This is supported by the recovery of P by P-ox from P added with struvite (i.e. 105%) and DAP (i.e. 82%) to the fertiliser zone of the sandy soil and the loamy soil (see Section S2; Table 3.S5). The lower P recovery from DAP can, in part, be explained by P uptake by maize (Table 3.S5), which was significantly higher for the DAP-amended soils than for the struvite-amended soils (Figure 3.2B). Hence, P-ox and the resulting P loading α may have been overestimated for the struvite-amended soils and the DAP-amended loamy soil, similar to P-AL. These results show the shortcoming of traditional soil P

extraction methods like P-$CaCl_2$ and P-AL for soils treated with poorly soluble mineral P fertilisers, as they are unable to separate P desorbed from soil itself from P dissolved during the extraction of soil from remnants of mineral P fertiliser. The consequences of this problem will be further elaborated in the next section where the results of these soil P tests are related to the aboveground biomass production and P uptake by maize.

3.5 Diagnostic Value of Soil P Tests for Struvite-amended Soils

For the fertiliser zone samples, both P-$CaCl_2$ and P-AL are highest for the struvite treatment of the sandy soil and the loamy soil (Figure 3.3). However, this is not at all reflected in the aboveground biomass production and P uptake by maize, which are highest for the control[+] and DAP treatments of both soils (Figure 3.1A and Figure 3.2B). Indeed, when establishing the relationship between biomass and P uptake on the one hand and P-$CaCl_2$ measured in the fertiliser zone soil samples from the four P treatments on the other, the data points of the struvite treatment of both soils and the data points of the DAP treatment of the loamy soil deviate strongly from the empirical relationship fitted to the results of the control[-] and control[+] treatments of both soils (Figure 3.5). Similar results were found for P-AL (Figure 3.S4). Hence, these soil P tests have less diagnostic value when classifying the agronomic soil P status for soil samples taken from zones of the soil where struvite has been placed as in our pot experiment. This can be explained by the dissolution of remnants of struvite in both soils and DAP in the loamy soil during the extraction of soil when determining P-$CaCl_2$ and P-AL, as discussed in Section 3.4. Dissolution of struvite has been shown as well for other established agronomic soil P tests like Mehlich-3, Bray-1, and Olsen P (Gu et al., 2021). Although the maize plants grown in the DAP and struvite treatments proliferate their roots in the direction of the fertiliser zone for both soils (Figure 3.1B), they do form some roots in the bulk soil of these P treatments. As such, the biomass and P uptake by maize does not depend only upon the level of plant-available P in the fertiliser zone soil but depend on the plant-available P level in the bulk soil as well. However, the levels of P-$CaCl_2$ and P-AL in the bulk soil of the DAP and struvite treatments were much lower than those in the fertiliser zone soil (Figure 3.3). Consequently, the use of P-$CaCl_2$ and P-AL measured in soil samples taken from zones of the soil where P fertiliser has been placed would lead to an overestimation of the level of plant-available P for crops. The importance of this effect would depend upon the ability of crops to adapt to P-deficient growth conditions via proliferation of their roots in zones of the soil where the level of plant-available P is elevated as a result of placed P fertilisation (Grant et al., 2001).

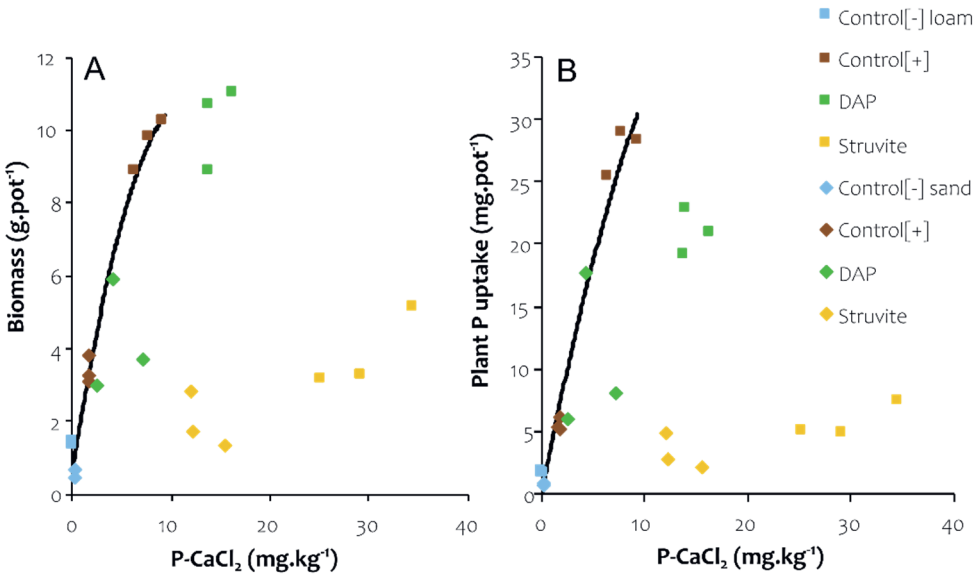

Figure 3.5. The aboveground biomass of maize (A) and P uptake (B) plotted against the P-$CaCl_2$ measured in the fertiliser zone samples, taken from the four P treatments of the sandy soil and the loamy soil (n = 3). The P-$CaCl_2$ is based on the prescribed shaking time of 2 h (Houba et al., 2000). To guide the eye, a quadratic relationship was fitted to the results of the control[-] and control[+] treatments of the sandy soil and the loamy soil.

3.6 Agronomic Effectiveness of DAP and Struvite

The PUE of the three P treatments are presented for both soils in Table 3.2. For the DAP treatment of the loamy soil, the PUE is significantly higher than for the control[+] treatment. Although the PUE in the sandy soil did not show significant differences between treatment, the same pattern as in the loamy soil was observed. This demonstrates the beneficial effect for crops when placing P fertiliser in a limited volume of soil rather than mixing it through the entire volume of potted soil, even though less P was applied with DAP and struvite (Table 3.S2) (see Section 3.3). For the loamy soils, the PUE of the DAP treatment is significantly higher than the PUE of the struvite treatment, and the same pattern was observed for the sandy soil. This is due to the higher solubility and the quicker dissolution of DAP in soil, as compared to struvite (Talboys et al., 2016). The latter is supported by the results of the our additional batch experiment in which the initial sandy soil and loamy soil were amended with fresh struvite and fresh DAP (see Section S1; Figure 3.S5). Furthermore, the PUE of the DAP treatment is higher for the loamy soil than for the sandy soil, because P uptake by maize weas significantly higher for the loamy soil (Figure 3.2B). For the struvite treatment, the PUE of the sandy soil and the loamy soil are rather similar, because P uptake was the same for both soils (Figure 3.2B).

Since maize growth was more P-limited on the sandy soil (Figure 3.2C) and because the sandy soil had a lower pH (Table 3.1), struvite was expected to have a higher agronomic effectiveness for the sandy soil than for the loamy soil. This would have been in line with the higher solubility of struvite at lower pH (Bhuiyan et al., 2007; Talboys et al., 2016), which is an important soil property in determining the agronomic effectiveness of this P fertiliser (Degryse et al., 2017; Hilt et al., 2016). A larger part of the added struvite may have dissolved in the sandy soil than in the loamy soil, as suggested by the results of the additional batch experiment (see Section S1), yet the P released from struvite dissolution was probably bound to a greater extent in the sandy soil resulting from the higher P buffering capacity of this soil (Figure 3.4). So besides pH, the P buffering capacity of the soil as an important soil property for the agronomic effectiveness of struvite as a P fertiliser.

Table 3.2. Phosphorus use efficiency (%) for the three P treatments of the sandy and loamy soils. The standard error of the mean is indicated in brackets (n = 3). Results of the Tukey tests are indicated in letters for the loamy soil. The ANOVA was not significant for the sandy soil.

P treatment	Sandy soil	Loamy soil
Control[+]	1.0 (0.1)	4.0 (0.2) a
DAP	5.6 (2.1)	11.0 (0.6) b
Struvite	1.4 (0.5)	2.3 (0.5) a

4 Conclusions

Although placed DAP and struvite fertilisation in general were effective in increasing the aboveground biomass production and P uptake by maize as compared to the treatment in which P was withheld, struvite had a lower agronomic effectiveness than DAP. This was due to the higher solubility of DAP. Nearly all DAP had dissolved during the pot experiment in the acidic sandy soil, while some residual DAP was likely present in the loamy soil with a near -neutral pH. For struvite, however, remnants of struvite were visually detected at the end of the pot experiment. Nevertheless, a larger part of the added struvite had probably dissolved in the sandy soil than in the loamy soil. Yet the biomass was higher for the loamy soil, although there was no difference in P uptake between both soils. This was explained by the lower P buffering capacity of the loamy soil. Consequently, P released from the dissolution of struvite was more effective in raising readily plant-available P in the loamy soil than in the sandy soil. Furthermore, P-$CaCl_2$ and P-AL overestimated biomass and P uptake by maize when the soil contained remnants of struvite, due to struvite dissolution during the extraction procedures. These results necessitate a critical rethinking of how to interpret P-$CaCl_2$ and P-AL as a basis for P fertiliser recommendations for soils receiving struvite as a P fertiliser.

Acknowledgements

The authors are grateful for the support of Gerlinde Vink and Miranda Vlag during the laboratory analysis.

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Supplementary Information

S1. Supplementary Material and Methods

Table 3.S1. Chemical composition of struvite and DAP. The claimed contents of N, P, and Mg (struvite) and N and P (DAP) are given in brackets.

Element	Unit	Struvite	DAP
N	$g \cdot kg^{-1}$	57 (57)	178 (180)
P	$g \cdot kg^{-1}$	122 (127)	190 (201)
Mg	$g \cdot kg^{-1}$	102 (103)	-
C	$mg \cdot kg^{-1}$	493	-
Na	$mg \cdot kg^{-1}$	20[1]	-
Al	$mg \cdot kg^{-1}$	37[1]	-
S	$mg \cdot kg^{-1}$	106	-
K	$mg \cdot kg^{-1}$	812	-
Ca	$mg \cdot kg^{-1}$	435	-
Cr	$mg \cdot kg^{-1}$	4.2	-
Mn	$mg \cdot kg^{-1}$	24.6	-
Fe	$mg \cdot kg^{-1}$	967	-
Ni	$mg \cdot kg^{-1}$	0.3[1]	-
Cu	$mg \cdot kg^{-1}$	3	-
Zn	$mg \cdot kg^{-1}$	8	-
As	$mg \cdot kg^{-1}$	0.06[1]	-
Cd	$mg \cdot kg^{-1}$	0.01[1]	-
Pb	$mg \cdot kg^{-1}$	0.5	-

[1]Values below detection limits of the ICP-OES.

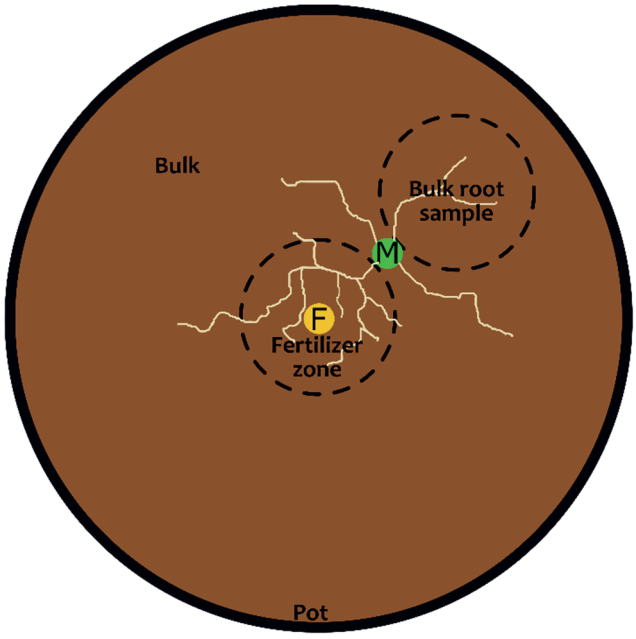

Figure 3.S1. Location of the root samples from the "fertiliser zone" and the bulk soil. F: Fertiliser application point. M: Maize plant.

Table 3.S2. Fertilisation regime of the pot experiment for each of the four P treatments.

Treatment		P fertiliser	P dose (kg P·ha^{-1})	P dose (g P·pot^{-1})	N dose (kg N·ha^{-1})	Mg dose (kg Mg·ha^{-1})
Control[-]		-	0	0.00	120	28
Control[+]	Sand	$NaH_2PO_{4(aq)}$ and $Na_2HPO_{4(aq)}$[1]	47	0.47	120	28
	Loam		64	0.64	120	28
DAP		$(NH_4)_2HPO_{4(s)}$	17	0.17	16+104[2]	28
Struvite		$Mg(NH_4)PO_4{\cdot}6H_2O_{(s)}$	17	0.17	8+115[2]	14+14[2]

[1]Applied in soluble form.
[2]Total N application split between DAP or struvite and N-containing solutions listed in Section 2.2.

S2. Supplementary Results

Table 3.S3. The results of the 0.01 *M* $CaCl_2$ extraction method of the soil samples taken from the bulk soil and fertiliser zone of the four P treatments of the sandy soil and the loamy soil. The standard shaking time of 2 h was used for the 0.01 *M* $CaCl_2$ extraction method, as prescribed by Houba et al. (2000). Statistical analyses were performed on sandy and loamy soils independently. P-values are for potential fertiliser effect and letters denote the results of the Tukey HSD test. Standard errors of the means are reported in brackets.

Sample	Soil	Treatment	pH	TDP (mg P·kg^{-1})	N-NH_4 (mg N·kg^{-1})	N-NO_3 (mg N·kg^{-1})	Nts (mg N·kg^{-1})	Mg (mg Mg·kg^{-1})	DOC (mg C·kg^{-1})
Bulk	Sandy	Control[-]	5.23 (0.02)	0.6 (0.03) a	10.0 (5.6)	212.4 (7.3)	219.7 (5.3)	162.4 (4.3) b	101.7 (10.0)
		Control[+]	5.23 (0.03)	2.1 (0.1) b	2.0 (0.4)	220.4 (8.8)	222.4 (10.2)	158.4 (4.8) b	97.7 (7.0)
		DAP	5.10 (0.03)	0.7 (0.03) a	3.7 (1.7)	212.4 (9.5)	212.0 (9.8)	161.7 (0.4) b	90.7 (4.0)
		Struvite	5.44 (0.14)	0.7 (0.03) a	6.1 (1.7)	228.7 (18.1)	233.0 (17.2)	139.0 (3.5) a	92.0 (6.2)
		P-value	*0.0752*	*<0.0001*	*0.3430*	*0.7300*	*0.6480*	*0.0062*	*0.279*
	Loamy	Control[-]	6.25 (0.02) ab	1.3 (0.03) a	2.4 (0.2)	490.6 (18.1) c	506.9 (23.2) c	241.6 (6.7) b	156.6 (2.6) a
		Control[+]	6.33 (0.03) b	10.4 (0.3) b	3.2 (0.1)	369.0 (24.9) ab	381.0 (25.4) ab	214.3 (6.2) a	183.7 (4.1) b
		DAP	6.29 (0.02) b	1.7 (0.2) a	3.2 (0.4)	326.0 (15.6) a	332.0 (14.1) a	221.0 (3.5) ab	161.4 (1.5) a
		Struvite	6.20 (0.02) a	1.4 (0.03) a	3.8 (0.5)	439.3 (1.2) bc	451.6 (4.8) bc	212.3 (2.6) a	161.7 (1.2) a
		P-value	*0.0075*	*<0.0001*	*0.1050*	*0.0011*	*0.0008*	*0.0125*	*<0.0001*
Fertiliser zone	Sandy	Control[-]	5.21 (0.01)	0.6 (<0.01) a	2.0 (0.2) a	152.0 (6.1)	152.3 (9.4) a	125.7 (6.4) a	92.7 (9.2)
		Control[+]	5.20 (0.02)	2.0 (0.03) ab	1.9 (0.2) a	171.0 (14.4)	169.7 (15.2) a	132.3 (4.3) a	87.3 (6.5)
		DAP	5.16 (0.03)	5.0 (1.4) b	4.3 (0.1) a	174.0 (16.4)	176.0 (15.5) a	158.3 (12.1) a	97.3 (6.5)
		Struvite	5.42 (0.20)	14.6 (1.3) c	87.8 (10.5) b	173.4 (20.6)	264.1 (18.5) b	306.1 (19.5) b	105.4 (16.3)
		P-value	*0.3540*	*<0.0001*	*<0.0001*	*0.713*	*0.0032*	*<0.0001*	*0.261*
	Loamy	Control[-]	6.40 (0.11) ab	1.2 (<0.01) a	2.4 (0.2) a	315.0 (20.3) b	329.3 (23.8) b	207.0 (5.1) b	157.3 (4.2) a
		Control[+]	6.37 (0.02) ab	9.4 (0.8) b	2.3 (0.1) a	251.0 (9.6) a	260.3 (8.9) a	188.3 (1.3) a	175.6 (1.2) b
		DAP	6.16 (0.02) a	17.5 (1.0) c	5.3 (0.7) a	282.4 (10.6) ab	295.4 (10.6) ab	221.0 (1.6) b	189.7 (2.3) c
		Struvite	6.60 (0.04) b	31.7 (2.3) d	42.6 (2.4) b	303.4 (9.8) ab	356.4 (11.2) b	398.4 (4.8) c	186.7 (6.0) c
		P-value	*0.0056*	*<0.0001*	*<0.0001*	*0.0412*	*0.0090*	*<0.0001*	*<0.0001*

Table 3.S4. The results of the 0.2 *M* acid ammonium oxalate extraction (Schwertmann, 1964) of the soil samples taken from the bulk soil and fertiliser zone of the four P treatments of the sandy soil and the loamy soil. The P-loading α was calculated as α=P-ox/[Fe+Al]-ox, with P-ox and [Fe+Al]-ox expressed in mmol/kg (van der Zee and van Riemsdijk, 1988). Statistical analyses were performed on the sandy soil and the loamy soil separately. P-values are for potential fertiliser effect and letters denote the results of the Tukey test. Standard errors are reported in brackets.

Sample	Soil	Treatment	P-ox (mg P·kg^{-1})	Al-ox (mg Al·kg^{-1})	Fe-ox (mg Fe·kg^{-1})	α
Bulk	Sandy	Control[-]	299.4 (8.7) a	1614.9 (49.8) a	650.5 (51.9)	0.13 (<0.1) a
		Control[+]	449.3 (1.8) b	1707.3 (11.8) a	649.7 (4.0)	0.19 (<0.1) c
		DAP	315.7 (8.1) a	1574.1 (13.9) a	601.6 (7.3)	0.15 (<0.1) b
		Struvite	328.8 (8.5) a	1742.4 (52.7) a	665.1 (19.1)	0.14 (<0.1) ab
		P-value	*<0.0001*	*0.0417*	*0.4490*	*<0.0001*
	Loamy	Control[-]	282.2 (5.1) a	802.3 (18.5)	4137.1 (107.2)	0.09 (<0.1) a
		Control[+]	477.9 (4.2) b	856.6 (10.9)	4475.2 (59.9)	0.14 (<0.1) b
		DAP	305.2 (15.5) a	828.7 (16.7)	4248.9 (119.2)	0.09 (<0.1) a
		Struvite	296.9 (4.0) a	836.4 (11.1)	4364.4 (48.1)	0.09 (<0.1) a
		P-value	*<0.0001*	*0.1500*	*0.1170*	*<0.0001*
Fertiliser zone	Sandy	Control[-]	281.9 (4.9) a	1509.2 (17.0) a	574.1 (7.6)	0.14 (<0.1) a
		Control[+]	409.9 (6.0) ab	1567.5 (19.9) ab	587.2 (13.2)	0.19 (<0.1) a
		DAP	572.7 (39.5) b	1569.6 (8.5) ab	592.6 (4.5)	0.27 (0.02) b
		Struvite	653.0 (28.4) c	1658.7 (49.2) b	628.8 (20.3)	0.29 (0.02) b
		P-value	*<0.0001*	*0.0338*	*0.0779*	*0.0002*
	Loamy	Control -	284.0 (3.3) a	833.2 (12.2)	4231.2 (38.2)	0.09 (<0.1) a
		Control +	451.8 (3.3) b	835.9 (19.9)	4175.0 (83.3)	0.14 (<0.1) b
		DAP	618.9 (25.5) c	872.6 (9.3)	4211.9 (33.3)	0.19 (0.1) c
		Struvite	714.0 (59.3) c	839.4 (7.8)	4206.2 (24.7)	0.22 (0.02) c
		P-value	*<0.0001*	*0.1990*	*0.883*	*<0.0001*

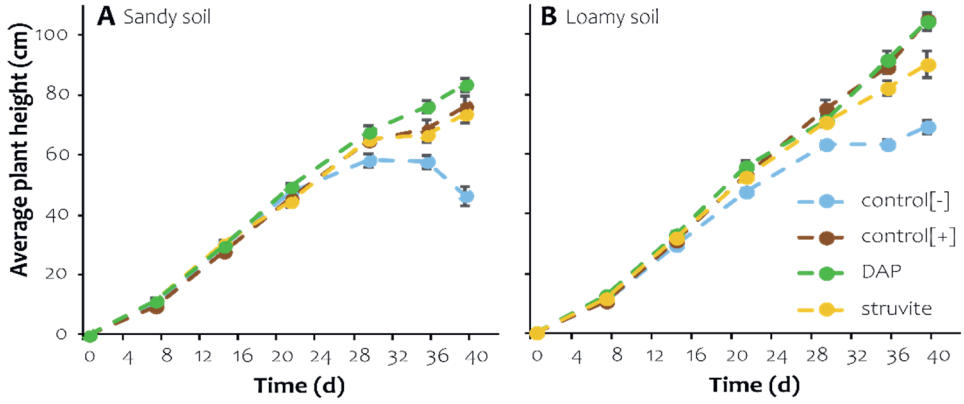

Figure 3.S2. Height of the maize plants of the four P treatments of the sandy soil (A) and the loamy soil (B) during the pot experiment (n = 5). For day 40 of the control[-] treatment of the sandy soil, one maize plant was no longer able to support its own weight (n = 4).

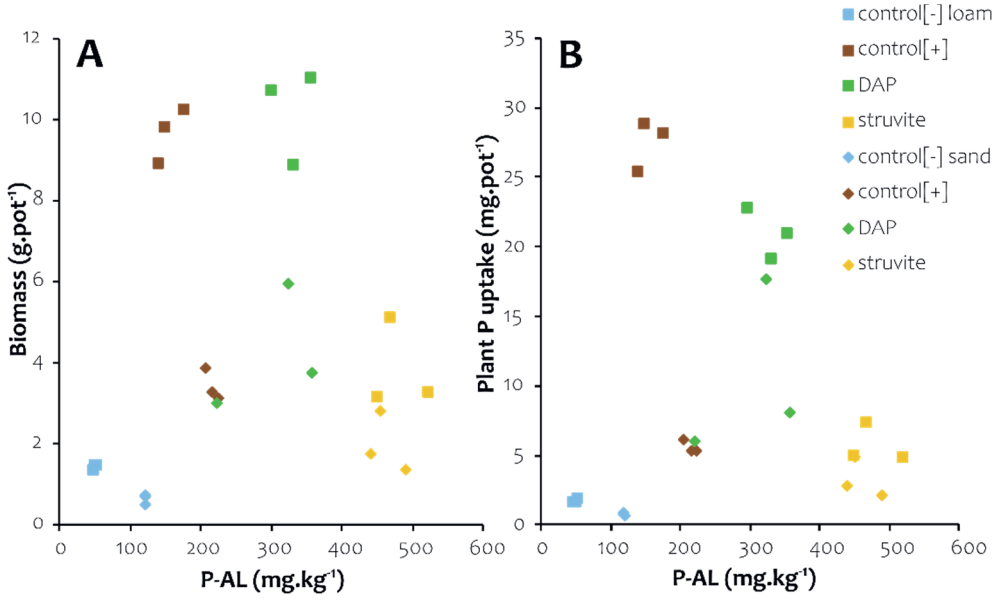

Figure 3.S4. The aboveground biomass of maize (A) and P uptake (B) plotted against the P-AL measured in the fertiliser zone samples, taken from the four P treatments of the sandy soil and the loamy soil (n = 3). To guide the eye, logarithmic relationships were fitted to the results of the control[-] and control[+] treatments of the sandy soil and to the results of the control[-] and control[+] treatments of the loamy soil.

S3. Batch Experiment

S3.1 Experimental Setup

We conducted an additional batch experiment to better understand the dissolution behaviour of struvite and DAP in 0.01 *M* $CaCl_2$ for the sandy soil and the loamy soil. For this experiment, soil samples taken at the end of the pot experiment from the fertiliser zone of the four P treatments of both soils were used. For each P treatment, one soil sample was used from the first block out of the in total five blocks of the pot experiment, without replication. Two additional treatments were added to the batch experiment as subsamples from the two initial soils were amended with fresh struvite and fresh DAP. For the sandy soil, dosages of 3.15 g of fresh struvite and 2.00 g of fresh DAP were mixed with one kg of soil resulting in a soil P content of on average 400 $mg \cdot kg^{-1}$, based on the claimed P contents of these fertilisers (Table 3.S1). For the loamy soil, dosages of 2.91 g of fresh struvite and 1.83 g of fresh DAP were mixed with one kg of soil, leading to a soil P content of on average 368 $mg \cdot kg^{-1}$. For the batch experiment, 4 g of each soil sample was suspended in 40 mL of 0.01 *M* $CaCl_2$. The batches were shaken for a prolonged shaking time: instead of the standard shaking time of 2 h as prescribed by Houba et al. (2000), a shaking time of 1, 2, 4, 8, and 24 h was used. For each shaking time, a separate batch was made for each soil sample. With six treatments (four P treatments and initial soil amended with either fresh struvite or fresh DAP) times two soils (sandy soil and loamy soil) times five shaking times, the total number of batches amounted to 60. The batches were shaken using a horizontal shaker with 160 $strokes \cdot min^{-1}$. Next, the suspension of each sample was centrifuged at 1800 x g for 10 minutes and filtered over a 0.45 µm-filter membrane (Aqua 30/0.45 CA Whatman). The remaining solution was acidified with 25 µL 5 *M* HCl for sample conservation. The $P\text{-}PO_4$ and $N\text{-}NH_4$ concentrations in solution were measured with an SFA.

S3.2 Results and Discussion

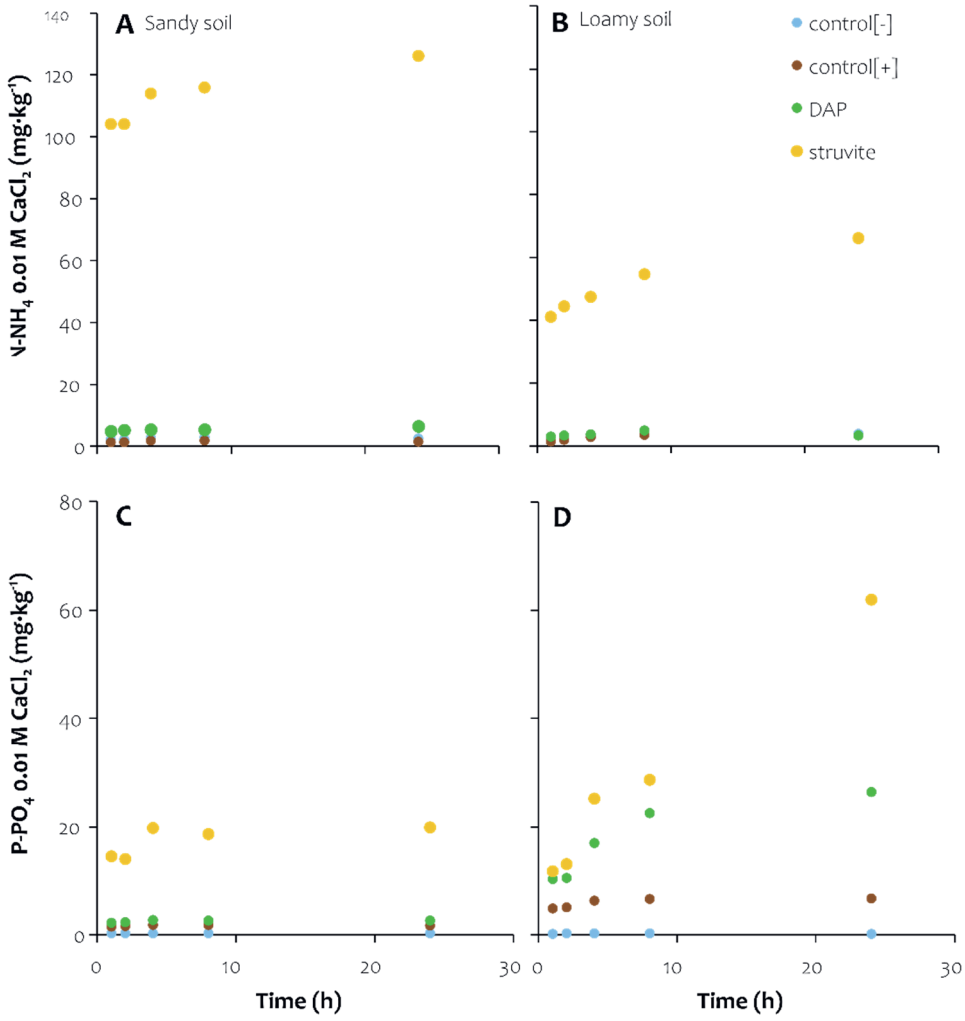

Figure 3.S3. Results of the batch experiment in which the release of N-NH_4 and P-PO_4 from the fertiliser zone soil samples of the four P treatments of the sandy soil and the loamy soil in 1:10 (w:v) 0.01 *M* $CaCl_2$ extracts was quantified as a function of shaking time of 1, 2, 4, 8, and 24 h. In these extracts, N-NH_4 (A and B) and P-PO_4 (C and D) were measured for the sandy soil (A and C) and the loamy soil (B and D). The standard shaking time of the 0.01 *M* $CaCl_2$ extraction method is 2 h (Houba et al., 2000).

First we will discuss the results of the batch experiment with the fertiliser zone samples, taken from the four P treatments of the sandy soil and the loamy soil, as presented in Figure 3.S3. After 24 h of shaking, the P-PO_4 and N-NH_4 contents as extracted by 0.01 *M* $CaCl_2$ from the fertiliser zone samples from the control[-] and [+] treatments of both soils did not change or hardly changed as compared to 2 h of

shaking, suggesting equilibrium had been established. This is in agreement with results (van Erp et al., 1998)of van Erp et al. (1998) where the prescribed shaking time of 2 h from Houba et al. (2000) was sufficient to reach equilibrium for multiple nutrients including $N\text{-}NH_4$ and $P\text{-}PO_4$. For the fertiliser zone sample from the struvite treatment of the sandy soil, the $P\text{-}PO_4$ content at a shaking time of 2 h was 5.9 times higher than the $P\text{-}PO_4$ content of the DAP-amended sandy soil at the same shaking time. This difference can, at least in part, be explained by the difference in P loading α between the two fertiliser zone soil samples used in the batch experiment: the α value of the struvite-amended sandy soil was 0.33 whereas it was 0.24 for the DAP-amended sandy soil. The relative increase in the $P\text{-}PO_4$ content of the struvite-amended sandy soil at a shaking time of 24 h as compared to 2 h of shaking amounted to a factor of 1.4, corresponding to an absolute increase of 5.8 mg $P\text{-}PO_4 \cdot kg^{-1}$. For $N\text{-}NH_4$, this factor was 1.2 (i.e. 22.1 $mg \cdot kg^{-1}$). For the DAP-amended sandy soil, the $P\text{-}PO_4$ and NH_4 contents at a shaking time of 24 h compared to 2 h of shaking increased by a factor of 1.1 (i.e. 0.3 mg $P\text{-}PO_4 \cdot kg^{-1}$) and 1.3 (i.e. 1.3 mg $N\text{-}NH_4 \cdot kg^{-1}$), respectively. The $P\text{-}PO_4$ content at a shaking time of 2 h was rather similar for the fertiliser zone samples from the struvite and DAP treatments of the loamy soil, i.e. 13.1 versus 10.5 $mg \cdot kg^{-1}$, respectively. This can again be explained by the P loading α, which was nearly the same for the two fertiliser zone samples used in the batch experiment: the α value of the struvite-amended loamy soil was 0.19, whereas it was 0.20 for the DAP-amended loamy soil. The $P\text{-}PO_4$ content of the DAP-amended loamy soil increased by a factor of 2.5 (i.e. 15.9 $mg \cdot kg^{-1}$) when prolonging the shaking time from 2 to 24 h, whereas the NH_4 content did not change. For the struvite-amended loamy soil, the $P\text{-}PO_4$ and $N\text{-}NH_4$ contents increased by a factor of 4.7 (i.e. 48.8 $mg \cdot kg^{-1}$) and 1.5 (i.e. 21.6 $mg \cdot kg^{-1}$), respectively. Clearly, struvite dissolution was not completed yet after 24 h for the loamy soil, because the $P\text{-}PO_4$ content increased with shaking time in a linear manner, whereas the $P\text{-}PO_4$ content of the DAP-amended loamy soil levelled off at a shaking time of 24 h.

The measurement of $P\text{-}PO_4$ in solution does not provide a good quantitative estimate of the extent to which residual DAP and struvite would dissolve during 0.01 *M* $CaCl_2$ extraction as a function of shaking time, because $P\text{-}PO_4$ generally binds strongly to the soil solid phase (Hesterberg, 2010). This is especially the case for the sandy soil as this soil has a higher P buffering capacity than the loamy soil (Figure 3.4). To estimate how much DAP and struvite dissolved during the batch experiment, it is better to use $N\text{-}NH_4$ in solution as a proxy because $N\text{-}NH_4$ generally binds less strongly to the soil solid phase than $P\text{-}PO_4$ (Chapter 4). To get a quantitative estimate of how much DAP and struvite did dissolve, we calculated the increase in the $N\text{-}NH_4$ content from a shaking time of 2 to 24 h as a percentage of the amount of N initially

added in the form of DAP and struvite to the fertiliser zone of both soils. For these calculations, we used the claimed N contents of DAP and struvite (Table 3.S1). To express the amount of N initially added with DAP and struvite per unit mass of soil for the fertiliser zone, we needed the mass of the cylindrical soil cores taken from both soils at the end of the pot experiment (Figure 3.S1). Unfortunately, the mass of the soil cores was not recorded. Therefore, we estimated the soil bulk density using the empirical equation from Lexmond and Edelman (1987) with the SOM content of the sandy soil and the loamy soil as input (Table 3.1), enabling us to calculate the soil mass of the cylindrical cores. Based on this approach, the increase in $N\text{-}NH_4$ expressed as a percentage of the amount of N added with DAP was 0.4% for the sandy soil and zero for the loamy soil. For struvite, this percentage was 13% for the sandy soil and 12% for the loamy soil. The latter agrees with our visual observation of residual struvite grains present in the soil sampled from the fertiliser zone of the sandy and loamy soils. However, DAP and struvite dissolution may have been underestimated with the approach of Chapter 4, especially for the loamy soil. Since $N\text{-}NH_4$ is known to bind to clay mineral particles (Chunying Wang et al., 2021; Wang and Alva, 2000), the release of $N\text{-}NH_4$ from the dissolution of residual DAP and struvite may have been underestimated as the loamy soil had a higher clay content than the sandy soil (Table 3.1). Using the measurements of $P\text{-}PO_4$ instead of those of $N\text{-}NH_4$ as a proxy to quantify the dissolution of residual DAP and struvite for the loamy soil even led to a higher percentage of DAP dissolved, i.e. 4% versus 0%, respectively. For the percentage of struvite dissolved, the outcome of the calculation for the loamy soil was the same, regardless of whether the $P\text{-}PO_4$ measurements were used or those of $N\text{-}NH_4$ (i.e. 12%). Either way, both the use of $P\text{-}PO_4$ and $N\text{-}NH_4$ measurements as a proxy to quantify DAP and struvite dissolution falls short for the loamy soil. One other reason why struvite dissolution may have been underestimated is because its dissolution was clearly not complete yet within the shaking time of 24 h, as discussed above. The same could apply to the struvite-amended sandy soil, although this was more difficult to assess from the $N\text{-}NH_4$ measurements for this soil.

To better quantify the dissolution of DAP and struvite in both soils, we included two additional treatments in the batch experiment. For these treatments, we knew how much N and P was present in soil in the form of DAP and struvite at the start of the 0.01 *M* $CaCl_2$ extraction as subsamples from the two initial soils were amended with known amounts of fertiliser. In Figure 3.S5, the $P\text{-}PO_4$ and $N\text{-}NH_4$ contents measured at the different shaking times are expressed as a percentage of the amounts of P and N added with DAP and struvite to the sandy and loamy soils, based on the claimed contents of these nutrients for both fertilisers (Table 3.S1). The $N\text{-}NH_4$

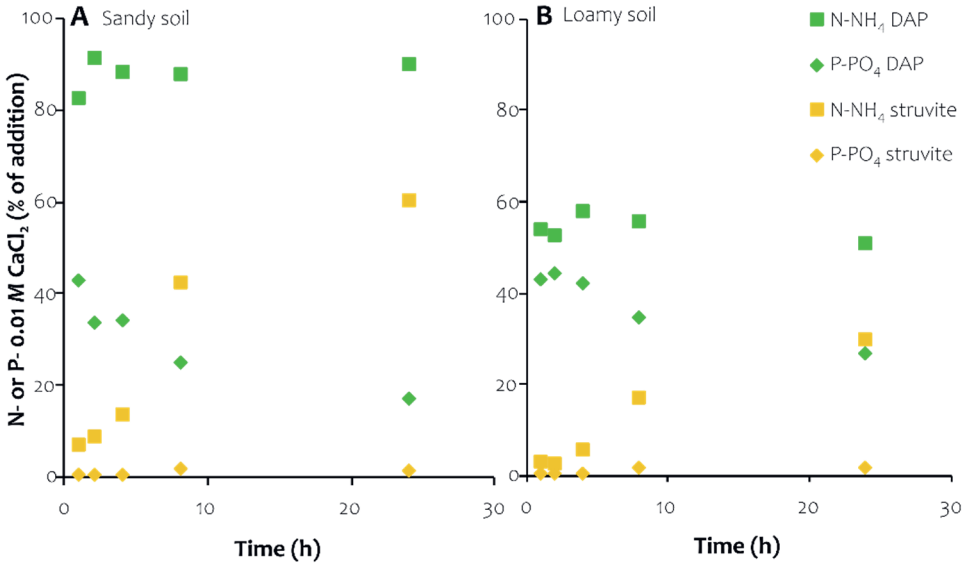

Figure 3.S5. Results of the batch experiment in which the release of N-NH_4 and P-PO_4 from the initial sandy soil and the initial loamy soil amended with fresh DAP and fresh struvite in 1:10 (w:v) 0.01 *M* $CaCl_2$ extracts was quantified as a function of a shaking time of 1, 2, 4, 8, and 24 h. The extracted amounts of N-NH_4 and P-PO_4 were expressed as a percentage of the amounts of N and P added with fresh DAP and fresh struvite to the initial soils. The standard shaking time of the 0.01 *M* $CaCl_2$ extraction method is 2 h (Houba et al., 2000).

contents measured at the different shaking times were corrected by subtracting the initial 0.01 *M* $CaCl_2$-extractable N-NH_4 content of the sandy soil and the loamy soil (Table 3.1). For the sandy soil, nearly all DAP dissolved within a shaking time of only 2 h, because the measured N-NH_4 content represented 91% of the amount of N added with DAP. The N-NH_4 content remained constant when prolonging the shaking time from 2 to 24 h, corroborating the observation of near-complete DAP dissolution within a shaking time of 2 h. Interestingly, the measured P-PO_4 content at a shaking time of 2 h represented only 33% of the amount of P added with DAP, because P-PO_4 is much more reactive than N-NH_4 for a sandy soil (Chapter 4). The P-PO_4 content decreased even further with shaking time as the percentage of P-PO_4 at a shaking time of 24 h was 17%. This can be attributed to a gradual increase in the binding of P-PO_4, released from the fast dissolution of DAP within the first two hours of the batch experiment. Clearly, the measurement of N-NH_4 in solution as a proxy to quantify DAP dissolution works reasonably well for the sandy soil, as postulated before by Ferron et al. (2023). For the loamy soil, however, the measured N-NH_4 content at a shaking time of 2 h represented only 53% of the amount of N added with DAP. Similar to the sandy soil, the N-NH_4 content remained reasonably constant when prolonging the shaking time from 2 to 24 h. Hence, just over half of the DAP

would have dissolved according to the $N\text{-}NH_4$ measurements. However, the following is again valid here: binding of $N\text{-}NH_4$ to clay mineral particles in the loamy soil may have led to an underestimation of DAP dissolution, as discussed above. The measured $P\text{-}PO_4$ content at a shaking time of 2 h represented 43% of the amount of P added with DAP to the loamy soil, which eventually decreased to 27% at a shaking time of 24 h. For both soils, struvite dissolved with shaking time in a linear manner. The $N\text{-}NH_4$ content measured at a shaking time of 24 h represented 60% of the amount of N added with struvite to the sandy soil. For $P\text{-}PO_4$, this percentage was 1.5% at a shaking time of 24 h. Hence, struvite dissolved more slowly than DAP and its dissolution was not completed yet within 24 h of shaking for the sandy soil. For the loamy soil, the percentage of struvite dissolution was 30%, based on the measurement of $N\text{-}NH_4$ at a shaking time of 24 h. The $P\text{-}PO_4$ content at a shaking time of 24 h represented 0.7% of the amount of P applied with struvite. Yet again, struvite dissolution may have been underestimated for the loamy soil when using $N\text{-}NH_4$ as a proxy for the dissolution of this fertiliser, as discussed above. Furthermore, struvite dissolution was incomplete within 24 h of shaking for the loamy soil, because the $N\text{-}NH_4$ content increased with shaking time in a linear manner.

S3.3 Synthesis

DAP seemed to have dissolved almost completely in the sandy soil during the pot experiment, because hardly any dissolution of this fertiliser from the fertiliser zone sample was observed during the batch experiment: the increase in $N\text{-}NH_4$ in 0.01 *M* $CaCl_2$ from a shaking time of 2 to 24 h represented <1% of the amount of N initially added with DAP. This is in line with the fast and near-complete dissolution of DAP when the initial sandy soil amended with fresh DAP was extracted with 0.01 *M* $CaCl_2$. For the loamy soil, we did find some DAP dissolution from the fertiliser zone sample during the batch experiment. Using $P\text{-}PO_4$ as a proxy to estimate DAP dissolution, the increase in $P\text{-}PO_4$ from a shaking time of 2 to 24 h represented 4% of the amount of P initially added with DAP. This is likely an underestimation of DAP dissolution, because $P\text{-}PO_4$ generally binds strongly to the soil solid phase. Hence, some residual DAP may have remained present in the loamy soil when the pot experiment was terminated. However, it is difficult to quantify how much DAP remained present, because both $P\text{-}PO_4$ and $N\text{-}NH_4$ interact strongly with solid phase of the loamy soil. In contrast, the use of $N\text{-}NH_4$ measurements in 0.01 *M* $CaCl_2$ as a proxy for DAP dissolution works reasonably well for the sandy soil, as shown by the results of the batch experiment in which the initial sandy soil was amended with fresh DAP. Clearly, dissolution of residual struvite from the fertiliser zone samples taken from the sandy soil and the loamy soil was observed during the batch experiment. These

observations were corroborated by the visual detection of residual struvite grains in the fertiliser zone samples from both soils. For the sandy soil, the increase in $N\text{-}NH_4$ in 0.01 *M* $CaCl_2$ from a shaking time of 2 to 24 h represented 13% of the amount of N initially added with struvite. For the loamy soil, a rather similar percentage of 12% was calculated. However, struvite dissolution was probably underestimated for the loamy soil, because $N\text{-}NH_4$ interacts with the clay mineral particles in the loamy soil. Furthermore, struvite dissolution was incomplete within 24 h of shaking, because the $P\text{-}PO_4$ content increased with shaking time in a linear manner. The same could apply to the struvite-amended sandy soil, although this was much less clearly visible. Either way, struvite dissolution must have been incomplete in both soils during the pot experiment resulting from the slow dissolution behaviour of this fertiliser in soil, explaining why residual struvite was present in the fertiliser zone samples of both soils. This corresponds to the results of the batch experiment where the initial sandy soil and the loamy soil were amended with fresh struvite: struvite dissolution in 0.01 *M* $CaCl_2$ still continued at a shaking time of 24 h. The amount of residual struvite was probably larger in the loamy soil than in the sandy soil. This would agree with the lower pH of the sandy soil (Table 3.1), because a lower pH is known to promote the dissolution of struvite (Bhuiyan et al., 2007). Even though the loamy soil had a lower initial P loading α than the sandy soil (Table 3.1), still the P buffering capacity of the sandy soil is higher as the adsorption isotherm of the latter soil has a higher slope at any given $P\text{-}CaCl_2$ than the isotherm of the loamy soil (Figure 3.4). In other words, the sandy soil is able to maintain a lower $P\text{-}PO_4$ concentration in solution upon the addition of P with struvite, which may have stimulated struvite dissolution. The same arguments would explain why DAP seemed to have dissolved completely in the sandy soil, whereas some residual DAP was likely to be present in the loamy soil.

S4 Recovery of P

Table 3.S5. Recovery of P (%) from the P fertilisers initially added in the pot experiment by 0.2 acid ammonium oxalate-extractable P (P-ox). For the sandy soil and for the loamy soil. For the P recovery + P uptake, P uptake was included in the calculation of the P recovery. For an explanation of the P recovery calculations, see Section S3.

P treatment	Sandy soil	Sandy soil + P	Loamy soil	Loamy soil + P
Control[+]	105	106	88	93
DAP	80	85	85	96
Struvite	101	103	109	111

Here we explain how we have calculated the recovery of P from the P fertilisers used in our pot experiment by the amount of P reversibly adsorbed to poorly crystalline Fe- and Al-(hydr)oxides (P-ox), as extracted from soil with 0.2 *M* acid ammonium oxalate (Schwertmann, 1964). For the control[+] treatment of the sandy soil and the loamy soil, we calculated the difference in P-ox between the control[-] treatment and the control[+] treatment for the bulk soil and for the fertiliser zone. Next, the increase in P-ox was scaled against the amount of P initially applied with P fertiliser, expressed in mg P·kg^{-1}. Finally, the average of the bulk soil and the fertiliser zone was taken as the P recovery for both soils. To calculate the P recovery for the DAP and struvite treatments, we had to express the amount of P initially added with these P fertilisers per unit mass of soil for the fertiliser zone. Hence, we needed the mass of the cylindrical soil cores taken from both soils when the pot experiment was terminated. As explained in Section S1, we estimated the soil bulk density using the empirical equation from Lexmond and Edelman (1987) to calculate the mass of the soil cores. Furthermore, we used the claimed P content of DAP and struvite (Table 3.S1). For the DAP and struvite treatments of both soils, we calculated the difference in P-ox between the bulk soil and fertiliser zone soil. The increase in P-ox was scaled against the amount of P initially applied with DAP and struvite, expressed in mg P·kg^{-1}. The results of the P recovery calculations are presented in Table 3.S4. We have added an extra calculation to account for the effect of P uptake on the P recovery. In this calculation for the DAP and struvite treatments, all P uptake was assigned to the fertiliser zone in which these P fertilisers were placed. Although the maize roots preferentially grew in this zone for the DAP and struvite treatments, some roots were growing in the bulk soil as well (Figure 3.1B). As such, not all P will have been taken up by maize from the fertiliser zone. This leads to an overestimation of the P recovery when taking P uptake into account for the DAP and struvite treatments.


S

Chapter 4

Nitrous Oxide Emissions After Struvite Application in Relation to Soil P Status

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Abstract

Purpose Although struvite ($MgNH_4PO_4 \cdot 6H_2O$) is mostly considered to be a novel phosphorus (P) fertiliser, it does contain a significant amount of nitrogen (N). Yet, relatively little is known about the soil N dynamics in struvite-amended soils. Here, we focus on how struvite application impacts emissions of the greenhouse gas nitrous oxide (N_2O), in relation to soil P status.

Methods We conducted a 54-day greenhouse pot experiment on two similar soils with different P status ("low-P soil"; "high-P soil") seeded with *Lolium perenne* L. We applied seven fertiliser treatments (Control; Struvite granules; Struvite powder; Urea; Triple superphosphate (TSP); TSP+Struvite granule; TSP+Urea). Except for the unfertilised Control and the TSP treatments, N application rate was 150 kg $N \cdot ha^{-1}$. Nitrous oxide (N_2O) fluxes, aboveground yield, plant N and P uptake and readily plant-available soil N and P contents were measured.

Results In the low-P soil, none of the fertiliser treatments induced a significant increase in N_2O emission compared to the control. In the high-P soil, struvite application resulted in lower emissions than urea application, statistically not different from the control treatment. Struvite powder significantly increased both plant N and P uptake compared to granular struvite and the resulting yield was similar to conventional fertilisation (TSP and Urea). Any struvite application also resulted in lower readily plant-available soil nitrate contents than urea.

Conclusion Our results suggest that struvite fertilisation can reduce the risk of gaseous N losses without compromising agronomic performance. Pulverizing struvite granules further promotes its dissolution, which could be useful for crops with early nutrient needs.

Keywords

Struvite · Nitrous oxide · Agronomic performances · N uptake · P uptake

1 Introduction

Phosphorus (P) is an essential element for plant growth. Since many soils are P-deficient and unable to supply P to plants at a sufficient rate, P fertilisation is needed to overcome P deficiency in plants (Hinsinger, 2001). Agricultural food production heavily relies on rock phosphate as a source to produce mineral P fertiliser (Nesme et al., 2018). However, global rock phosphate reserves are limited (Childers et al., 2011; Elser and Bennett, 2011) and might be exhausted within a century (Cordell and White, 2013). This causes an increasing need for an alternative, more sustainable management of P in agriculture. To achieve this, circularity needs to be restored through the recycling of nutrients from waste products, especially for P (Muhmood et al., 2019; Tonini et al., 2019). For a more sustainable P management, the recovery of P from wastewater rich in nutrients such as ammonium (NH_4) and orthophosphate ($P\text{-}PO_4$) through the precipitation of struvite ($MgNH_4PO_4{\cdot}6H_2O$) is a promising option (Liu et al., 2011; Muhmood et al., 2019; Uysal, 2015). Struvite has a high potential for replacing conventional synthetic mineral P fertilisers (Huygens and Saveyn, 2018; Rahman et al., 2014).

Since the solubility of struvite in water is low when compared to conventional mineral P fertilisers such as monoammonium phosphate and triple superphosphate (TSP), it is considered a slow-release P fertiliser (Degryse et al., 2017; Rech et al., 2019). The agronomic efficiency of struvite depends therefore on the rate at which struvite dissolves in soil as well as the subsequent diffusion of $P\text{-}PO_4$ to the surface of the plant roots where uptake occurs. Kratz et al (2019) suggested that soils with a different P status could result in a different agronomic efficiency for a given struvite, with a lower P status soil leading to a higher efficiency due to an increase in struvite dissolution. Indeed, the solubility of struvite depends, among others, on the equilibrium concentrations of PO_4, ammonium (NH_4) and magnesium (Mg) ions in soil pore water, with lower concentrations of these ions driving the dissolution of struvite, and the pH, with a more acidic pH favouring dissolution (Bhuiyan et al., 2007). Furthermore, struvite granular size has been reported to have an impact on its dissolution rate in soil. In a pot experiment conducted by Talboys et al. (2016), struvite granules did not provide P to plants during early growth as fast as conventional diammonium phosphate fertiliser. Degryse et al. (2017) reported that when struvite was ground to powder and mixed with soil, the struvite dissolved significantly faster than granular struvite, due to the increase in soil:struvite contact area. It is also reported that struvite powder had similar agronomic performances in terms of wheat yield, P content, and P uptake as monoammonium phosphate (Degryse et al., 2017). Indeed, the dissolution rate of struvite is highly dependent on its specific surface area (SSA) (Ariyanto et al., 2017). Compared to struvite granules,

struvite powder has higher SSA, leading to faster dissolution in soil pore water. In most literature-reported studies, powdered struvite rather than granular struvite was used to compare the agronomic efficiency of struvite with conventional mineral P fertilisers, which may lead to a bias in the estimation of agronomic performance (Hertzberger et al., 2020). As struvite is mostly sold on the market in the granular form (farmer's equipment being adapted to spread fertiliser as granules), it is important not to overlook the agronomic efficiency of granular struvite.

Although struvite is used primarily as a P fertiliser, it also contains significant amounts of nitrogen (N). Yet, soil N dynamics following struvite application, including nitrous oxide (N_2O) emissions, have received little attention. Urea, accounting for 57% of global N fertiliser demand in 2013/2014 (Heffer and Prud'homme, 2016), leads to on average 0.56% of the applied N being lost as N_2O (Luo et al., 2007). Although this is a small part of the N budget, the strong global warming potential of N_2O compared to CO_2 makes it a significant contributor to global warming (IPCC, 2022). Some studies indicated that the highest anthropogenic emissions of N_2O are derived from arable land (Reay et al., 2012; Tian et al., 2020). It is therefore valuable to study how struvite application impacts the soil N turnover including N_2O emissions. Liu et al. (2011) estimated the cumulative N_2O emission from soil columns amended with struvite by multiplying the measured total N leaching loss with a N_2O emission factor from the IPCC. The estimated N_2O emissions were lower for the struvite-treated soil than for the urea-treated soil. However, to our knowledge, no study has directly measured the N_2O fluxes from soil after struvite application. It is unknown how powdered and granular struvite affect N_2O emissions.

Interactions between P fertilisation and soil N_2O emission have previously been studied but results were inconsistent. In a greenhouse pot experiment, P application significantly improved plant N uptake by alleviating plant P shortage. This resulted in a lower N surplus in the soil, thereby mitigating soil N_2O emissions after N fertiliser application (Baral et al., 2017). However, Wang et al. (2015) found that P addition increased the soil microbial biomass during wet seasons, potentially leading to a higher microbial activity. The addition of N and P led to a 60% of increase in soil N_2O emission (compared to the control without fertilisation), while the addition of only N increased soil N_2O emission by 39% as compared to the control (Wang et al., 2014). Some studies also showed an increase in soil N_2O emissions after P application, especially on P-limiting soils (He and Dijkstra, 2015; Mehnaz et al., 2018; Mehnaz and Dijkstra, 2016). Given the fact that struvite results in slow release of both N and P, it is not yet clear how struvite addition would impact the soil N cycle.

In order to address the research gaps identified above, we conducted a greenhouse pot experiment with grass to measure N_2O fluxes from two acidic sandy soils (low and high-P status) amended with powdered or granular struvite. Control treatments with conventional P and N fertilisers were used as a reference. We hypothesised that:

- H1: Granular struvite dissolves at a higher rate in a soil with a lower P status. This leads to a higher plant N uptake and thereby to lower cumulative soil N_2O emissions in low-P soil than high-P soil;
- H2: Under the same N application rate, struvite application in the granular form leads to lower cumulative N_2O emissions compared to urea application, while having the same agronomic performance;
- H3: The soil amended with powdered struvite has higher cumulative N_2O emissions and agronomic performance compared to that of the soil fertilised with granular struvite;
- H4: Addition of P in the form of TSP reduces cumulative soil N_2O emissions derived from both urea and granular struvite, as the addition of extra P in both cases will improve the nitrogen use efficiency of struvite and urea.

To test these hypotheses, we applied the following treatments: a control without fertiliser, struvite granules (H1, H2, H3), urea (H2), struvite powder (H3), struvite and TSP combined (H4), urea and TSP combined (H4) and TSP only (H4).

2 Materials and Methods

2.1 Soil Description

Two acidic sandy soils were used in the greenhouse pot experiment, i.e., a soil with a low soil P status (low-P soil) and a soil with a high soil P status (high-P soil). The low-P soil was collected from the subsoil (30-60 cm) of an arable land in Achterberg, the Netherlands (51°59′32.28”N, 5°35′1.37”E), which had not received P fertilisation for 30 years. The high-P soil was collected from the topsoil of an arable field in Wageningen, the Netherlands (51°59′15.70”N, 5°39′32.46”E) and was regularly fertilised using both organic and mineral fertilisers following local practices prior to sampling. Both soils were air-dried and homogenized for the pot experiment. The properties of the two soils are shown in Table 4.1. They have a similar texture. However, the low-P-soil had a slightly lower pH and both soils had a different soil organic matter (SOM) content as well as different N and P characteristics. The high-P soil also had a higher SOM and higher mineral N content than the low-P soil. The distinction between the low-P soil and high-P soil was made based on the amount of P that can be extracted from soil by using acid ammonium acetate lactate (P-AL)

Table 4.1 Initial physico-chemical properties of the two acidic sandy soils.

Property	Unit	Low-P	High-P
Clay	%	<1	<1
Silt	%	15	12
Sand	%	84	87
SOM	%	1.9	3.6
pH[1]	-	5.05	5.66
$P\text{-}PO_4$[1]	$mg \cdot kg^{-1}$	bd[2]	1.1
$N\text{-}NH_4$[1]	$mg \cdot kg^{-1}$	2.1	4.7
$N\text{-}NO_3$[1]	$mg \cdot kg^{-1}$	1.7	21.1
Total dissolved N[1]	$mg \cdot kg^{-1}$	6	41
Mg[1]	$mg \cdot kg^{-1}$	28.8	35.6
P-AL	$mg \cdot kg^{-1}$	23	300

[1]Measured in 0.01 *M* $CaCl_2$ extract (Houba et al., 2000). [2] bd=below detection limit of 0.4 $mg \cdot kg^{-1}$. A description of the 0.01 *M* $CaCl_2$ soil extraction method can be found in Section 2.6 and the analytical details of the other methods can be found in the Supplementary Information.

(Egnér et al., 1960). 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. Based on P-AL, the soil P status of the low-P soil is considered as very low whereas the soil P status of the high-P soil is considered as high (Reijneveld et al., 2010).

2.2 Experimental Design

An eight-week greenhouse pot experiment was set up at Wageningen University Campus (Bornsesteeg 48, Wageningen, the Netherlands, 51°59'16.3"N, 5°39'48.5"E), as a fully randomized block design with seven fertilisation treatments and five replicates which were distributed over the five blocks, amounting to a total of 70 PVC pots of 19.5 cm diameter and 23 cm depth. Details of the fertiliser treatments are shown in Table 4.2. Nitrogen fertilisation rates followed the Dutch fertiliser advice for intensively managed grassland (Commissie Bemesting Grasland en Voedergewassen, 2017). To calculate the application rate of struvite, we used the theoretical composition (12.6% of P, 5.7% of N and 9.9% of Mg), which was quite close to the actual composition (Table 4.S1). For all treatments except the control and TSP treatments, we provided 75 kg $N \cdot ha^{-1}$ at each growing cycle (two growing cycles in total). TSP was applied to ensure a surplus of soil available P at a rate of 50 kg $P \cdot ha^{-1}$ per growing cycle. All treatments including the control received K

Table 4.2 The composition and rate of fertiliser applied in the different treatments during the entire pot experiment.

Treat-ment	Fertiliser use [g·pot^{-1}]				Fertilisation rate [kg·ha^{-1}]		
	Struvite (12.6% P; 5.7% N)	Urea (46% N)	TSP (TSP) (45% P)	Potassium sulphate (25% K)	N	P	K
C	-	-	-	1.12	-	-	100
Sg	7.37	-	-	1.12	150	332[1]	100
Sp	7.37	-	-	1.12	150	332[1]	100
U	-	0.91	-	1.12	150	-	100
T	-	-	0.62	1.12	-	100	100
TSg	7.37	-	0.62	1.12	150	332+100[2]	100
TU	-	0.91	0.62	1.12	150	100	100

[1] 332 kg P·ha^{-1} from struvite,
[2] 332 kg P·ha^{-1} from struvite and 100 kg P·ha^{-1} from TSP.
The treatment abbreviations refer to the control (C), struvite granule (Sg), struvite powder (Sp), urea (U), TSP (T), TSP+struvite granule (TSg) and TSP+urea (TU).

fertilisation at 50 kg·ha^{-1} per growing cycle. In short, we had two treatments using struvite only, either as granule (Sg) or as powder (Sp). We compared these treatments with urea (U) and an unfertilised control (C). To assess the effect of P addition on soil N_2O emission, we added TSP to both a struvite granule treatment (TSg) and an urea treatment (TU). Finally, we had a P-fertilised control without N but with TSP added (T). Urea, TSP and potassium sulphate were applied in conventional granular form. The struvite used in this study was recycled from a potato peels sludge (NuReSys, Deerlijk, Belgium) and its average granular diameter was 2 mm.

2.3 Pot Preparation and Maintenance

During the pot filling (24th July 2019), potassium nitrate, ammonium nitrate and micronutrient solutions were mixed thoroughly with 6.2 kg of air-dry soil per pot and the application rate of N and K were the same at 50 kg·ha^{-1} for all treatments including C, to ensure a proper grass cover before the start of the experiment. The soil was packed in the pot in two layers to ensure a homogeneous bulk density of 1.29 g·cm^{-3}. Estimations of field bulk density following the method of Rawls (1983) were 1.39 and 1.24 g·cm^{-3} for the low and high-P soils, respectively. A watering tube of 5 cm diameter was inserted in the middle of each pot to avoid artificially modifying the topsoil structure and thus impacting gaseous emissions or increasing the dissolution of fertilisers through more frequent watering in the greenhouse than under field conditions. Ryegrass (*Lolium perenne* cv Barforma) seeds were sown on the soil

surface three times until the grass cover was sufficient and homogeneous for all pots (2 x 8 g·m^{-2} on the 25th July and two weeks after, and 16 g·m^{-2} a month before the start of the experiment).

The experiment started on 28th October 2019 (day 1). On the first day of the experiment, the aboveground biomass was cut at 7 cm above the soil surface. Fifty percent of the fertiliser application rates listed in Table 4.2 were homogeneously applied on the soil surface (broadcasting) to start the first growing cycle. On 25th November 2019 (day 29), the first growing cycle ended and grass was harvested for plant analysis and soil samples were collected for intermediate soil analysis. Subsequently, the rest of the fertilisers was applied to start the second growing cycle. On 20th December 2019 (day 54), grass was harvested, and soil samples were collected again for analysis and the experiment was terminated. On the two sampling days, the grass was cut at a height of 7 cm above the soil surface. Soil samples were taken within each pot from two randomly selected places (*i.e.*, not purposefully avoiding struvite remnants). The soil sampling depth was 10 cm. After the first growing cycle, the drilling holes were filled with quartz sand after soil sampling. Soil N_2O fluxes were measured 27 times during the 54 days. All pots were watered through the watering tube and the moisture content of the soil was maintained gravimetrically at 117 ml water·kg^{-1} dry soil (*i.e.*, 60% of water holding capacity) on a daily basis. Two 15 mm-rainfall events were simulated by spraying demi-water on the surface of the whole pot on 20th November 2019 (day 24) and 13th December 2019 (day 47), temporarily bringing the soils beyond 100% of water holding capacity.

2.4 N_2O Flux Measurements and Calculations

N_2O fluxes were measured overall 27 times during the 54-day pot experiment. The fluxes were determined with the flux-chamber method described by Velthof and Oenema (1995). During the measurement, the top of each pot was sealed by a PVC chamber and the watering tube was closed with a rubber stopper. The headspace volume of the chamber was approximately 4.2 L. The N_2O concentration in the headspace was measured after closing the chamber for 30-40 minutes, with the exact closing time being recorded, using a photoacoustic gas monitor (Innova, Type 1302) for N_2O measurement. A soda-lime filter was connected before the inlet to eliminate the interference of carbon dioxide. We calculated the increase of N_2O concentration by subtracting the ambient N_2O concentration from the N_2O concentration inside the chamber. The emission rate of N_2O from soil was calculated based on the assumption that the increase of the concentration in the chamber during the closing period was linear (Velthof and Oenema, 1995). To calculate the cumulative

emissions, we assumed that the emission rates changed linearly between two measurement dates.

2.5 Plant Analysis

The plant samples were dried at 70°C for 48 hours in order to determine dry yields. The dried plant samples were finely ground for N and P content analysis. A mixture of sulfuric acid and hydrogen peroxide was used to digest the plant material (Novozamsky et al., 1983). The N and P concentrations in the digests were measured by a segmented flow analyser (SFA; Skalar, SAN^{++}). Although some N and P must have been present in the grass roots and stubbles, we hereafter refer to plant uptake as the amount of N and P in the harvested grass shoots. The nitrogen use efficiency (NUE) of the grass was calculated as:

$$\text{NUE}\ (\%) = \left[\frac{U - U_0}{F}\right] \times 100$$

in which NUE is the N use efficiency expressed as a percentage, U is the amount of N taken up by the grass in the fertiliser treatment for both harvests, U_0 is the amount of N taken up in the control treatment for both harvests, and F is the total amount of N applied (Table 4.2), with U, U_0 and F all expressed in kg $N \cdot ha^{-1}$.

In this study, we defined the agronomic performance of a fertiliser as the effect of a given fertiliser on the yield and the nutrient uptake of the grass.

2.6 Soil Analysis

All soil samples collected on day 29 and day 54 of the pot experiment were air-dried (40°C) and sieved through a 2 mm-screen. To determine the readily plant-available amounts of N and P, soils were extracted with 0.01 *M* $CaCl_2$ using a soil-to-solution ratio of 1:10 (g:ml) and a shaking time of 2 h (Houba et al., 2000). After centrifugation at 3000 rpm for 10 min and subsequent 0.45 µm filtration (Aqua 30, Whatman) of the soil extracts, the total dissolved Mg concentration was measured by an inductively coupled plasma-optical emission spectrometry (ICP-OES, Thermo scientific, ICAP 6000) whereas the concentrations of $N\text{-}NH_4$, $N\text{-}NO_3$ and $P\text{-}PO_4$ were measured by SFA (Skalar, SAN++). Strictly speaking, where we report the $N\text{-}NO_3$ content, the sum of $N\text{-}NO_3$ and $N\text{-}NO_2$ is meant.

Furthermore, an additional experiment was performed in which we quantified the dissolution of struvite in 0.01 *M* $CaCl_2$ solution, separately and in combination with soil, up to an extraction time of 96 h. A description and short discussion of the results of the experiment can be found in the Supplementary Information.

2.7 Statistical Analysis

Statistical analysis of data was performed within SPSS 25.0 software (SPSS Inc., Chicago, IL, USA). Before conducting any statistical test, normality of residuals and homogeneity of variances were examined. Normality was firstly checked with the Shapiro-Wilk test. If the assumption of normality was violated, we further checked the normality visually with a Normal Quantile-Quantile plot. The homogeneity of variances was examined with the Levene test. If the variances were homogeneous, we conducted one way ANOVA with Tukey HSD post hoc test. In the case of heterogeneity of variances, Games-Howell test was the alternative. When the p-value was smaller than 0.05, we rejected the null hypothesis of equal means. As for dealing with the violation of normality, data transformation (Log transformation and Box-Cox transformation) would be applied. If data transformation methods failed to normalize data, the bootstrap module in SPSS would be used. In this case, statistical significance was obtained by examining the 95% confidence interval of the difference between two means. If the confidence interval was intersected with zero, two means were not statistically different. Relations between soil parameters and plant parameters with cumulative N_2O emission were probed by Pearson Bivariate Correlation. Pearson *r* was used to interpret the magnitude of correlation. In order to control the confounding variables, Partial Correlation was also performed as a supplement to the bivariate correlation. The interpretation of the magnitude of correlation was in line with the guideline proposed by (Gignac and Szodorai, 2016).

3 Results

3.1 Grass Performance

Figure 4.1a shows the aboveground yield of grass. For the low-P soil, the largest yield was observed for the Sp and TU treatments. The sole application of TSP did not increase yield compared to that of the C treatment whereas the application of urea without any P addition (U treatment) significantly increased yield. The yield of the Sp treatment was significantly larger than that of the Sg treatment, and the yield of the Sg treatment was significantly higher than that of the U treatment. The yields of the TSg and TU treatments were not statistically different. In the high-P soil, similar to that of the low-P soil, the two lowest yields were observed for the C and TSP treatments that did not receive any N fertilisation. The application of struvite powder and urea resulted in a significantly larger yield compared to the C treatment. The yields of the Sg, TSg, and TU treatments were not statistically different from each other and that of the C treatment. The two soils resulted in very different yields for the same treatments. When comparing the yields of the C treatments, there is an average yield difference of 3301 $kg \cdot ha^{-1}$ between the low-P soil and the high-P soil.

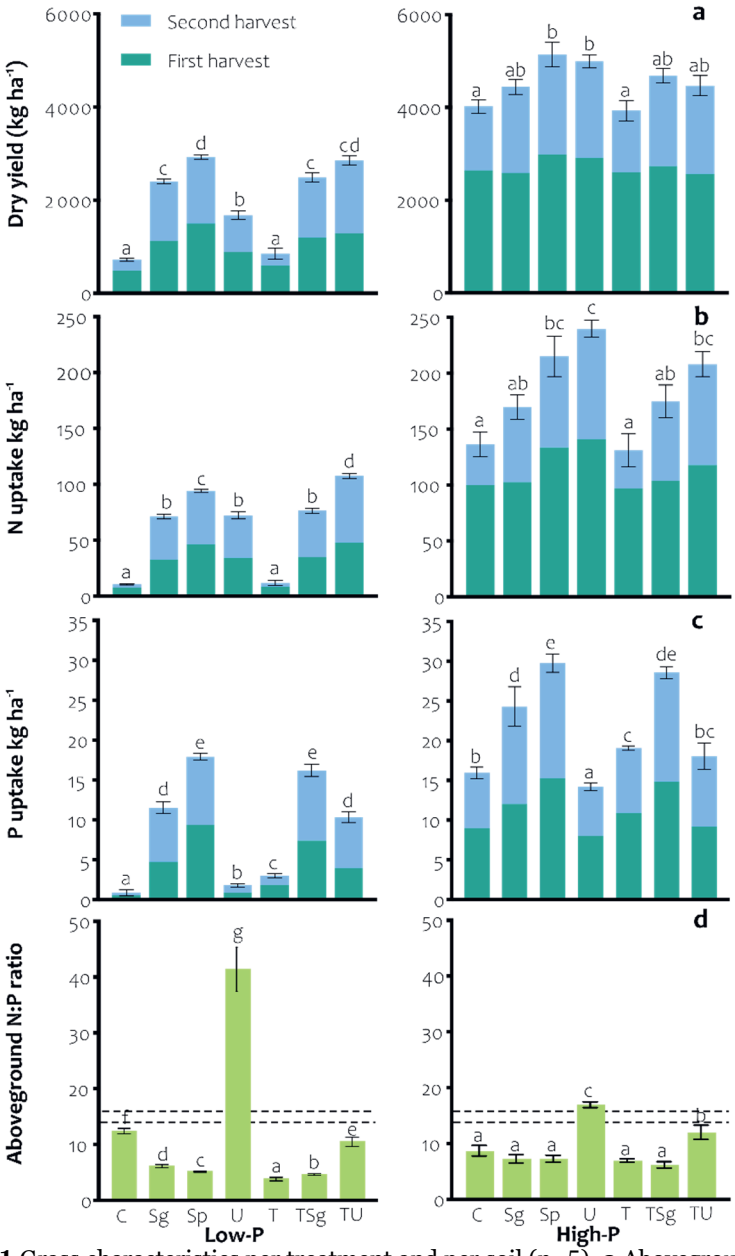

Figure 4.1 Grass characteristics per treatment and per soil (n=5). **a** Aboveground dry yield. **b** Aboveground N uptake. **c** Aboveground P uptake. **d** Aboveground N:P ratio, dashed line represent commonly acknowledged N and P limitation thresholds (14 and 16 respectively). The bars in a, b and c are split by harvest; error bars denote the standard errors of the means of the sum of both harvests. d is averaged for both harvests and error bars are standard errors of the means. Letters indicate significant differences in overall dry yields. Significant differences for a and b were obtained by the Tukey HSD test ($p<0.05$). Significant differences for c and d were obtained by the bootstrapped Games-Howell test (the 95%-confidence interval of the difference of two means does not intersect with zero). The seven treatments are control (C), struvite granule (Sg), struvite powder (Sp), urea (U), TSP (T), TSP+struvite granule (TSg) and TSP+urea (TU).

Aboveground N uptake of grass is shown in Figure 4.1b. In the low-P soil, the TU treatment had the largest N uptake, followed by that of Sp. Similar to the yield, the two treatments with the lowest amounts of N taken up were the C and T treatments. For the Sg, U and TU treatments, the N uptake was not statistically different from each other. However, in the high-P soil, N uptake of the U treatment was significantly higher than that of the Sg and TSg treatments. The lowest N uptake was also observed for the C and TSP treatments which did not include any N fertilisation. For each treatment, N uptake was higher for the high-P soil than for the low-P soil. For example, N uptake in the C treatment of the high-P soil was higher than 135 kg·ha^{-1}, but it was only about 10 kg·ha^{-1} in the low-P soil.

For both soils, aboveground P uptakes of both Sg and Sp treatments were significantly higher than that of the C treatment (Figure 4.1c). On both soils, the highest P uptake was realised in the Sp and TSg treatments, followed by the Sg treatment. On the high-P soil, the T treatment had lower P uptake than the Sg treatment. The TU treatment was not different from that in the C treatment, while the U treatment had the lowest P uptake. On the low-P soil, the TU treatment had the same P uptake as the Sg treatment. Interestingly, the C, U and T treatments all had a much lower P uptake than the treatments in which a fertiliser source with both N and P was provided. Again, the P uptake differed strongly for all treatments of the two soils. For example, the P uptake of the control in the high-P soil was higher than 15 kg·ha^{-1}, but it was lower than 5 kg·ha^{-1} in the low-P soil.

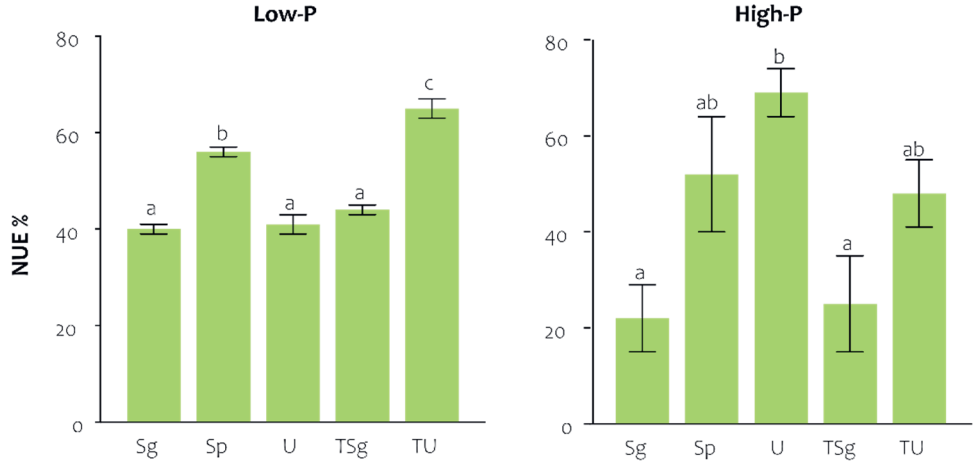

Figure 4.2 NUE (%) for the treatments receiving N fertilisation for the two soils. Error bars are standard errors of the means. Lowercase letters indicate significant differences. The significant differences were obtained by the Tukey HSD test ($p<0.05$). Seven treatments are control (C); struvite granule (Sg); struvite powder (Sp); urea (U); TSP (T); TSP+struvite granule (TSg) and TSP+urea (TU).

The U treatment led to the largest aboveground N:P ratio which was even above 30 in the low-P soil (Figure 4.1d). The second largest N:P ratio was found in the C treatment without any fertilisation. In addition, the Sp treatment resulted in a significantly lower aboveground N:P ratio than that of the Sg treatment. The lowest aboveground N:P ratio was observed with the T treatment. All treatments with P addition had a lower aboveground N:P ratio than that of the C treatment. In the high-P soil, the largest N:P ratio was still obtained for the U treatment. However, the second largest value was from the TU treatment. Struvite application did not significantly change the aboveground N:P ratio compared to the control treatment. In both soils, the application of only urea strongly increased the aboveground N:P ratio.

A similar pattern in NUE (Figure 4.2) for the different treatments receiving N fertiliser was found as that of aboveground N uptake (Figure 4.1b). For the low-P soil, the TU treatment had the highest NUE followed by that of Sp. The treatment with only urea application (U) led to the lowest NUE, which was not statistically different from that of the other two treatments of struvite granules application (Sg and TSg). For the high-P soil, the U treatment had a significantly higher NUE compared to that of Sg and TSg. The P use efficiency (PUE) was calculated in the same manner as the NUE; results are shown in the Supplementary Information (Table 4.S2).

3.2 Readily Plant-available Soil Nutrients

Table 4.3 shows the results of the extraction of the soil samples taken at the two sampling dates with 0.01 *M* $CaCl_2$. For the treatments with struvite addition (Sg, Sp and TSg) of both soils, the $CaCl_2$-extractable amount of $N\text{-}NH_4$ was significantly higher on days 29 and 54 compared to the C treatment. The same is true for the extracted amounts of $P\text{-}PO_4$. Interestingly, the extracted amounts of $N\text{-}NH_4$ and $P\text{-}PO_4$ for the treatments receiving powdered struvite (Sp) and granular struvite (Sg and TSg) of the low-P soil did not differ significantly on day 29. Likewise, no significant differences were found for the Sg and Sp treatments of this soil on day 54. However, the extracted amounts of $N\text{-}NH_4$, and $P\text{-}PO_4$ for the Sp treatment of the high-P soil were significantly higher than for the Sg treatment on day 29. These differences remained intact for the extracted amounts of $P\text{-}PO_4$ on day 54. With respect to $N\text{-}NO_3$ for the low-P soil, the largest amount was extracted for the U, TU and TSg treatments on day 29. On day 54, the U treatment still had the largest extractable amount of $N\text{-}NO_3$, but we did not observe significant differences in the extracted amounts of $N\text{-}NO_3$ across the other treatments. For the high-P soil, the Sp, U and TU treatments had the highest amounts of extracted $N\text{-}NO_3$ on day 29. The

Table 4.3 Results of 0.01 *M* $CaCl_2$ extraction of soil samples in the seven treatments of the low-P soil and the high-P soil (soil samples collected on day 29 and day 54). The results of pH and Mg are presented in supplementary Information (Table 4.S3).

		Day 29			Day 54		
		$N\text{-}NH_4$ $mg \cdot kg^{-1}$	$N\text{-}NO_3$ $mg \cdot kg^{-1}$	$P\text{-}PO_4$ $mg \cdot kg^{-1}$	$N\text{-}NH_4$ $mg \cdot kg^{-1}$	$N\text{-}NO_3$ $mg \cdot kg^{-1}$	$P\text{-}PO_4$ $mg \cdot kg^{-1}$
Low-P	C	0.5(0.1) b	0.1(0.0) a	0.00(0.00) a	0.3(0.0) a	0.2(0.0) a	0.03(0.03) ab
	Sg	13.6(1.7) d	0.3(0.1) b	1.02(0.38) c	14.0(5.1) de	0.1(0.0) a	7.59(3.37) cd
	Sp	6.9(3.0) cd	0.2(0.0) b	0.74(0.26) c	3.4(0.5) d	0.2(0.0) a	1.12(0.40) c
	U	7.3(2.8) cd	2.3(0.9) c	0.00(0.00) a	7.6(2.7) d	3.1(1.4) b	0.00(0.00) a
	T	0.2(0.0) a	0.1(0.0) a	0.02(0.02) b	0.5(0.1) b	0.1(0.0) a	0.12(0.04) b
	TSg	10.3(3.2) cd	0.5(0.2) bc	0.99(0.26) c	16.3(2.2) e	0.1(0.0) a	9.37(2.35) d
	TU	5.3(1.3) c	1.9(0.9) c	0.00(0.00) a	1.8(0.2) c	0.1(0.1) a	0.02(0.02) a
High-P	C	2.6(0.5) a	9.3(3.4) a	1.00(0.04) a	1.4(0.1) a	0.9(0.3) a	0.82(0.04) a
	Sg	13.4(0.8) c	9.8(4.8) a	3.57(0.50) c	11.3(2.8) bc	2.1(0.8) ab	5.20(1.53) d
	Sp	23.9(3.8) d	19.4(7.0) ab	12.52(2.49) d	20.1(6.4) c	2.3(0.7) b	14.93(3.85) e
	U	3.4(0.9) ab	45.5(18.4) b	1.10(0.09) ab	3.0(0.3) b	29.2(13.2) c	0.98(0.07) b
	T	3.3(0.4) a	5.5(2.0) a	1.00(0.07) ab	1.8(0.2) a	0.7(0.2) a	1.57(0.22) c
	TSg	21.4(4.3) d	7.5(3.2) a	11.33(6.21) cd	22.5(6.3) c	1.5(0.9) ab	43.29(21.30) e
	TU	9.8(4.0) bc	35.2(13.3) b	1.30(0.13) b	8.4(2.9) b	19.6(9.9) c	1.76(0.39) c

Data show average values with standard errors in brackets (n=5). Letters indicate significant differences. Significant differences were obtained by the bootstrapped Games-Howell test (The 95% confidence interval of the difference between two means does not intersect with zero).

amount of extractable $N\text{-}NO_3$ for all three treatments with struvite addition did not differ significantly from the amount of $N\text{-}NO_3$ extracted from the C treatment. For day 54, still no differences were found for these treatments. Similar to the low-P soil, the U and TU treatments again resulted in the largest amounts of extracted $N\text{-}NO_3$ on day 54.

3.3 Cumulative Soil N_2O Emissions

The two soils showed distinctive cumulative soil N_2O emission patterns (Figure 4.3). In general, the N_2O fluxes measured from the low-P soil were substantially lower than those from the high-P soil. Rain events on day 24 and 47 led to an increase in N_2O fluxes for both soils, especially for the U and TU treatments on the high-P soil. For the low-P soil, none of the fertiliser treatments resulted in a significant increase in cumulative N_2O emission (one-way ANOVA, $F=0.453$, $p=0.837$). For the high-P soil, however, both treatments receiving urea (U and TU) resulted in a significant

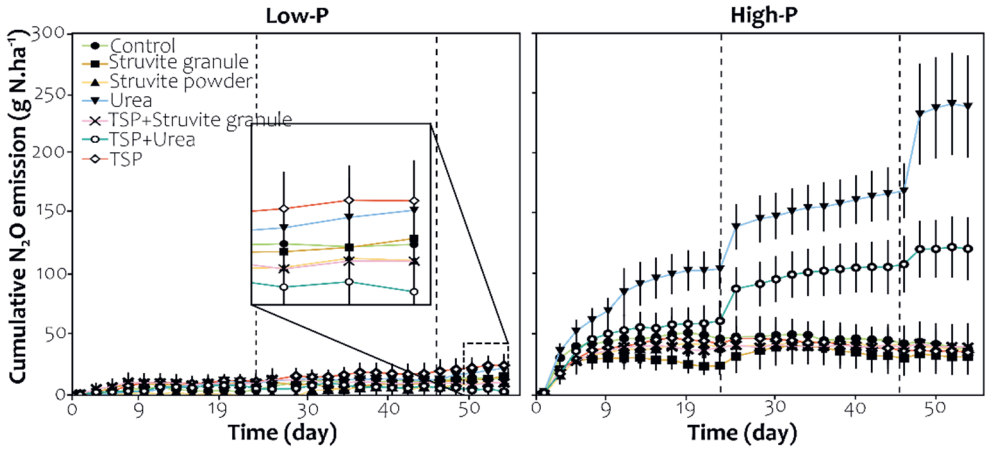

Figure 4.3 Cumulative N_2O emissions from two soils. Error bars represent standard errors of means (n=5). Dotted lines indicate two simulated rain events (day 24 and day 47). Fertilisers were applied on day 1 and day 29. For the low P soil, the results of the last three measurements are amplified in the inset figure.

increase in the cumulative N_2O emission, with the N_2O emission peaking at 239 g N·ha^{-1} for the U treatment. In both soils, neither Sg nor Sp led to a significant increase in the N_2O emission compared to the C treatment, meaning that the grinding of struvite did not have a significant effect on the N_2O emissions. We also calculated the N_2O emission factors, with the application of the soluble N fertiliser (urea) resulting in significantly higher N_2O emission factors than the other treatments in the high-P soil (Table 4.S4). The daily N_2O fluxes from each measurement are presented in Table 4.S5. For the high-P soil, we found a significant positive correlation ($r=0.59$, $p<0.001$; $n=35$) between the cumulative N_2O emission and $CaCl_2$-extractable N-NO_3 content (Figure 4.6a). However, no such correlation was found in the low-P soil ($p=0.277$). Furthermore, there was a significant positive correlation between the cumulative N_2O emission and the N:P ratio of the aboveground biomass in the high-P soil ($r=0.68$, $p<0.001$, $n=35$).

4 Discussion

4.1 Agronomic Performances of Struvite in Relation to Soil P Status

We hypothesized that granular struvite would dissolve at a higher rate on a soil with a lower P status. This would then lead to a higher plant N uptake and thereby lower cumulative soil N_2O emissions from the low-P soil than that from the high-P soil. However, we did not measure the actual dissolution rate of struvite as this would be

technically challenging because any P released to solution upon struvite dissolution is to a large extent immediately captured through adsorption by the soil solid phase. By using labelled 33P, a diffusion test showed that most P released by a single struvite granule could not travel more than 1 mm distance in the soil, while the P released by TSP travelled more than 25 mm, indicating that any P dissolved from struvite that has a low solubility would be captured by the soil (Rech et al., 2019). The fast adsorption of P by the soil solid phase after struvite dissolution is further supported by the results of the test that we performed to quantify the dissolution of struvite during the soil extraction with 0.01 *M* $CaCl_2$ (Supplementary Information, Section 8.2). The concentration of P-PO_4 in 0.01 *M* $CaCl_2$ kept increasing over time (up to 96 h) when struvite was extracted in the absence of soil. However, when the struvite-amended soil was extracted, hardly any P-PO_4 could be detected in the solution (Figure 4.S1A).

Nevertheless, we did extract soil samples taken at two sampling times from all treatments of the two soils with 0.01 *M* $CaCl_2$ (Table 4.3) to mimic soil pore water conditions (McDowell and Sharpley, 2001). The results were surprising because of the large amounts of N-NH_4 extracted compared to those of N-NO_3. The ratios between N-NH_4 and N-NO_3 observed in the $CaCl_2$ soil extracts of the Sg-treated soils on day 29 were on average 45.3 and 1.3 for the low and high-P soils respectively. Treatments without struvite showed considerably lower ratios, ranging from 2 to 5 and >0.1 to 0.8 for the low and high-P soils respectively (Table 4.3), which is comparable to the ratios of the soils before the start of the experiment (Table 4.1) as well as ratios in cultivated soil with similar texture (Łukowiak et al., 2017). The difference in the ratio between N-NH_4 and NO_3 for the Sg-treated soils and for the soils that did not receive struvite is even more pronounced at day 54 than at day 29. The Sg-treated soils had average ratios of 140 and 5.3 for the low and high-P soils respectively while the treatments without struvite stayed within similar range as on day 29, with ratios from 1.5 to 18 and 0.1 to 2.5 for the low and high-P soils respectively (Table 4.3). Under normal conditions, it is expected that soil NH_4 from fertiliser application would be shortly nitrified. Moreover, these apparently high mineral N-NH_4 contents in the soil were contradictory to plant N limitations in the treatments with struvite (Figure 4.1d). We visually observed that not all of the applied struvite dissolved during the greenhouse pot experiment. Unfortunately, these undissolved struvite particles could not be separated from soil during sample preparation due to their small diameters. Thus it is suspected that some of the remaining struvite must have at least partially dissolved during the 2 h $CaCl_2$ extraction procedure of our soil samples. Struvite dissolution does occur during 2 h $CaCl_2$ extraction (Supplementary information, Section 8.2) as well as during other

commonly used soil P tests (Gu et al., 2021). Thus, for the treatments containing struvite in our pot experiment, the $CaCl_2$ extraction most likely did not reflect the actual readily plant-available soil N-NH_4. Since grass growth in the treatments containing struvite was N-limited (Figure 4.1d), it is likely that the actual level of readily plant-available N-NH_4 was low and that any dissolved NH_4 from struvite during the growing period would have been quickly taken up by the grass. The residual struvite in soil poses a substantial analytical challenge in evaluating the true nutrient availability of the soil. Especially when struvite is applied under field conditions, separating undissolved struvite residues from soil is difficult. By comparing various soil P extraction methods, Gu et al (2021) suggested that sink-based P test might be the most reliable method to assess the plant-available P

Although the $CaCl_2$ extraction method failed to realistically reveal the readily plant-available nutrient derived from struvite in the soil, the calculated NUE could be an alternative proxy. Indeed, the grass would have taken up any plant-available N as both soils were N-limited and a comparison between the difference in N uptake of the C and Sg treatments in each soil can give some quantitative idea to which extent struvite dissolved during the pot experiment. Struvite granules (Sg treatment) led to a NUE twice as high on the low-P soil than on the high-P soil (Figure 4.2). Assuming that any N from dissolved struvite would have been taken up, this suggests that struvite granules did indeed dissolve faster in the low-P soil than in the high-P soil. However, this did not lead to a higher N uptake in the Sg treatment for the low-P soil than for the high-P soil as the amount of N supplied by the latter without any fertilisation overcompensated this difference in struvite dissolution. The N uptake of grass in the C treatment of the high-P soil was more than 12 times higher than in the low-P soil (Figure 4.1b). This can be explained by the higher amounts of readily plant-available N, as well as the higher SOM in the high-P soil before the start of this experiment (Table 4.1). We can only partially accept H1 as struvite granules seem to dissolve faster on the low-P soil but this apparently did not translate into a higher N uptake for the very same reason: a soil with low levels of plant-available N and P would lead to a faster dissolution and thus a better performance of struvite granules but would also intrinsically provide less N for uptake by the plants. In this respect, struvite granules can be considered as an “on demand” fertiliser *i.e.,* a mineral fertiliser with a low solubility, providing more N when the soil is lacking N, and less N when the soil is already quite fertile.

4.2 Agronomic Performances and N_2O Emission: Struvite vs Urea

Our second hypothesis stated that under the same N application rate, struvite application in the granular form would lead to lower cumulative N_2O emissions compared to urea application, while having the same agronomic performances.

In the high-P soil, Sg led to a statistically similar yield as U, while in the low-P soil struvite led to a higher yield than U (Figure 4.1a). This can be explained by the strong P limitation of the U treatment in the low-P soil (Figure 4.1d). When TSP was combined with urea in the TU treatment for the low-P soil, this limitation was alleviated (Figure 4.1d) and a similar yield as with struvite was reached (Figure 4.1a). Additionally, in the low-P soil, Sg and U led to the same N uptake but this was not the case in the high-P soil where U led to a significantly higher N uptake than Sg (Figure 4.1b). However, since the high N uptake of the U treatment did not lead to an increased yield (Figure 4.1a) and showed P limitation (Figure 4.1d), this hints at the possibility that the plant had more N than what it could actually use. In other words, part of the N that was taken up by the grass in the U treatment is due to luxury uptake. This is further supported by the effects of applying TSP combined with urea in the TU treatment of the high-P soil: the TU treatment had a N uptake comparable to the Sg treatment (Figure 4.1b). With respect to P, the Sg treatment had a clear advantage over U in both soils. When TSP was combined with urea in the TU treatment, similar levels of P uptake were found in the low-P soil as compared to the Sg treatment but not in the high-P soil where Sg had a more positive effect on P uptake than TU. Although surprising, this result could be explained by the nature of the P fertilisers. From literature it is known that most of the TSP dissolves in soil within 24 hours after application while struvite dissolves slowly over 60 days or even more depending on soil properties like pH (Degryse et al., 2017; Lawton and Vomocil, 1954). Dissolution of struvite in the high-P soil may have proceeded at a lower rate than in the low-P soil, because the initial pH and concentrations of N-NH_4, P-PO_4, and Mg in the $CaCl_2$ soil extracts of the high-P soil were higher than for the low-P soil (Table 4.1). For P uptake by plants, it may be more advantageous to have a low but regular flow of P arriving from soil in the pore water than one large flush of P: in the first case, plant roots have a higher chance to intercept this P before it binds to the soil while in the second case, plants cannot take up that much P within a given short period of time meaning that the excess P will be bound by the soil and as such be less available for uptake. Overall, Sg showed a rather similar agronomic performance as U. The slow release of P from struvite dissolution over time may be considered as an advantage to provide P to the soil, as compared to TSP.

N_2O emissions originate from nitrification and denitrification and thus depend on the availability of $N\text{-}NH_4$ and $N\text{-}NO_3$ in soil. Because of residual struvite dissolving during $CaCl_2$ extraction (Supplementary information section 8.2), we cannot use the measured readily plant-available $N\text{-}NH_4$ contents as a reliable source of information for how much $N\text{-}NH_4$ was actually present in these soils. The $CaCl_2$-extractable $N\text{-}NO_3$ contents, however, are not impacted by struvite dissolution during $CaCl_2$ extraction (Supplementary information, Section 8.2). In both soils, Sg showed lower $N\text{-}NO_3$ concentrations than the U treatment (Table 4.3). This is consistent with the findings of another pot experiment where the total amount of N leached from soil amended with struvite was about three times less than that of urea in a column experiment (Liu et al., 2011). This seems to suggest that the actual soil $N\text{-}NH_4$ concentration for Sg was also lower than for U. This is in line with the N_2O emissions we observed for the high-P soil: we found a significantly lower cumulative N_2O emission from the Sg treatments compared to that of U (Figure 4.3). Thus, in the high-P soil, the slow release of nutrients originating from struvite did not increase N_2O emissions. This is in line with studies reporting that slow-release N fertilisers such as slow-release urea can significantly decrease N_2O emission compared to that of traditional urea, as used in our study (Awale and Chatterjee, 2017; Trinh et al., 2017). In the low-P soil, the average cumulative emission of Sg was lower than that of U (15.7 and 22.5 g $N\text{-}N_2O \cdot ha^{-1}$ respectively) but this was not significantly different. The emissions were low because the soil had very little N to start with (Table 4.1) and a large part of the fertiliser added was taken up by the plants, especially when addition of N was combined with addition of P (Figure 4.3). This is further supported by the significant positive correlation we found in the high-P soil between $N\text{-}NO_3$ and cumulative N_2O emissions, in line with many previous studies that identify denitrification as the main source of N_2O emissions following N application (Ji et al., 2020; Mehnaz and Dijkstra, 2016; Zhou et al., 2020). Although we did not find significant differences in the cumulative N_2O emissions for the low-P soil, we did find a significantly lower $N\text{-}NO_3$ concentration for Sg than for U. This does suggest that N_2O emissions may in general be lower for Sg than for U.

With Sg having a rather similar agronomic performance as U, and N_2O emissions being lower than for the former, we can accept our second hypothesis.

4.3 Agronomic and Climatic Impact of Powdering Struvite

Our third hypothesis stated that the soil amended with powdered struvite would have higher cumulative N_2O emissions and agronomic performance compared to that of the soil fertilised by granular struvite. In the low-P soil, the Sp treatment led to higher yield, N uptake and P uptake compared to that of the Sg treatment (Figure

4.1a, b, c). In comparison to TU, Sp had a similar yield, a slightly lower N uptake and a higher P uptake. A similar pattern was observed in the high-P soil, although results of yield and N uptake were not statistically different between Sg and Sp. The higher NUE for Sp in both the low and the high-P soil (significant difference for the low-P soil only) (Figure 4.2) indeed seems to suggest a faster dissolution of the struvite powder. Grinding struvite increases its SSA i.e, surface area per unit of mass. Similar to phosphate rocks, struvite with a higher SSA will dissolve faster than the one with a lower SSA, all other soil properties being equal (Degryse et al., 2017). This is because the dissolution of struvite occurs at the particle surface, which comes into contact with soil pore water as the solvent. A higher SSA means that there is a higher mineral surface area exposed to the soil pore water.

As the N-NO_3 concentrations in the Sg- and Sp-treated soils were similar in both soils (Table 4.3), we can argue that N dissolved from the struvite, in granules or in powder, was taken up in similar amount by the grass, which resulted in identical N_2O emissions for Sp and Sg from both soils. Therefore, contrary to our hypothesis, the dissolution of powdered struvite does appear to have gone faster than that of granular struvite, leading to a better agronomic performance of the fertiliser, but without impacting N_2O emissions. Therefore, our third hypothesis was rejected. Our results seem to suggest that reducing the granular size could increase the agronomic performance of struvite fertiliser without sacrificing its ability to mitigate N_2O emission. Some studies have suggested that in order to deal with the low solubility of struvite, it could be applied in combination with soluble fertilisers such as mono- or di-ammonium phosphate, especially in the early growth stage when plants may require a faster nutrient supply (Ackerman et al., 2013; Talboys et al., 2016). Combining struvite with conventional fertiliser may increase risks of nutrient leaching and N_2O emissions. Our results demonstrate that a good alternative solution to a lack of nutrients in the early growth stage of plants can be to provide (at least some of) the struvite in powdered form. This could be done by pelletising powdered struvite so that it could be applied with conventional fertiliser application equipment. In this form it would still be more soluble than granular struvite. Alternatively, regular equipment could be adapted to apply powder instead of granules. Research on how powdered struvite can be applied in practice needs further attention.

4.4 Effects of P Addition on N_2O Emissions

Our fourth hypothesis stated that the addition of P in the form of TSP would reduce cumulative soil N_2O emissions derived from both urea and granular struvite, as the addition of extra P in both cases would improve the NUE of struvite and urea. In the

low-P soil, TSP addition did not have any measurable influence on N_2O emission in either of the treatments because the emissions were all very low, even for the U treatment (Figure 4.3). However, TSP addition did increase the N uptake of grass fertilised with urea (Figure 4.1b). As mentioned in Section 4.2, plants of the U treatment in the low-P soil probably accumulated luxury N and did not leave sufficient N available in the soil to result in significant N_2O emissions. The combination of TSP and urea meant that plants could use this N to invest in growth (Figure 4.1a). In the high-P soil, TU had much lower N_2O emissions than U (Figure 4.3). However, we did not observe such an effect for struvite granules (TSg vs Sg). This can be explained by the slow release of N from struvite granules as compared to urea (see Section 4.2). Interestingly, although adding TSP to urea reduced N_2O emissions, we did not find that P addition led to a higher aboveground N uptake in the high-P soil (Figure 4.1b). We also did not find that the N was significantly immobilized by the soil microbes in the treatments with TSP (Table 4.S6). Thus, it is possible that the rest of N might be present in the plant roots, which were not samples in this study.

Koerselman and Meuleman (1996) proposed to use the N:P ratios of vegetation to represent nutrient limitation on a community level. Although they used a N:P ratio of 14 and 16 as the thresholds for N (< 14) and P limitation (> 16), a study has shown that indicating nutrient limitation by a canonical N:P ratio was questionable (Yan et al., 2017). Using more conservative thresholds of 10 and 20, instead of the commonly used thresholds of 14 and 16, was leading to better estimation of N and P limitation. The N:P ratio should thus be used with caution. Yet, in general a high N:P ratio of vegetation indicates P limitation for the plant community, while a low ratio implies N limitation. In both our soils, any form of struvite fertilisation led to a low N:P ratio of the grass (< 10), which shows that grass growth under struvite fertilisation was possibly N-limited (Figure 4.1d). U and TU resulted in P-limited or less N-limited conditions compared to struvite fertilisation, although only U in the low-P soil had a N:P ratio higher than 20. Since N was still a limiting resource to grass after the struvite fertiliser application, grass might utilise various strategies to acquire as much N as they can take up from soil (Gutschick, 1981; Tolley and Mohammadi, 2020). In the high-P soil where urea application led to an increase of cumulative N_2O emission, a significant positive correlation was found between aboveground N:P ratio and the cumulative N_2O emission (partial correlation; soil surplus NO_3 as the control variable). This demonstrates that the more severe the P shortage was for plant growth (or the less N-limiting the system was), the more N was lost through N_2O. The reason for struvite mitigating N_2O emission is therefore that it potentially shifts the system towards N limitation.

5 Conclusion

This study reports the first measurement of soil N_2O fluxes after struvite fertilisation. Under the same N application rate, struvite has lower cumulative N_2O emissions compared to those of urea. The direct application of granular struvite did not lead to yield penalties on either soil. Increasing the contact area between struvite and soil through grinding resulted in higher N and P uptake in comparison to regular struvite granules. Our results show that the powdered struvite application could increase the agronomic performance of this fertiliser without increasing N_2O emissions. Although struvite fertilisation may pose a concern of N deficiency if applied in regular doses, on both soils the aboveground dry yield from the struvite powder treatment was the highest of all treatments. This greenhouse study showed that the use of struvite led to lower N_2O emission as compared to a conventional N fertiliser like urea and these results need further confirmation in the form of a field experiment.

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Supplementary Information

S1. Initial Soil and Struvite Characterisation

For the determination of the relevant physico-chemical properties of the the low and high-P soils used in the pot experiment, a subsample was dried at 40°C and sieved (2 mm). Soil texture was determined by Eurofins Agro (Wageningen, the Netherlands) through near infrared spectroscopy. Soil organic matter (SOM) was measured through loss-on-ignition in a muffle furnace (550°C) (Hoogsteen et al., 2015). For determining the soil P status, soil was extracted with acid ammonium acetate lactate (P-AL) (Egnér et al., 1960). 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.

Table 4.S1 Struvite composition

Element	Value
P [g·kg^{-1}] *	126
N [g·kg^{-1}] *	52.5
Mg [g·kg^{-1}] ‡	102
Na [mg·kg^{-1}] ⁂	<100
Al [mg·kg^{-1}] ‡	<40
S [mg·kg^{-1}] ‡	<30
K [mg·kg^{-1}] ‡	5973
Ca [mg·kg^{-1}] ‡	307
V [mg·kg^{-1}] ⁂	<0.1
Cr [mg·kg^{-1}] ⁂	4.1
Mn [mg·kg^{-1}] ‡	127
Fe [mg·kg^{-1}] ‡	103
Ni [mg·kg^{-1}] ⁂	<1.6
Cu [mg·kg^{-1}] ⁂	<0.6
Zn [mg·kg^{-1}] ‡	5
As [mg·kg^{-1}] ⁂	<0.1
Mo [mg·kg^{-1}] ⁂	<0.3
Cd [mg·kg^{-1}] ⁂	<0.05
Pb [mg·kg^{-1}] ⁂	<0.3

* Struvite was destructed with sulfuric acid and hydrogen peroxide (Novozamsky et al., 1983), and the concentration of the elements in the extracted solution were measured with a segmented flow analyzer (SFA). All the other elements were extracted using an Aqua Regia destruction (Sastre et al., 2002). ‡Elements were measured with an inductively coupled plasma-mass-spectrometry (ICP-MS). ⁂Elements were measured with an Inductively coupled plasma - optical emission spectrometry (ICP-OES).

S2. Dissolution of Struvite During 0.01 *M* $CaCl_2$ Extraction

We performed an experiment to quantify struvite granule dissolution during $CaCl_2$ extraction as described by Houba (2000). We used struvite recycled from soybean residues sludge (NuReSys, Deerlijk, Belgium) that is similar to the one used in the pot experiment and a similar low-P sandy soil, as described in Vos et al (2019). The soil was dried at 40 °C and sieved over a 2 mm screen. We used five treatments without replication: only struvite granules (*i*), only soil (*ii*), soil and struvite granules freshly mixed (*iii*) and soil with addition of an ammonium sulphate solution at either 5 (*iv*) or 10 mg $N\text{-}NH_4.kg{\cdot}soil^{-1}$ (*v*). The ammonium sulphate addition to soil allowed us to determine on whether any nitrification occurred during $CaCl_2$ extraction of soil . In 1 L containers, we added 80 g of dried soil and, if applicable, either 9.2 mg of struvite or ammonium according to the application mentioned above. To each container, we added 800 mL 0.01 *M* $CaCl_2$ solution to realize a soil-to-solution ratio of 1:10 (g:ml). The containers were closed and placed on a linear shaker (72 $strokes{\cdot}min^{-1}$). At 2, 4, 24, 48, 72 and 96 hours after the start of shaking, 20 mL of suspension was taken from each container and transferred to a 50 mL-polypropylene tubes. The suspensions in Greiner tubes were then centrifuged at 3000 rpm for 10 min and the supernatants were filtered through a 0.45 µm membrane (Aqua 30, Whatman). In these filtrates, we measured $P\text{-}PO_4$, $N\text{-}NH_4$ and $N\text{-}NO_3$ with a SFA as described in Houba (2000). For treatments including a combination of soil and fertiliser (*iii*, *iv* and *v*), we further processed the data by subtracting the concentration realised by the "only soil" treatment (*i*).

Figure 4.S1 presents the concentrations of the $P\text{-}PO_4$, $N\text{-}NH_4$ and $N\text{-}NO_3$ in the $CaCl_2$ solutions over time. When pure struvite was extracted in $CaCl_2$ over time, there was a sharp increase in $P\text{-}PO_4$ (Figure 4.S1A) and $N\text{-}NH_4$ (Figure 4.S1C) but no significant amounts of $N\text{-}NO_3$ could be detected (Figure 4.S1E). Furthermore, the addition of ammonium sulphate to the soil, even at the highest dosage, did not result in the production of NO_3 (Figure 4.1F). The $N\text{-}NO_3$ concentration in the struvite-amended soil was practically nil after subtracting the concentration of $N\text{-}NO_3$ of the soil without struvite addition. This proves that *(a)* struvite is dissolving in the $CaCl_2$ solution and that *(b)* the NH_4 during the extraction released does not nitrify. However, when struvite was mixed with the soil, the $P\text{-}PO_4$ that should have dissolved seemed buffered by the soil (Figure 4.S1A). Indeed, the $P\text{-}PO_4$ concentrations for the soil with struvite and the soil without struvite were the same. Once the $P\text{-}PO_4$ concentration of the soil without struvite was subtracted from the struvite-amended soil, the resulting $P\text{-}PO_4$ concentration was close to zero. On the other hand, the $N\text{-}NH_4$ concentration increased in the $CaCl_2$ solution (Figure 4.S1C). We suspect that NH_4 is less strongly absorbed to the soil solid phase than $P\text{-}PO_4$. Our

results demonstrate that $P\text{-}PO_4$ is not a good proxy for how much struvite has dissolved during the duration of the pot experiment. In addition, the high concentration of $N\text{-}NH_4$ is mainly the result of struvite dissolving during the $CaCl_2$ extraction. The concentration of $N\text{-}NO_3$, however, is only impacted by the NO_3 present in the soil before the start of the extraction and is not impacted by the struvite dissolving during the extraction.

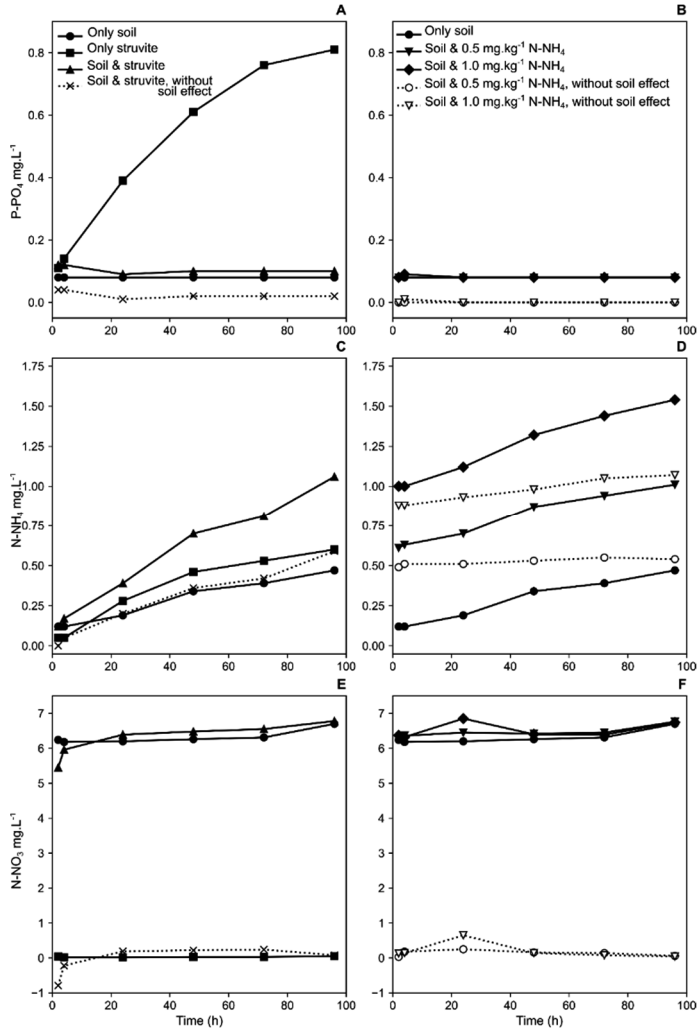

Figure 4.S1 Concentrations of $P\text{-}PO_4$ (A, B) , $N\text{-}NH_4$ (C, D) and $N\text{-}NO_3$ (E, F) over time during the 0.01 *M* $CaCl_2$ extraction experiment. Plots A, C and E show the results for the struvite alone and struvite amended soil, with and without the soil effect. Plots B, D and F show the results for soil amended with ammonium sulphate, with and without the soil effect. Dotted lines indicate the "soil & fertiliser" treatments without soil effects, which were calculated by subtracting the $P\text{-}PO_4$, $N\text{-}NH_4$ and $N\text{-}NO_3$ measured in the treatment only soil from the treatment with struvite-amended soil.

S3. Additional Results

Table 4.S2 Average PUE (%) in treatments with P fertilisation

	Sg	Sp	T	TSg	TU
Low-P	3.2(0.2) b	5.1(0.1) c	2.2(0.3) a	3.6(0.2) b	9.5(0.6) d
High-P	2.5(0.8) ab	4.2(0.3) b	3.1(0.3) a	2.9(0.2) a	2.1(1.7) ab

Data show average values with standard errors in brackets (n=5). Lowercase letters indicate significant differences. The significant differences were obtained by the Tukey HSD test ($p<0.05$). Seven treatments are control (C); struvite granule (Sg); struvite powder (Sp); urea (U); TSP (T); TSP+struvite granule (TSg) and TSP+urea (TU). The phosphorus use efficiency (PUE) was calculated in the same way as the NUE (see Section 2.5).

Table 4.S3 Additional results of 0.01 *M* $CaCl_2$ extraction of soil samples in seven treatments of the low-P soil and the high-P soil (soil samples collected at day 29 and day 54)

		Day 29		Day 54	
		pH	Mg $mg \cdot kg^{-1}$	pH	Mg $mg \cdot kg^{-1}$
	C	5.08(0.02) a	39(2) b	5.10(0.03) b	37(2) a
	Sg	5.39(0.03) d	106(14) c	5.42(0.09) dc	150(34) cd
	Sp	5.36(0.04) d	105(12) c	5.29(0.07) c	101(11) c
Low-P	U	5.17(0.02) c	32(2) a	5.22(0.04) bc	41(3) ab
	T	5.12(0.02) ab	36(2) b	5.13(0.02) b	48(3) b
	TSg	5.36(0.06) d	94(15) c	5.49(0.06) d	190(14) d
	TU	5.12(0.01) b	36(3) ab	4.99(0.03) a	33(4) a
	C	5.71(0.04) b	43(3) a	5.75(0.05) ab	62(11) a
	Sg	5.83(0.06) bc	99(3) c	5.76(0.03) b	107(18) b
	Sp	5.90(0.05) c	155(10) d	5.95(0.06) c	175(25) c
High-P	U	5.60(0.04) a	63(10) b	5.56(0.07) a	65(4) a
	T	5.77(0.03) b	53(6) ab	5.68(0.02) ab	64(10) a
	TSg	5.83(0.04) bc	131(22) cd	5.84(0.04) bc	194(36) c
	TU	5.73(0.03) b	56(4) b	5.66(0.01) a	55(7) a

Data show average values with standard errors in brackets (n=5). Letters indicate significant differences. Significant differences were obtained by the bootstrapped Games-Howell test (the 95% confidence interval of the difference between two means does not intersect with zero). Seven treatments are control (C); struvite granule (Sg); struvite powder (Sp); urea (U); TSP (T); TSP+struvite granule (TSg) and TSP+urea (TU). Soil pH was measured in the 0.01 *M* $CaCl_2$ solution.

Table 4.S4 Emission factors (%) in N fertilised treatments from two soils.

	Sg	Sp	U	TSg	TU
Low-P	0.00(0.01) a	0.00(0.01) a	0.01(0.01) a	0.00(0.01) a	0.01(0.01) a
High-P	0.00(0.01) a	0.00(0.01) a	0.13(0.03) c	0.00(0.01) a	0.06(0.02) b

Data show average values with standard errors in brackets (*n=5*). Significant differences were obtained by the bootstrapped Games-Howell test (The 95% confidence interval of the difference of two means does not intersect with zero). Seven treatments are control (C); struvite granule (Sg); struvite powder (Sp); urea (U); TSP (T); TSP+struvite granule (TSg) and TSP+urea (TU). Emission factors were calculated as Velthof and Oenema (1995)

Table 4.S6 Soil microbial N ($mg \cdot kg^{-1}$) for the seven treatments of the two soils used in the pot experiment (soil samples were collected at day 54)

	C	Sg	Sp	U	T	TSg	TU
Low-P	-0.4(0.4)	0.2(1.4)	1.3(2.1)	3.2(1.6)	0.7(1.0)	-0.3(0.8)	1.0(0.8)
High-P	7.8(0.6)	9.4(3.1)	8.7(4.0)	7.2(1.8)	4.7(1.3)	9.2(4.8)	7.1(1.2)

Data show average values with standard errors in brackets (*n=5*). 2*7 ANOVA was conducted to examine interactions and differences (no differences across treatments $p=0.916$, soil effect $p<0.001$, no interaction effect $p=0.753$). Seven treatments are control (C); struvite granule (Sg); struvite powder (Sp); urea (U); TSP (T); TSP+struvite granule (TSg) and TSP+urea (TU). Microbial soil N analysis was only done for the soil samples collected on day 54 following Paul et al. (1999). For this analysis, 0.5 *M* K_2SO_4 solution was used to extract N from soil samples before and after fumigation, respectively. Total N in the supernatant was measured by SFA. Soil microbial N was calculated with a standard correction factor of 0.54 to compensate for un-lysed compounds (Paul et al., 1999).

Table 4.S5 N_2O emission rates (g N-N_2O·day^{-1}·ha^{-1}) at each measurement day per treatment for the two soils (average ± standard error)

	Low-P soil							High-P soil						
Day	C	Sg	Sp	U	T	TSg	TU	C	Sg	Sp	U	T	TSg	TU
0	0.11±0.72	1.23±0.54	0.97±0.49	1.17±0.37	0.75±0.38	1.00±0.35	0.69±0.53	0.63±0.46	0.16±0.27	0.51±0.14	2.81±0.49	0.73±0.33	0.82±0.30	2.10±0.98
2	0.66±0.59	0.57±0.54	-0.47±1.00	-0.36±0.62	0.59±2.46	2.18±2.13	0.96±0.26	15.19±4.82	10.74±3.18	7.98±2.25	16.99±2.63	10.14±5.25	9.93±4.22	8.01±3.88
4	0.32±0.17	2.03±1.75	0.26±0.42	0.39±0.61	0.79±0.42	0.84±1.00	0.06±0.48	4.77±1.02	3.62±0.95	6.40±2.34	8.14±1.57	4.39±0.40	3.71±0.79	9.27±1.43
6	1.39±0.74	1.29±0.77	0.51±0.45	0.92±1.01	2.26±0.98	1.96±1.38	0.40±0.62	1.09±0.85	0.57±0.78	1.92±1.22	4.73±1.38	3.77±1.20	3.08±0.94	4.91±1.20
8	0.48±0.26	0.18±0.86	0.34±0.43	0.05±0.40	1.08±0.42	-0.33±0.31	-0.01±0.52	0.75±0.92	0.33±1.02	1.18±0.92	3.74±1.13	0.93±0.71	1.30±0.38	2.25±1.05
10	0.27±0.57	-0.11±0.85	-0.64±0.64	0.84±0.95	0.44±0.08	-0.03±0.18	1.75±0.45	1.50±0.50	0.12±0.75	1.30±1.03	7.72±4.73	0.72±0.43	1.57±0.49	1.40±0.27
12	-0.76±0.40	-0.32±0.75	0.82±0.40	1.43±0.48	-0.60±0.32	-0.03±0.47	-0.02±0.78	-0.24±0.47	0.08±0.39	0.09±0.53	3.40±1.48	1.16±0.41	0.32±0.57	1.24±0.14
14	-0.48±0.65	-0.36±0.26	-1.09±0.86	-0.07±0.71	-0.11±0.32	-0.79±0.52	-0.38±0.13	0.76±0.72	-0.85±0.58	-0.02±0.29	2.34±1.23	1.61±0.57	0.28±0.57	-0.36±0.11
16	-0.27±0.41	1.03±0.86	0.36±0.93	1.07±0.92	0.38±0.60	0.71±0.57	0.99±0.23	1.18±0.72	-0.06±0.50	1.78±0.44	1.69±0.83	0.64±0.55	-0.54±0.39	1.52±0.91
18	0.49±1.19	-0.21±0.59	0.16±0.89	1.15±1.14	0.91±0.52	-0.33±0.26	0.03±0.93	0.60±0.66	-1.89±0.44	0.46±0.70	1.55±0.58	-0.82±1.03	-1.19±0.82	0.27±0.61
20	-0.74±0.40	0.65±0.40	-0.83±0.79	-0.46±0.36	0.02±0.44	0.04±0.82	0.08±0.53	-0.94±0.33	-0.74±0.46	-0.37±0.42	-0.01±0.75	-0.37±0.39	-0.33±0.87	0.20±0.91
22	0.84±0.89	0.02±0.38	-0.78±0.50	-0.70±0.77	-0.40±0.54	0.35±0.63	-1.39±1.18	-1.56±1.66	0.28±0.66	-0.83±0.74	0.66±0.54	-1.22±0.87	1.33±0.56	1.05±1.23
24	1.54±1.97	0.12±0.31	0.88±0.44	1.31±0.48	0.67±1.17	1.52±0.59	-0.24±0.65	0.85±1.03	3.89±3.78	0.43±0.44	17.45±3.18	2.24±1.80	0.83±0.79	13.31±2.95
26	1.01±0.54	-0.82±1.33	-0.19±0.45	0.04±0.68	1.10±0.33	-0.25±0.42	0.17±0.79	0.04±0.74	1.59±0.46	-0.01±0.96	2.22±0.51	-0.03±0.35	-0.55±0.69	1.35±0.46
29	0.96±0.43	0.59±0.82	2.28±1.04	1.07±0.97	-0.48±0.24	-0.05±0.40	1.22±0.55	0.56±0.82	1.29±0.87	0.30±0.88	1.06±1.10	-0.36±0.41	1.15±0.67	1.89±0.65
31	0.06±0.19	-1.58±0.55	0.24±0.43	-1.03±0.54	0.16±0.85	-1.13±0.30	1.15±0.48	0.31±0.48	0.68±0.52	0.98±0.35	1.96±0.37	-0.43±0.26	-0.70±0.84	1.95±0.28
33	-1.17±0.52	-0.98±0.66	0.44±1.02	0.29±0.53	0.78±0.51	0.04±0.90	-0.89±1.81	-0.23±0.84	-0.34±0.77	0.69±0.65	1.53±0.71	-0.97±0.21	-0.16±0.99	0.92±0.88
35	0.54±0.51	1.32±1.09	0.29±0.51	0.03±0.39	1.02±0.99	0.64±0.64	-0.97±0.58	-2.25±0.60	-0.20±0.20	-0.93±0.93	0.38±0.90	0.27±0.58	0.15±0.53	0.65±0.65
37	-0.47±0.63	1.01±0.61	0.48±0.23	0.07±0.35	-0.10±0.21	-0.28±0.53	0.17±0.35	0.50±0.40	-1.85±0.54	0.54±0.74	1.38±0.65	-0.74±0.35	0.40±0.79	0.72±0.68
39	0.89±0.41	0.05±0.53	-0.07±0.55	0.43±0.45	-0.41±0.26	-0.63±0.57	-0.33±0.17	0.36±0.14	-0.20±0.38	-0.30±0.45	1.58±0.50	-0.96±0.76	-0.32±0.55	0.55±0.23
41	-0.09±0.60	-0.26±0.42	-0.99±0.57	-0.66±0.62	-0.26±0.34	0.07±0.57	-0.29±0.80	-0.48±0.88	-1.59±0.47	-1.67±1.02	1.36±0.61	-0.70±0.51	-0.55±0.34	0.29±0.37
43	-0.65±0.60	0.62±0.46	0.84±0.35	-0.10±0.89	0.27±0.29	0.69±0.66	1.01±0.37	-0.24±0.41	-0.03±0.82	0.75±0.41	1.07±0.52	-0.06±0.52	1.16±0.62	-0.01±0.85
45	0.46±0.45	0.62±0.95	0.02±0.81	0.23±0.75	1.04±0.48	-0.71±0.95	-1.03±1.01	-1.39±0.43	-0.90±0.39	0.28±0.88	1.21±0.69	-1.03±1.01	-1.29±0.52	1.13±0.63
47	1.21±0.70	0.57±1.18	0.97±0.85	2.08±0.63	0.74±1.40	0.36±0.71	0.27±1.38	0.65±0.96	1.76±0.96	-0.87±0.84	31.78±11.16	0.05±0.63	1.75±0.84	6.29±1.22
49	0.17±1.02	0.09±0.48	0.20±1.00	0.54±0.76	0.61±0.36	-0.96±1.14	-1.04±1.03	-0.93±0.63	-0.63±0.21	-0.63±0.72	2.56±0.58	-0.31±0.57	-1.43±0.57	0.07±1.12
51	-0.33±0.50	0.50±0.51	1.08±0.47	1.24±0.52	1.00±0.27	0.92±0.36	0.63±0.61	-1.12±0.30	-0.68±0.28	-0.44±0.50	1.69±0.66	0.18±0.59	0.12±0.64	0.80±1.27
53	0.26±1.17	1.07±0.72	-0.26±0.70	0.85±0.33	-0.06±0.60	-0.01±0.96	-1.19±0.42	-0.77±0.25	0.17±1.09	-0.90±0.76	-1.14±0.60	-0.70±1.27	-0.21±0.66	-0.72±0.38


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Chapter 5

Can Earthworms and Root Traits Improve Struvite-P Uptake in Grasslands? A Field Study

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Abstract

The availability of conventional mineral phosphorus (P) fertilisation will become increasingly challenging as phosphate rock stocks are limited and strongly concentrated in a few locations. Therefore, we need to increase agronomic P use efficiency and find alternative, recycled sources of P. Two possible solutions mentioned in the literature are *(i)* using struvite, a mineral circular P fertiliser; and *(ii)* making use of earthworm activity, which has been shown to increase P availability. Here, we study the interaction between these two approaches, with the hypothesis that earthworms could increase the P availability from the poorly soluble struvite. We set up a field-based mesocosm experiment in a sandy soil with a low agronomic P status with 13 different treatments combining three earthworms species (*Lumbricus rubellus* Hoffmeister, *Aporrectodea caliginosa* Savigny and *A. longa* Ude alone or in a threes species mixture) and different P fertilisers (no P, Triple Super Phosphate (TSP) and struvite). The experiment lasted 13 months (five fertilisation-harvest cycles). We found that, in field conditions, the yield and P uptake of *Lolium perenne* did not differ between fertilisation with struvite or TSP. Earthworms only played a minor role in explaining ryegrass P uptake compared to fertilisation. We did not see either positive nor negative interactions between earthworms and struvite. The equal performances of struvite and TSP are explained by an enhanced effort from plants to actively take up P through a modification of root traits. This includes increased arbuscular mycorrhizal fungi colonisation and the production of finer and longer roots. Our results show that struvite performs comparably to TSP under realistic field conditions, making it a viable alternative to phosphate rock-based fertilisers.

Keywords

Circular P fertilizer, Arbuscular mycorrhizal fungi, Root acquisition strategies, Triple superphosphate, *Lumbricus rubellus*, *Aporrectodea caliginosa*

1 Introduction

Plant phosphorus (P) limitation is a major issue in many agroecosystems. Although a substantial amount of P can be present in soils, the large majority of it is bound to metal-(hydr)oxides and clay mineral edges or included in primary minerals and soil organic matter, making it unavailable for uptake by plants (Hesterberg, 2010). Most soils need P fertilisation to reach an optimum crop production, but current P fertilisation practices are not sustainable in the long term. Indeed, most mineral P fertiliser is extracted from phosphate rocks, which are a finite resource predicted to run out in the coming centuries (Koppelaar and Weikard, 2013). Moreover, phosphate rocks are concentrated in a limited number of countries, as more than 80% of the global phosphate rock stock is located in only four countries (U.S. Geological Survey, 2022). While there is a strong monopoly and dependency on phosphate rock-based fertiliser, P use in agriculture needs to become both more efficient and more circular (Nesme et al., 2018; Schneider et al., 2019).

Struvite ($NH_4MgPO_4 \cdot 6\ H_2O$) is one of the key products for a more circular P fertilisation and can be recycled from wastewater. This mineral is one of the most promising circular P fertilisers to date with several industrial recovery units already running worldwide (Nageshwari and Balasubramanian, 2022). Struvite has a low solubility in water (Bhuiyan et al., 2007), which allows for a reduced environmental impact of the fertiliser, but also makes plant P uptake more challenging than with conventional P fertilisers. The literature mentions environmental benefits including less nitrogen (N) and P leaching (Ahmed et al., 2018), lower nitrous oxide emissions (Chapter 4), and less P becoming less directly available due to adsorption to reactive soil particles (Degryse et al., 2017). The agronomic effectiveness of struvite compared to conventional P fertilisers is still debated (Ahmed et al., 2018), partly because of the unrealistic experimental designs used in studies (*e.g.*, short experimental times, high plant density, confounding factor of soil P tests or high struvite application rates) (Hertzberger et al., 2020). To date, greenhouse experiments still make up the majority of struvite fertilisation trials; this may contribute to the perceived poorer agronomic performance of struvite as compared to conventional P fertilisers (Hertzberger et al., 2020; Huygens and Saveyn, 2018).

Although in agricultural systems plants rely largely on fertiliser for P supply, biotic factors can also play an important role in P uptake in agricultural grasslands. Plants have strategies themselves to increase their P uptake, for instance through increased root exploration or symbiosis with arbuscular mycorrhizal fungi (AMF) (Richardson et al., 2009b). There is a trade-off between the various strategies, meaning that plant species will usually favour one strategy over another (Honvault et al., 2021; Wen et

al., 2019), although some plants like *Lolium perenne* seem to equally use multiple strategies to alleviate soil P deficient growth conditions (Ros et al., 2018; Turner and Newman, 1984). The trade-off between nutrient uptake strategies can be best illustrated by the "Root Economics Space", a concept that describes the root system according to two gradients (Bergmann et al., 2020). The first is a conservation gradient that distinguishes fast-growing plants with a high root N concentration from slow ones with a high Root Tissue Density (RTD). The second gradient relates to collaboration with "Do-it-yourself" plants exploring the soil for nutrients by themselves, having a high Specific Root Length (SRL) as opposed to plants that rely more on AMF to acquire nutrients and thus having a larger root diameter (Bergmann et al., 2020). AMF are linked to increased plant P uptake in managed grasslands (Oelmann et al., 2021) and the collaboration between plants and AMF is highest under intermediate levels of P fertilisation (Liu et al., 2016). Arbuscular mycorrhizal fungi increased the plant P uptake under struvite fertilisation in a greenhouse study with tomato plants, likely indirectly by increasing the benefit of soil exploration in a situation where the soil solution P is low, albeit produced in constant flow through the slow dissolution of struvite, as opposed to the peaks of readily available P provided by conventional and highly soluble fertilisers (Di Tomassi et al., 2021). Although a few studies have explored the effect of struvite fertilisation on the plant root system (Di Tomassi et al., 2021; Omidire et al., 2023; Valle et al., 2022b), to our knowledge none have placed their results within the frame of the P uptake strategies belowground.

Soil biota play an important role in the soil P cycle (Alori et al., 2017; Le Bayon and Milleret, 2009) and may have an impact on plant P uptake from struvite. Struvite fertilisation led to a more diverse and active microbial community compared to Mono-Ammonium Phosphate (MAP) and Simple Superphosphate (SSP) in a lettuce pot experiment (Mancho et al., 2023). Another relevant group of soil biota that can affect P availability is earthworms. Earthworm presence can increase in soil P availability through mineralisation of organic P source and desorption of mineral P source in relation to their effect on the soil pH and reactive surface area (Le Bayon and Milleret, 2009; Vos et al., 2022a), but this is highly context and species-dependent. Earthworms are commonly categorized into three distinct groups reflecting their feeding guilds: epigeic species, eating and leaving in the litter at the soil surface; endogeic, geophagic species living buried in the soil; and anecic species, leaving in deep permanent galleries but feeding on the surface litter (Bouché, 1977; Örley, 1885). While casts from earthworms belonging to the three feeding guilds were richer in readily available P than the bulk soil (Vos et al., 2019), under field conditions only two species (*Lumbricus terrestris* Linnaeus and *Aporretodea longa*

Ude) have been proven to improve grass P uptake (Vos et al., 2022b). Beyond mobilising soil P, earthworms are able to enhance the availability of P from mineral fertilisers with a low solubility such as phosphate rock (Atmaca, 2021; Mackay et al., 1983). Their composted casts (EcoTea, Canada) have been shown to increase the effectiveness of struvite to fertilise oat in controlled conditions (Hernández Jiménez et al., 2020). The chemical impact of their casts is one way that earthworm could impact dissolution. Another way could be linked to the bioturbation of the soil they inhabit which could move struvite potentially closer to plant's root as shown with other less soluble fertilisers (Mackay et al., 1983). Although soil biota seem to improve the fertilising performances of struvite in controlled conditions, to our knowledge the link between struvite and biota has not been studied under field conditions before.

This study has the objective to understand what biotic factors influence struvite effectiveness as a fertiliser. We hypothesised that: (1) in the field conditions, struvite is as effective as Triple Superphosphate (TSP) to fertilise plants; (2) earthworms enhance struvite dissolution and thus its effectiveness; and (3) struvite fertilisation stimulates plants to develop different P acquisition strategies, thus affecting their root traits. We tested these hypotheses with ryegrass in a 13-month field-mesocosm experiment using a sandy soil with a low agronomic P status and three different earthworm species.

2 Materials and Methods

2.1 Soil

The soil was collected from the topsoil of a field in Achterberg, the Netherlands (51°59′32.28" N, 5°35′1.37" E) that received no phosphorus fertilisation for a period of 25 years. Over the last few years, this field has been conventionally managed with respect to fertilisation and crop rotation but still remains P-deficient. Prior to excavation, it was used to grow potatoes. Details on the soil characteristics are given in Table 5.1. Briefly, it was a noncalcareous sandy soil with a pH of 5.8 The agronomic soil P status was classified as "low" in the Dutch P fertiliser recommendation system (Commissie Bemesting Grasland en Voedergewassen, 2017), which is based on P-$CaCl_2$ as an intensity soil P test method and P-AL as a quantity method (Reijneveld et al., 2022). For P-$CaCl_2$, soil was extracted with 0.01 *M* $CaCl_2$ to determine the size of the readily available soil P pool (Houba et al., 2000), whereas soil was extracted with ammonium lactate (P-AL) (Egnér et al., 1960) to determine the sum of readily available P plus labile soil P (van Doorn et al., 2023). The P-$CaCl_2$ of the soil used here was 0.2 $mg \cdot kg^{-1}$ and P-AL was 116 $mg \cdot kg^{-1}$. The

amount of reversibly adsorbed P (P-ox) as determined by soil extraction with 0.2 acid ammonium oxalate (Schwertmann, 1964) was rather low with 268 $mg \cdot kg^{-1}$. The summed amount of Fe- and Al-(hydr)oxides ([Fe+Al]-ox), which are simultaneously extracted with P-ox by acid ammonium oxalate, was 69.3 $mmol \cdot kg^{-1}$. The P loading α, which was calculated as the molar ratio of P-ox versus [Fe+Al]-ox (van der Zee and van Riemsdijk, 1988), was 0.12. This P loading α can be considered as low when compared to a large set of representative Dutch agricultural topsoils (Koopmans et al., 2006). The soil texture was measured using the pipet method (Houba et al., 1997). Soil organic matter was measured through loss on ignition at a temperature of 500 °C (Hoogsteen et al., 2015). Ammonium, nitrate, dissolved organic nitrogen and magnesium in the soil solution were extracted with a 0.01 *M* $CaCl_2$ solution and measured with a SFA (Houba et al., 2000).. After excavation, the soil was gamma-irradiated (9 kGy, Steris, Ede, the Netherlands) to kill all earthworms and their cocoons (Nakamori et al., 2009; Zhang et al., 2016). To facilitate the recolonisation of soil by native microorganisms, the irradiated soil was inoculated with 3% non-irradiated soil sieved over 1 mm to ensure the absence of earthworms and cocoons (Davis, 1975; Edwards and Bohlen, 1996).

Table 5.1 Characteristics of the soil used here. DON: Dissolved organic nitrogen.

Property	Extractant	Unit	Value
Sand	-	%	87.7
Clay	-	%	1.6
Silt	-	%	6
Soil organic matter	-	%	3.8
pH	Calcium chloride	-	5.8
$N\text{-}NH_4$	Calcium chloride	$mg \cdot kg^{-1}$	2.0
$N\text{-}(NO_3\text{-}NO_2)$	Calcium chloride	$mg \cdot kg^{-1}$	3.9
N-DON	Calcium chloride	$mg \cdot kg^{-1}$	5.1
Mg	Calcium chloride	$mg \cdot kg^{-1}$	88.9
$P\text{-}PO_4$	Calcium chloride	$mg \cdot kg^{-1}$	0.2
P-AL	Ammonium lactate	$mg \cdot kg^{-1}$	116
P-ox	Ammonium oxalate	$mg \cdot kg^{-1}$	268
Fe-ox	Ammonium oxalate	$mg \cdot kg^{-1}$	1553
Al-ox	Ammonium oxalate	$mg \cdot kg^{-1}$	656
P loading-α	-	-	0.12

2.2 Experimental Design

We followed a full randomised block design (five replicates) with 13 treatments (Table 5.2). Eight treatments included earthworms, with either a single earthworm species (*Lumbricus rubellus* Hoffmeister, epigeic "*Lrub*"*; Aporrectodea caliginosa* Savigny, endogeic "*Acal*"; or *A. longa*, anecic, "*Alon")* or a combination of these three species ("Multi"), fertilised with struvite ("Stru") or left without P fertilisation and always fertilised with N in the form of Calcium Ammonium Nitrate (CAN). In addition, we had five earthworm-free treatments: one without any N or P fertiliser ("control"), one with conventional N and P fertilisation ("TSP+CAN"), a treatment with only TSP to induce a N limitation ("Nlim"), a similar treatment with only CAN to induce P limitation ("Pli") and a treatment of struvite supplemented with CAN ("Struvite"). We used granular struvite ($NH_4MgPO_4 \cdot 6\ H_2O$) harvested from the wastewater of an experimental plant transforming soy into a low-P feed for cattle (Nuresys, Deerlijk, Belgium). The chemical composition of struvite is presented in Table 5.S1. The struvite fertilisation was supplemented with calcium ammonium nitrate (CAN) and potassium sulphate to ensure adequate N and K availability. The alternative P fertiliser for the TSP+CANand N-lim treatments was TSP. All fertilisers were applied in a granular form, broadcasted at the soil surface. The earthworms

Table 5.2 List of treatments of the field-based mesocosm experiment.

Treatment	*L. rubellus* (indiv·mesocosm^{-1})	*A. caliginosa* (indiv·mesocosm^{-1})	*A. longa* (indiv·mesocosm^{-1})	P fertiliser	N fertiliser	K fertiliser
Control	0	0	0	-	-	-
TSP+CAN	0	0	0	TSP	CAN	K_2SO_4
Nlim	0	0	0	TSP	-	K_2SO_4
Plim	0	0	0	-	CAN	K_2SO_4
Lrub	15	0	0	-	CAN	K_2SO_4
Acal	0	46	0	-	CAN	K_2SO_4
Alon	0	0	5	-	CAN	K_2SO_4
Multi	15	46	5	-	CAN	K_2SO_4
StruLrub	15	0	0	struvite	CAN	K_2SO_4
StruAcal	0	46	0	struvite	CAN	K_2SO_4
StruAlon	0	0	5	struvite	CAN	K_2SO_4
StruMulti	15	46	5	struvite	CAN	K_2SO_4
Struvite	0	0	0	struvite	CAN	K_2SO_4

were introduced at the rate of 15, 46 and 5 individuals per mesocosm for *L. rubellus*, *A caliginosa* and *A. longa* respectively, corresponding to the density reported per feeding guild (Frazão et al., 2017). The three species-mixture used the same earthworm density for each of the species as those as in the respective single species treatments, as these three species would inhabit different ecological niches. The earthworms were collected from a grassland in Wageningen as well as from the "earthworm hotel" (van Groenigen, 2022) and stored in a climate-controlled room (16 °C) in buckets containing a moist sandy soil similar to the one used in the field-based mesocosm experiment, which was mixed with fresh litter. Preference was given to adult individuals but sub-adults were also used to reach the desired density. Earthworms were weighed, with voided gut (filter paper method for 48 h at 16 °C (Dalby et al., 1996)), per species and mesocosm before introduction.

The mesocosm design is shown in Figure 5.1. It was composed of a 35x35x35cm PET felt bag (PLANT!T Dirt Pots, Coventry, UK) sown to a 30 cm high insect mesh. To prevent earthworms from leaving the mesocosms, a 2 cm-wide Velcro band was sown at the top, on the inside of the insect net. Each mesocosm was filled with 40 kg of the earthworm-free soil mix described above. The mesocosms were placed in the field in June 2020. The soil surrounding the mesocosms was replaced by coarse sand to reduce the possibility of earthworms invading mesocosms. A drip irrigation system was established, with the drip set at the soil surface to prevent the acceleration of the fertiliser granules dissolving due to a splash effect from the irrigation. The irrigationsystem was used during the dryer months of the summer to balance the draining and drying effect of the surrounding coarse sand. In July 2020, seeds of *Lolium perenne* diploid (8 g·m^{-2}) were sown and the grass cover was left to grow for eight weeks, after which the grass was cut 5 cm aboveground and the treatments

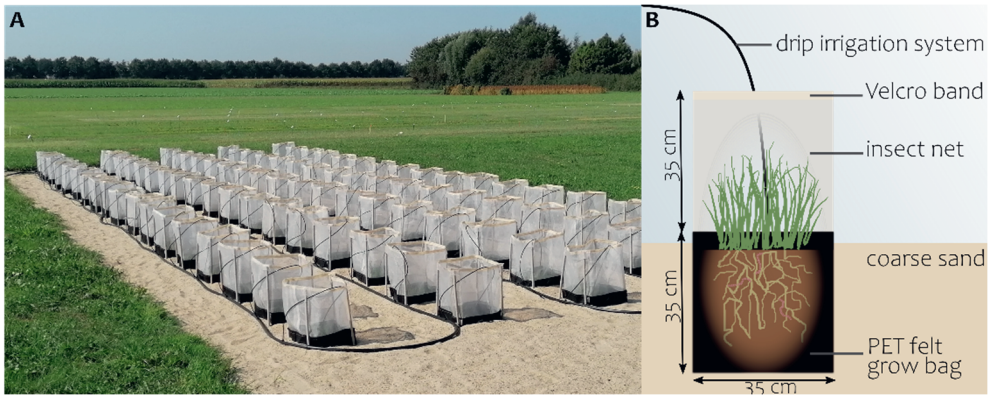

Figure 5.1 Visualisation of the field-based mesocosm experiment. A: Photograph of the experiment in May 2021. B: Schematic cross-section of a mesocosm buried in the sand bed.

were applied. Earthworms were introduced two days prior to the fertiliser application to minimise the risk of salt stress. The fertilisation was 150% of the national recommendation for N, P and K (Commissie Bemesting Grasland en Voedergewassen, 2017), and was split per harvest cycle over the two years. Details about the fertilisation rate are given in Table 5.3.

Table 5.3 Fertilisation rates used in the field-based mesocosm experiment. Fertilisers were applied in granular form at the soil surface.

Time	N (kg N·ha^{-1})	P (kg P·ha^{-1})	K (kg K·ha^{-1})
October 2020	63	40.1	19.5
April 2021	201	40.1	19.5
May 2021	160.5	40.1	19.5
July 2021	127.5	40.1	19.5
September 2021	84	40.1	19.5

The grass was harvested five times after application of the fertilisers and earthworms: in November 2020 as well as in May, July, September and October 2021. After the last harvest, the mesocosms were excavated. A soil sample of 11 cm diameter and 25 cm depth was taken in the middle of each mesocosm and washed over a 1 mm-sieve to separate the roots from the bulk of the soil. The root sample and the soil remaining in the mesocosm were hand-sorted to recover any earthworms, including mesocosms that were not inoculated with earthworms. Their guts were voided (Dalby et al., 1996) and the weight per species was subsequently measured. Each individual was also assigned to an age class (juvenile, sub-adult, or adult) depending on its morphological characteristics.

2.3 Plant Analysis

The harvested plant material from the five harvests was dried at 70 °C for 48 h. It was subsequently ground and digested using a mixture of H_2SO_4, salicylic acid, and H_2O_2 with Se (Novozamsky et al., 1983). In the digest, total N and total P were measured using a fully automated segmented flow analyzer (SFA; Skalar, SAN^{++}). Root samples of the first three blocks were carefully washed removing all soil and organic matter particles. A first subsample was weighted fresh and stained with neutral red and scanned (600 dpi, Epson V700 Photo, Suwa, Japan). The resulting images were batch analysed with Winrhizo Pro 2013e (Regent Instrument, Québec, Canada, objects smaller than 0.5 mm^2 or with a length:width ratio of less than 4 removed). The analysis was conducted on 10 diameter classes of 0.1 mm to separate finer roots from larger ones. The subsample was then dried at 70 °C for 48 h, followed by the determination of the dry weight. A second subsample was weighed fresh and

stored in 60% ethanol for subsequent AMF colonisation rate determination. The roots were stained with the ink and vinegar method (Vierheilig et al., 1998) and the level of colonisation of 100 observations across the root sample was scored from 0 to 5. The resulting colonisation intensity measurement is the weighted average of the scores according to the following formula:

$$AMF\ colonisation\ intensity = \frac{95n5\ +\ 70n4\ +\ 30n3\ +\ 5n2\ +\ n1}{100}$$

where n*i* represent the number of observation having received the *i* score (Trouvelot et al., 1986). The dry weight of this sample was estimated using the moisture content of the first subsample. The remaining bulk of the root sample was dried at 70 °C for 48 h and weighed. The calculations of SRL and RTD were performed considering the cylinder bias (Rose, 2017).

Although a classical approach to study P fertilisation would call for soil P test, these are difficult to perform with soil that potentially still contains struvite. Indeed, the struvite remaining in the soil then partially dissolves during the extraction of many soil P tests (Gu et al., 2021, Chapters 3 and 4). Hence, we decided not to perform any soil P tests in this study, as the best proxy for struvite dissolution is ultimately the plant P uptake.

2.4 Statistical Analysis

The statistical analyses were conducted in R (R Core Team, 2022). Earthworm species biomass per treatment was tested with an ANOVA and Tukey's Least Significant Difference post-hoc test when normality assumption were respected (*L. rubellus*), or with the Kruskal-Wallis test followed by Dunn's post-hoc test with the Benjamini-Hochberg procedure when the assumption of normality was violated (*A. caliginosa*, *A. longa*). Since earthworm communities differed at the end of the experiment as compared to the communities we set at the beginning, we used a modelling approach to analyse the data. The total P uptake was modelled according to the earthworm biomass and density per species, as well as the N and P fertilisers with the *MuMIn* package (Bartoń, 2023). We ranked all the possible multiple linear regression models according to their Akaike Information Criteria (AIC). Any model with an AIC value within two units of the best AIC value is considered as good as the best model. We thus selected all models with an AIC value within two units from the best AIC value and averaged them into one model, as model averaging is considered the best practice (Lukacs et al., 2010). All models selected for the averaging were given the same weight. There was no block effect and so this was not further used in the analyses.

3 Results

3.1 Earthworm Biomass and Density

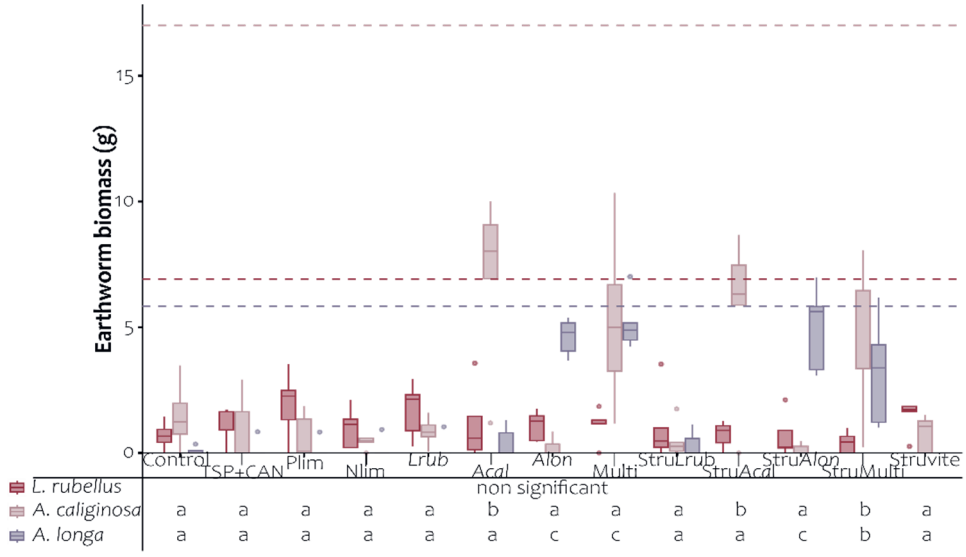

Figure 5.2 Earthworm biomass per treatment for the three species at the end of the field-based mesocosm experiment. The table shows the results of the ANOVA and Tukey test. Dashed lines correspond to the original average biomass of earthworms in treatments where they were applied. lim: limitation, *Lrub*: *L. rubellus*, *Acal*: *A. caliginosa*, *Alon*: *A. longa*, Multi: combination of the three earthworm species, Stru: struvite.

The earthworm biomass per species at the end of the field-based mesocosm experiment is shown in Figure 5.2. In the treatments they were introduced in, the biomass of earthworm species were 20, 30 and 80% of the original biomass at the end of the experiment for *L. rubellus*, *A. caliginosa* and *A. longa,* respectively. The density of earthworms followed the same pattern with 30, 50 and 90% of the original density remaining at the end of the experiment respectively for *A. caliginosa L. rubellus* and *A. longa* (Figure 5.S1). *A. longa* and *A. caliginosa* were mostly found back in the treatment were they were introduced. However, at the end of the experiment, *L. rubellus* biomass was distributed equally in all mesocosms, regardless whether it was initially introduced or not (Figure 5.2).

3.2 Plant Phosphorus Uptake

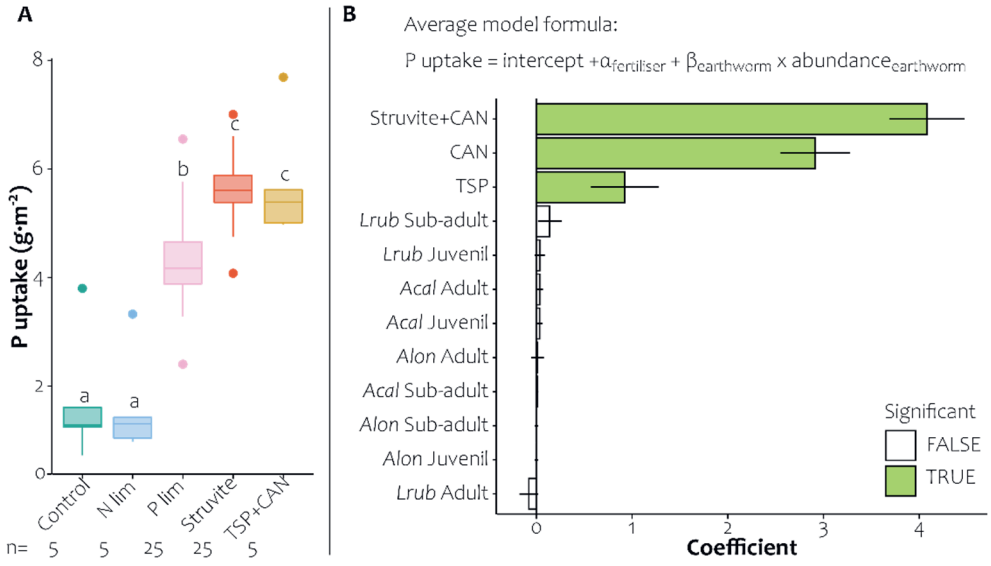

Figure 5.3 Phosphorus uptake of grass as impacted by fertilisation and earthworms density per age class. (a) Phosphorus uptake according to fertilisation regimes. (b) Coefficients of parameters included in the average model of P uptake. Significance refers to the t-test testing if coefficient are significantly different from 0. For further details, see section2.4. lim: limitation, *Lrub*: *L. rubellus*, *Acal*: *A. caliginosa*, *Alon*: *A. longa*

The plant P uptake over the course of the five harvests is shown in Figure 5.3. Struvite supplemented with CAN resulted in the same plant P uptake as the positive control combining TSP and CAN, reaching 5.6 $g \cdot m^{-2}$ on average, which was significantly higher than the P-limited treatment (Figure 5.3A). The N-limitation control had the same P uptake as the negative control, which was 30% of the uptake of the struvite and TSP+CAN treatments. The N:P ratio according to the fertilisation regime confirm that these two treatments were the most N-limited (Figure 5.S4). To assess the potential impact of earthworms on grass P uptake, we used an average model approach (see section 2.4). The average model presented in Figure 5.3B is the result of the weighted average according to the AIC value of a list of the best models. Our results highlight that fertilisers, but not earthworms, significantly contribute to the final model. Nonetheless, earthworm parameters were selected in individual models included in the average model and thus appear in that model. Thus, although none of the earthworm parameters significantly contributed to P uptake as modelled through the average model, their presence in it indicates that they do have an effect, albeit small and unsignificant in comparison to fertilisers. There was no significant

interaction between earthworm species and fertilisation treatments. Similar results were found for the aboveground grass biomass (Figure 5.S2).

3.3 AMF Colonization and Root Morphology

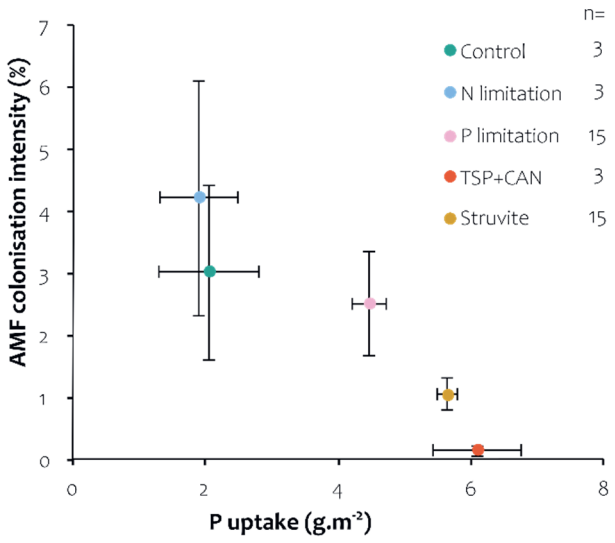

Figure 5.4 AMF colonisation intensity according to the total P uptake. Colours indicate the different fertilisation regimes.

The results of the AMF colonisation analysis are presented in Figure 5.4. Plants with adequate N and P fertilisation, *i.e.* the struvite and TSP+CAN treatments, were less colonised by AMF compared to plants that were limited by either of both elements, although this was not statistically significant. Still, the struvite treatment colonisation was 7.7 fold that of the TSP+CAN treatment, while having a similar P uptake. There was a significant negative correlation between AMF colonisation and the P uptake (Spearman rank correlation, rho= -0.367).

The "Root Economics Space" is a theoretical space drawn by the first two dimensions of a PCA build with, vertically, the RTD opposing the root N content and horizontally the SRL opposing the average root diameter of multiple plant species (Bergmann et al., 2020). Our dataset did not include the root N content so we used a variation of the RES built only with the SRL, RTD and average diameter to differentiate between ryegrass rooting strategies (Figure 5.5). Using these traits, we could identify the conservation and collaboration gradients, which together explain 98% of the variability of the three root traits included. Figure 5.5A shows the position of the various fertilisation treatments in the root economics space. While the control and

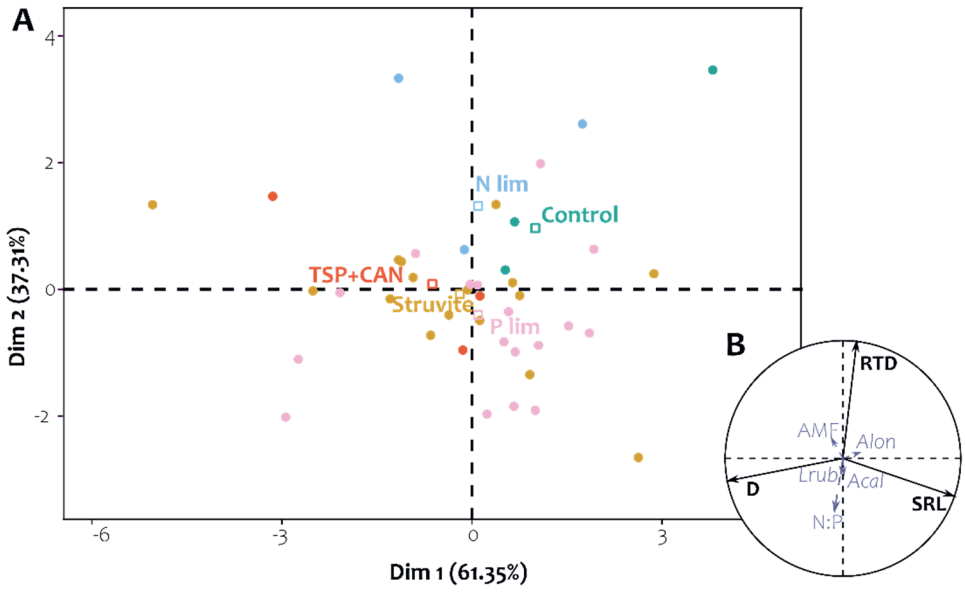

Figure 5.5 Fertilisation treatments on the root economics space. (a) Individual graph of the PCA according to their fertilisation regime. (b) Variable graph of the PCA. Active variables are in black and supplementary variables are in blue. D: average diameter, *Lrub*: *L. rubellus*, *Acal*: *A. caliginosa*, *Alon*: *A. longa*

N limitation treatments were in the "slower" part of the space, the other treatments that did receive N fertilisation were close to the middle of the conservation gradient, with the TSP+CAN, struvite and P limitation treatments ranging from slower to faster, respectively. The treatments range from TSP+CAN, struvite, N and P limitations and control along the collaboration gradient from "outsourcing" to "do-it-yourself", respectively. Figure 5.5B shows the correlation of active and passive variables with the two PCA axes. The N:P ratio in shoots was correlated with the conservation gradient towards the "fast" pole. Earthworm species biomasses' correlations to either PCA axis were negligible.

4 Discussion

4.1 Struvite Is Just as Effective a Fertiliser as TSP

We hypothesised that struvite would be just as effective a fertiliser as TSP in field conditions. This was indeed the case as there was no significant difference in P uptake between the two treatments (Figure 5.3). Field experiments with struvite tend to yield better results than with a conventional P counterpart regarding aboveground biomass but poorer regarding P uptake compared to greenhouse pot experiments (Hertzberger et al., 2020; Huygens and Saveyn, 2018). This could be

related to the slower plant growth in the field versus in the greenhouse. Although struvite dissolution is impacted by temperature, the parameter limiting its dissolution might be the surface of contact between struvite and the soil (Ariyanto et al., 2017; Bhuiyan et al., 2007; Degryse et al., 2017). Plants on the contrary, are heavily impacted by temperature and thus the environmental conditions from the greenhouse or the field will have a large impact on how fast they grow and how fast fertilizer need to be able to provide nutrients to avoid limitation (Sato and Ito, 1969).

Moreover, irrespectively of their growing conditions, grass trials tend to show a higher agronomic efficiency of struvite for biomass production, and a significantly higher P uptake than when using a conventional phosphate rock-based fertiliser (Huygens and Saveyn, 2018). Although *L. perenne* is very sensitive to P fertilisation compared to other grasses (Ros et al., 2018), it is less dependent on the timing of the P supply in the form of P fertilisation as opposed to other crops such as maize for which grains are the main production target and that require a high P uptake early on to reach optimum grain yield (Bindraban et al., 2020; van Duijnen et al., 2021). This explains why grasses usually reach a similar yield and a higher phosphorus use efficiency when fertilised with "precipitated phosphate salts" *sensu* Huygens and Saveyn, (2018) as compared to conventional fertilisers (Huygens and Saveyn, 2018). Our results thus demonstrate that slow release of P from struvite appears to be an effective fertilisation strategy for grass biomass production as it led to the same P uptake as TSP that has a high solubility. To understand the similar P uptake from these two fertilisers with different dissolution rate, we must investigate belowground.

4.2 Earthworms Only Played a Minor Role in Explaining Plant Performance

A critical point to assess any earthworm effect is the retrieval of earthworms from the mesocosms where they were inoculated (Vidal et al., 2023). The earthworm treatments we set up were not maintained as expected during the experiment (Figure 5.2). Earthworms were introduced in October, which is rather late in the season and may be quite close to their period of reduced activity for the winter (Edwards, 2004). Where *A. caliginosa* and *A. longa* seemed to have been fit enough to survive the winter, this might not have been the case for *L. rubellus*. As an epigeic species, *L. rubellus* has an ecological selection strategy of type r (Pianka, 1970), meaning the survival of the species relies on the production of a large number of individuals with short life spans. This could explain the decrease of the earthworm biomass in mesocosms where *L. rubellus* was introduced (i.e. high mortality). Because of the significant change in the distribution of *L. rubellus*, it is not appropriate to look at

results of our experiment through the lens of earthworm treatments as they are. Instead, we used the parameters describing the earthworm community at the end of the experiment to investigate possible earthworm effect on the system.

We hypothesised that earthworms would enhance the dissolution and thus the effectiveness of struvite. Although earthworms have been shown to indirectly increase the dissolution of phosphate rock pellets (Mackay et al., 1983) and the compost of their casts increases the effectiveness of struvite in greenhouse conditions (Hernández Jiménez et al., 2020), in our field-based mesocosm experiment earthworms did not appear to increase struvite dissolution. Toxicity tests have shown that *Eisenia fetida* does not avoid nor seek struvite (Rastetter et al., 2017) and unpublished observations in our group seem to confirm this for the three earthworm species used in this experiment. Mackay et al. (1983) described the interaction of earthworms with phosphate rocks via two pathways: the incorporation of pellets into the soil through bioturbation and the increased dissolution through ingestion. Although these two pathways significantly improved the availability of phosphate rocks in their pot study, the earthworm density per kg of dry soil of their incubation trial was 20 times higher. It is possible that at such an unrealistic density, earthworms might have an effect on struvite dissolution (Vidal et al., 2023), but they did not at field density, which we used in our experiment.

Although earthworms have been shown to increase P availability and plant P uptake in the greenhouse (Vos et al., 2014), in our field-based experiment their effect is small and not significantly contributing to the final average model as opposed to fertilisers (Figure 5.3). Yet, they were selected in the average model, meaning that they are important to explain plant P uptake. The lack of significance might be due to several factors. First, the co-linearity among earthworm variables: this was due to the nature of the variables included in the model (age classes) and to the presence of multiple earthworm species in several of the mesocosms. This co-occurrence was expected from the treatments where the three species were initially introduced, but was exacerbated by the invasion of *L. rubellus* and to some extent of *A. caliginosa* in many mesocosms. Reducing the number of earthworm variables would have reduced the co-linearity but also reduced the fit of the model, so we chose to keep the earthworm data detailed per species and life-stages in the model. Another factor influencing the lack of significance might have been the duration of the experiment. In a field mesocosm experiment, Vos et al (2022) did find a significant effect of *Lumbricus terrestris* and *A. longa* on the P uptake of *L. perenne* over a longer experimental period (three years and fifteen harvests). Increasing the length of the experiment might have revealed a significant earthworm effect, but it would also

have increased the risk of further homogenisation of the earthworm communities by invasion and reproduction in mesocosms that were initially earthworm-free.

To sum up, we reject our hypothesis: earthworms did not enhance struvite effectiveness in our field-based experiment. However, they did have a small (albeit not significant *stricto sensu*) effect on the plant P uptake.

4.3 Struvite's Effectiveness is Related to a Shift to a P-limited Plant Growth Strategy

We hypothesised that struvite fertilisation stimulates plants to develop different P acquisition strategies, thus affecting their root traits. Plants of the P-limitation treatment engaged more in collaboration with AMF (Figure 5.4) and had thinner, longer and lower-density roots (Figure 5.5) compared to roots of the TSP+CAN treatment. Although the P applied in the form of struvite and TSP+CAN were the same and resulted in equally similar P uptake (Figure 5.3), the plants treated with struvite had a significantly higher AMF colonisation, which was about half of that of P-limited plants but 7.7 times higher than that of the TSP+CAN treatment (Figure 5.4). Morphological root traits were also intermediate between those of the TSP+CAN treatment and the P-limitation treatment (Figure 5.5, 5.S3). The explanation for this likely resides in the properties of struvite. As opposed to TSP which has a high solubility thereby facilitating a fast released of P to the soil solution, struvite is poorly soluble leading to a lower rate of P release, which means that plants may also have to rely on other P sources such as the release of residual P residing within the soil solid phase to fully meet their P needs (Bogdan et al., 2021; Degryse et al., 2017). The soil used in our experiment had little readily available P for plants to take up (Table 5.1) as well as a relatively high P buffering capacity (Chapter 3), which probably resulted in a limiting amount of P available at time locally in the soil solution so strategies to increase both the soil exploration and the unlocking of phosphorus from the solid phase had to be used (Wen et al., 2022). Here we were able to demonstrate that struvite-treated plants relied more heavily on an enhanced soil exploration through mycorrhizal association as well as increased root length and so we accept our hypothesis. Plants may also have used some nutrient mining strategies *sensu* Lamber et al. (2008) although we did not measure it. Overall, struvite P fertilisation, by being released at a sub-optimal rate for the plants, actually promoted an increased reliance on plant-led P uptake whereas TSP+CAN did not promote the same self-reliance. Struvite, in addition to being a more sustainable fertiliser (Remy and Ruhland, 2006), leads to a more resilient grass crop against P shortages.

We used the "Root Economics Space" concept to visualise the root traits of *L. perenne* under different fertilisation regimes. This tool served the visualisation purpose well and although we could not include the root N content, the "fast" pole of the conservation gradient was still defined as intended since the N:P ratio was negatively correlated with the conservation gradient (Figure 5.5B). Omitting the root N content, however, has reduced the variability of the data included in the PCA and has likely resulted in the very high variability explained through the root economics space: 98% instead of 73.4% for the root economics space of global species (Bergmann et al., 2020). The variability of the data will have been further reduced by the inclusion of only one plant species (albeit under different fertilisation regimes) when the root economics space designed by Bergmann et al (2020) contained 748 plant species. Yet, the positions of the various fertilisation treatments on this gradient were still compelling here as they clearly ranged from fully readily available nutrients on the left to a strong nutrient limitation on the right. With the need to increase nutrient use efficiency in agrosystems, having a tool helping to visualise the root response to a given fertiliser is valuable, as plants relying more on their own nutrient acquisition strategies while still agronomically performing to the best standards is a step towards more sustainable agroecosystems.

5 Conclusions

Our field-based mesocosm experiment showed that struvite was just as good a P fertiliser as a conventional P fertiliser. Furthermore, struvite, by being intrinsically a slow-release fertiliser, triggered plant P acquisition strategies and thus resulted in more AMF colonisation and morphological root changes that are reflective of moderate P limitation without having an impact on either the yield or the P uptake of *L. perenne*. Although earthworms have been shown to impact plant P uptake in controlled conditions, their role was only minor in explaining grass P uptake in our field conditions in comparison to the major role of P fertilisers.

Acknowledgements

We are grateful to NuReSys for providing struvite for our experiment. We thank Angela Sievernich, Selma Moerland, Péter Garamszegi, and the Unifarm staff for their involvement in the execution of the field-based mesocosm experiment and laboratory analysis.

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Supplementary Information

S1 Struvite Chemical Composition

Table 5.S1 Struvite chemical composition. N total and P total were obtained through a destruction with sulfuric acid and hydrogen peroxide (Novozamsky et al., 1983). All other elements were obtained through an aqua regia destruction (Sastre et al., 2002). The EU limits are defined by regulation 2019/1009 (European Parliament and Council of the European Union, 2019)

Element	Unit	Value	EU limit
N total	$g \cdot kg^{-1}$	44	
P total	$g \cdot kg^{-1}$	122.12	
Mg	$g \cdot kg^{-1}$	103.54	
K	$g \cdot kg^{-1}$	16.41	
As	$mg \cdot kg^{-1}$	0.00	40
Cd	$mg \cdot kg^{-1}$	0.07	60
Cr	$mg \cdot kg^{-1}$	0.4	2
Cu	$mg \cdot kg^{-1}$	1.1	600
Ni	$mg \cdot kg^{-1}$	0.3	100
Pb	$mg \cdot kg^{-1}$	1.0	120
Zn	$mg \cdot kg^{-1}$	11	1500

S2 Earthworm Density

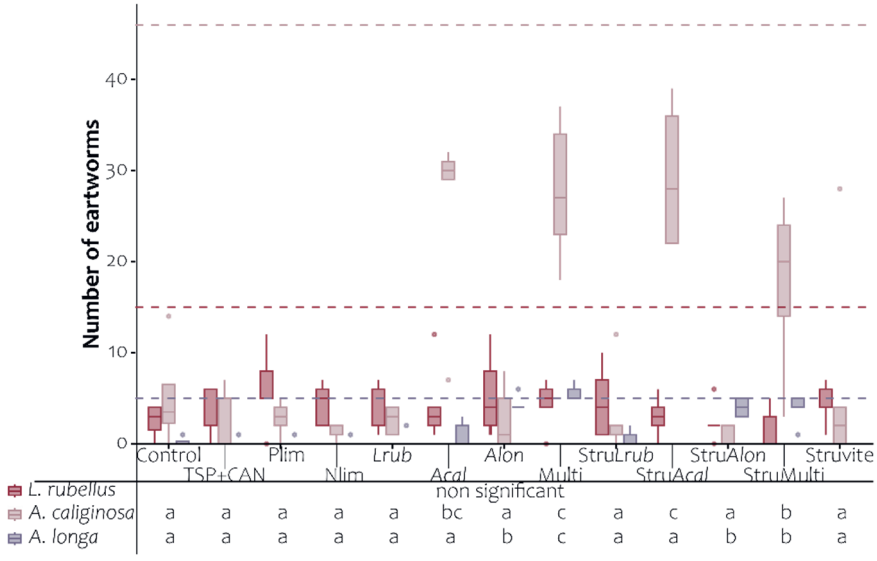

Figure 5.S1 Number of earthworms per treatment for the three species. The table shows the results of the ANOVA and Tukey test. Dashed lines correspond to the original numbers of earthworms in treatments where they were applied.

S3 Plant Biomass

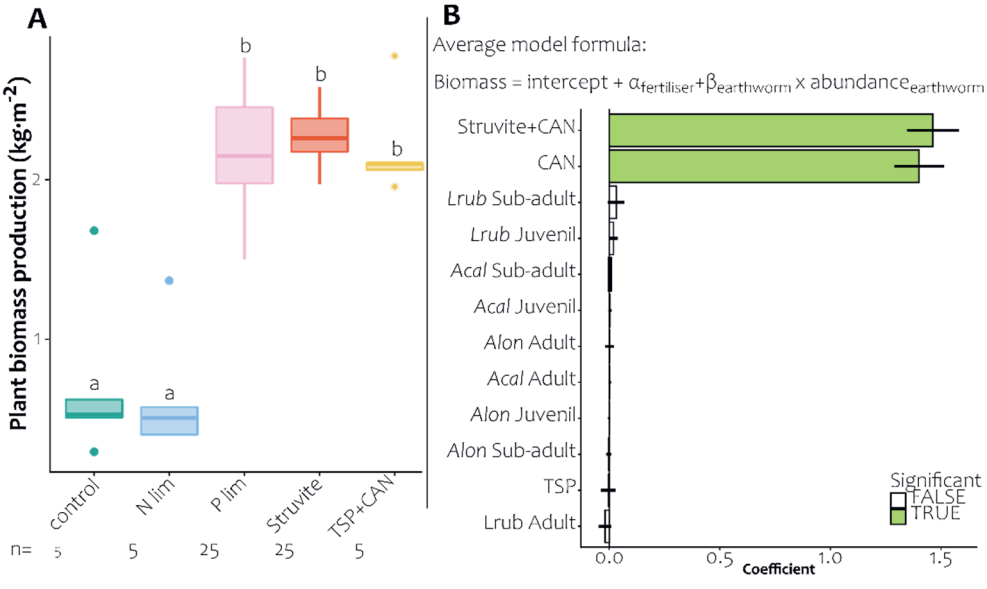

Figure 5.S2 Aboveground grass biomass as impacted by fertilisers and earthworms density per age class. (A) Aboveground grass biomass according to fertilisation. (B) Coefficients of averaged model of P uptake. lim: limitation, *Lrub*: *L. rubellus*, *Acal*: *A. caliginosa*, *Alon*: *A. longa*

S4 Root Traits

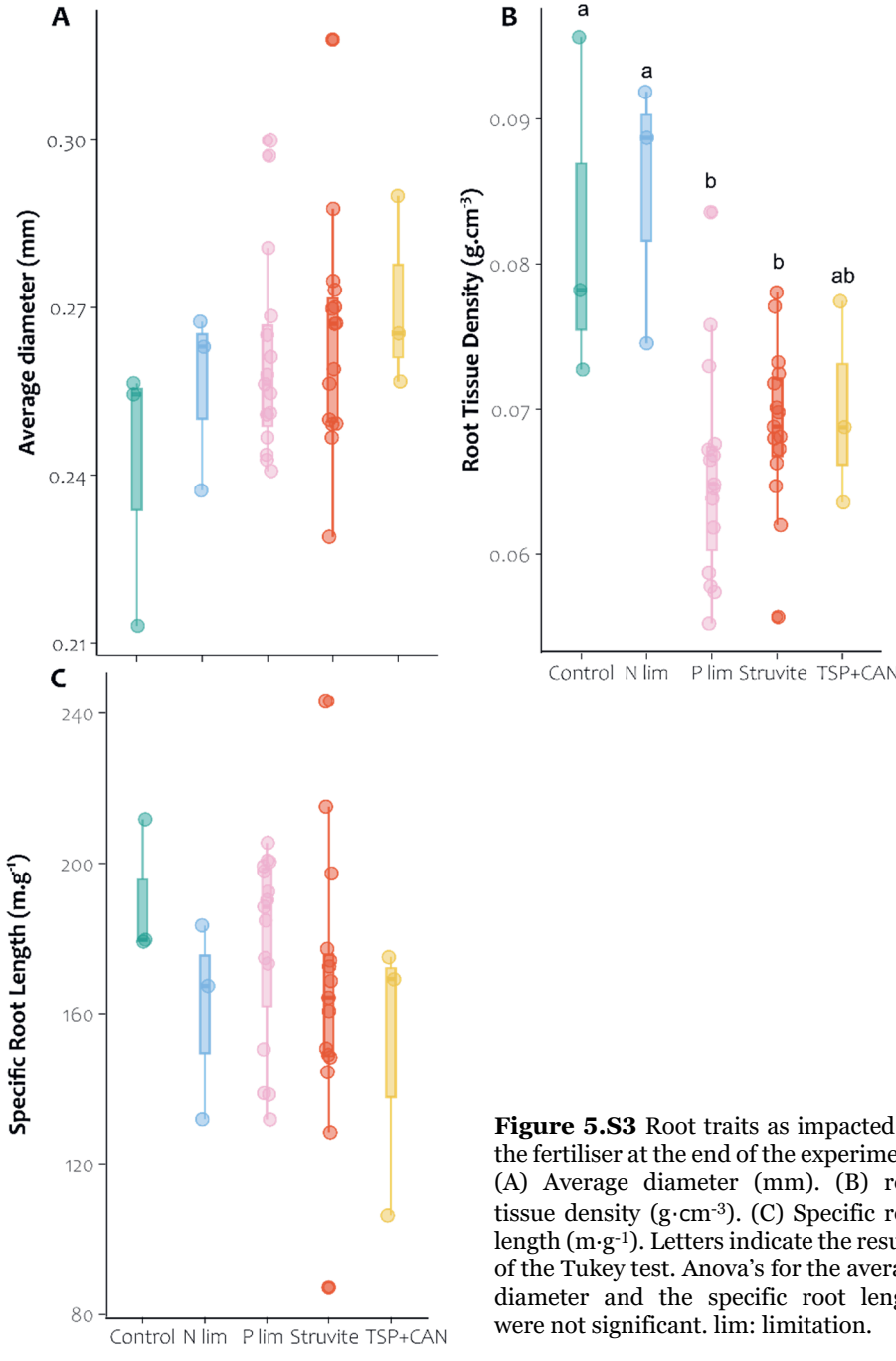

Figure 5.S3 Root traits as impacted by the fertiliser at the end of the experiment. (A) Average diameter (mm). (B) root tissue density ($g \cdot cm^{-3}$). (C) Specific root length ($m \cdot g^{-1}$). Letters indicate the results of the Tukey test. Anova's for the average diameter and the specific root length were not significant. lim: limitation.

S5 Plant N:P Ratio

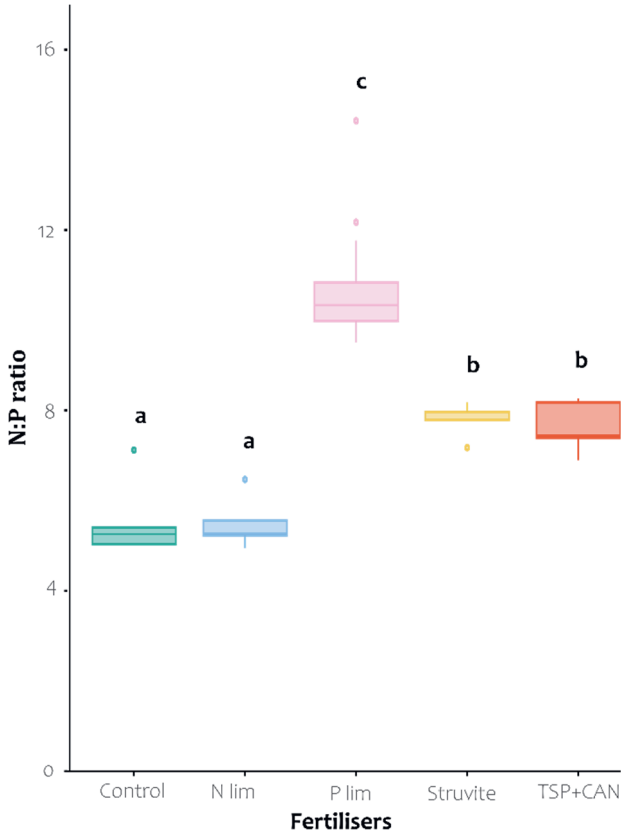

Figure 5.S4 N:P ratio of the grass over the whole experiment. Letters indicate the results of the Tukey test. lim: limitation.

Chapter 6

General Discussion

Laura M.E. Ferron

1 Introduction

Agroecosystems are in need of a redesign since they are unsustainable for both the environment and food sovereignty. The leakiness of current biogeochemical cycles of phosphorus (P) and Nitrogen (N) in agroecosystems leads to environmental issues such as eutrophication of surface waters (Correll, 1998; Werner, 2009) and emissions of nitrous oxide (N_2O), a potent greenhouse gas involved in climate change (Jones et al., 2023). The production of fertilisers itself has negative environmental impacts due to its high energy consumption (Daramola and Hatzell, 2023). Moreover, phosphate rock stock repartition causes geopolitical tensions (Drury, 2013). These issues were developed further in Chapter 1. Two solutions proposed and explored in this thesis to make agroecosystems more sustainable are (*i)* utilizing the benefits of increased biodiversity in agricultural fields and (*ii)* using circular fertilisers.

Various levers exist to increase biodiversity at the field scale of managed agroecosystems. One possibility is to increase the diversity of seeded plants (Chen and Chang, 2022); another one is to enhance soil biodiversity (Cozim-Melges et al., 2024). The first research objective of my thesis was to assess the potential of cultivated and soil biodiversity to further close the P cycle (RO1). The results related to this objective will be discussed in section 2 of this chapter.

One particularly promising circular fertiliser is struvite. Although the fertilisation properties of struvite have increasingly been studied in the last decade, its performance is still very much debated within the scientific community. Since struvite is typically considered a slow-release fertiliser, it is different from conventional mineral P fertilisers (Degryse et al., 2017; Talboys et al., 2016). To truly understand the fertilising value of struvite, we first need to understand the plant and soil responses of agroecosystems fertilised with struvite (RO2). I will discuss this in section 3 below. Once we understand these responses, we can define the agronomic conditions for successful struvite fertiliser use (RO3). This will be discussed in section 4 below. The experiments of this thesis made me encounter several methodological challenges. These are discussed in section 5, with advice on future experimental design including struvite and earthworms as well as tools for N and P limitations assessment.

2 *(RO1)* Biodiversity Can Contribute to Increasing the Phosphorus Use Efficiency of Agroecosystems

2.1 Plant Communities Under P-limited Conditions

In Chapter 2, I explored the impact of increasing the biodiversity of cultivated plant species in intensively managed grasslands to improve the overall P Use Efficiency (PUE) of these systems. Under P-limiting conditions, although the mixture of *Lolium perenne* L. and *Festuca arundinacea* Schreb. did lead to an over-yielding compared to the performance of these two species in monoculture their P uptake was not significantly different from the P uptake obtained in monocultures (Figure 2.2, Table 2.S4). When using a P fertiliser, the yield advantage of combining the two species disappeared (Figure 2.2). This experiment also contained all the other two-species combinations from the four grass species used in Chapter 2 (Annexe to Chapter 2). The other two species combinations did not always follow the same pattern as the two species combination showcased in Chapter 2 (Figure 2.A1). The combination of *L. perenne* tetraploid and *F. arundinacea* led to the most over-yielding under P-fertilised conditions, while this combination had the smallest relative yield total in P unfertilised conditions. Hence, grass species selection is crucial to sow multi-species grasslands in soils with low-P status.

The combination of four grass species (*L. perenne* diploid and tetraploid, *F. arundinacea*, and *Phleum pratense* L.) did not consistently over-yield compared to the performances of the four species in monocultures (Figure 2.2), nor did it bring a sizable advantage in terms of P uptake (Table 2.S4). This could be due to a larger overlap of the ecological niches of the four species, which is more likely to happen with increasing species richness (Tilman, 1999). Two-species combinations, when carefully selected, are more likely to associate divergent ecological niches, which would be more beneficial for agronomic performance (Tilman, 1999).

2.2 A Diversity of Nutrient Acquisition Strategies

The effect of the combination of the different niches of individual species is observable in the nutrient acquisition strategies of the different plant treatments. Through the root economics space (RES) concept (Bergmann et al., 2020), I could see how the combination of species is also a combination of different growing strategies (Figure 2.A3 and 4). Some of the rooting strategies seemed purely additive: their position on the RES reflects the average position of the species included in the mixture. For instance, this is the case for the combination of *L. perenne* diploid and *P. pratense*. For other combinations, though, the mixture is more than the addition of the individual rooting strategies: their positions on the RES deviate from the

average point. This is the case for the combination of *F. arundinacea* and *L. perenne* (either diploid or tetraploid). In both P conditions, the combination of *L. perenne* diploid and *P. pratense* did not over-yield (Figure 2.A1) and took up less P than other grass treatments (Figure 2.A2). *F. arundinacea* and *L. perenne*, however, did lead to some over-yielding (Figure 2.A1) as well as a relatively high P uptake (Figure 2.A2), although this depended on the level of ploidy from *L. perenne*. The diploid variant performed better in P-unfertilised conditions, while the tetraploid variant performed better in P-fertilised conditions. Indeed, *L. perenne* tetraploid may be less adaptable to conditions of stress in general compared to *L. perenne* diploid and this, together with its lower root biomass might explain its poorer performance in low-P conditions (Deru et al., 2014; Tozer et al., 2017). Aside from the level of ploidy, the performance of a given mixture could be explained by its competitive strength, which can be assessed by its position on the Competitor-Stress tolerator-Ruderal (CSR) plant spectrum (Grime, 1977). The position of a given species on the CSR space can be determined by leaf trait (Pierce et al., 2017). The positions of the three species used in Chapter 2 are all close to the centre of the space, in the CSR group (Wingler and Sandel, 2023). Their values regarding the competitor aspect as defined by Pierce et al. are 25.34, 19.38 and 15.69 for *F. arundinacea, P. pratense* and *L. perenne* respectively. The latter, however, might not be representative of a *L. perenne* variety that has been intensively bred for agricultural purposes and which could result in a higher competitor value as calculated by leaf traits (Loos, 1994). In that case, I would argue that the success of a mixture composition to reach maximum yield and P uptake is dependent on two conditions. The first condition is the matching of their competitor value, i.e. a fair competition aboveground. The second condition is the complementarity of their root ecological niche. As explained in Chapter 2, the root morphology and root distribution in the soil are optimum for the mixture of *L. perenne* and *F. arundinacea*. To sum up, this particular species combination experiences a high competition aboveground and a high compatibility belowground, which explains its success in P-limited conditions.

2.3 The Contribution of Soil Micro-organisms

The RES can help to visualise the rooting strategies of plants with respect to their collaboration with soil micro-organisms (Bergmann et al., 2020; Rutten and Allan, 2023; Semchenko et al., 2022; Wen et al., 2022). Although the four grasses studied in Chapter 2 did show contrasting root strategies with respect to the collaboration gradient (Figure 2.A3, dimension 2), they are all very closely genetically related and as such would position themselves close to each other on a global RES built with a wider variety of species (Bergmann et al., 2020). If the plant treatments studied in Chapter 2 are fundamentally close to each other on the global RES, then this might

explain why little difference was observed among the soil microbial parameters measured in each of the plant treatments (Table 2.3). Indeed, there was no difference in terms of microbial P biomass or phosphatase enzymes for the different grass treatments, even with increasing species richness, which is consistent with the lack of response to plant species richness of the P cycle in agricultural grasslands (Oelmann et al., 2021). This lack of response could also be linked to the P addition in some plots. Compared to agricultural grasslands where the organic carbon input is controlled by management such as organic amendments application, the organic carbon in natural grasslands is controlled by plant richness, which then impacts microbial P biomass (Oelmann et al., 2021). Possibly, such relationships played a role in my own work as in the plots without P addition, more β-glucosidase, a carbon-related enzyme, was detected in the four species treatment than in the monocultures of *L. perenne* and *F. arundinacea* (Table 2.3). Although it was not significant, the same trend was observable in the microbial P biomass. Consequently, less P fertilisation in biodiverse grasslands could result in more competition for resources, more exudation of molecules containing organic carbon, and thus a higher microbial activity leading to potentially a higher P mineralisation. This, however, should be further tested with additional plant species as well as soil diversity.

2.4 The Contribution of Earthworms

In addition to soil micro-organisms, larger soil biota can also impact the P biogeochemical cycle (Le Bayon and Milleret, 2009). In Chapter 5, I studied the impact of earthworms on plant P uptake in a field experiment in a low-P soil. *Lumbricus rubellus*, *Apporectodea longa* and *A. caliginosa* all had a small but positive impact on the plant P uptake. This had been reported in pot experiments for various earthworm species (Vos et al., 2019, 2014) and more recently in a field experiment for *A. longa* and *L. terrestris* (Vos et al., 2022b). Similar to my work, the experiments of Vos et al. (2014, 2019, 2022) were all caried out on noncalcareous sandy soils with a low soil P status for grasslands. Although the diversity and abundance of earthworms are mostly fixed by environmental constraints like climate and habitat (Phillips et al., 2019), agricultural practices such as soil tillage, crop residue management and fertilisation can have a large impact on earthworm communities (Edwards and Arancon, 2022). Increasing plant species richness, especially functional diversity, could be an agronomic way to promote earthworms in agricultural fields (Eisenhauer et al., 2009). This could be twice-beneficial for crop production as increasing plant diversity as well as promoting earthworms may lead to increased plant performance in soils with low P status and little P fertilisation (section 2.1). Other measures that have been proven to positively affect earthworm communities are the application of fertilisers, especially in organic form such as

manure, compost or slurry as well as the reduction of soil tillage and pesticide use (Cozim-Melges et al., 2024). These measures, like the increase of plant biodiversity, may have additional benefits with respect to the P cycle in agricultural systems, while others such as reduced tillage might have adverse effects (Tatewaki et al., 2023; Xomphoutheb et al., 2020). Therefore, the implementation of agricultural practices needs to be assessed according to their effects on the whole agroecosystem, rather than only on the earthworm community.

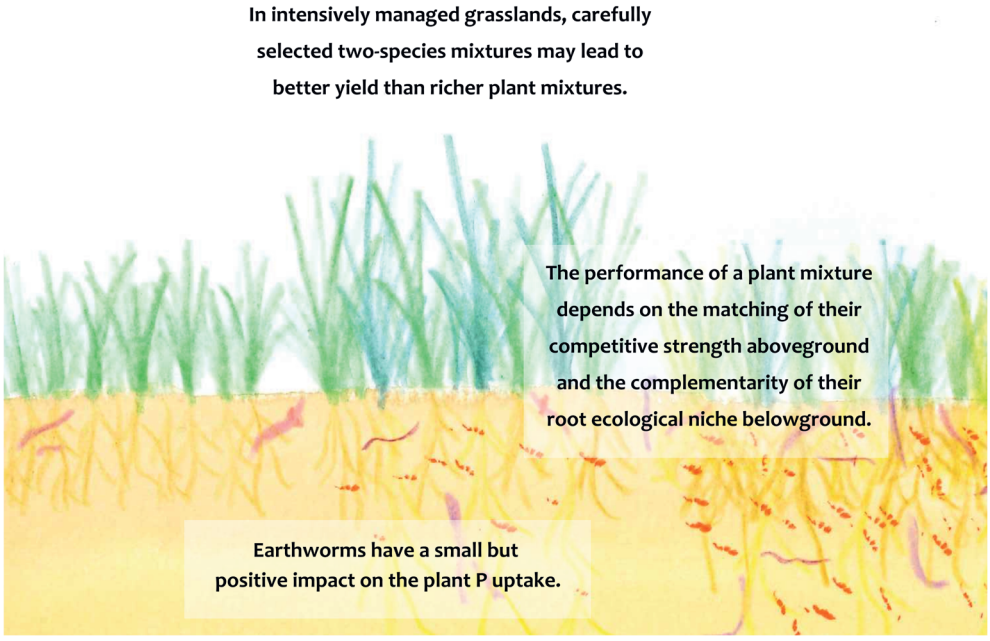

Figure 6.1 Highlights of Research Objective 1.

The first objective of my thesis was to assess the potential of plant and earthworm biodiversity to further close the P cycle (RO1, Figure 6.1). Although in Chapter 2 we did not find a significant effect of increasing grass species' richness on the plant P uptake, there was a yield benefit to using specific pairs of grass species in a soil with a low P status and likely a stronger activation of the microbial community. In Chapter 5, three different species of earthworms had a small but positive impact on plant P uptake. Under P-limiting conditions such as those occurring under struvite fertilisation, increased biodiversity therefore has the potential to improve phosphorus fertilisation use.

3 *(RO2)* Struvite Fertilisation Triggers Plant P-acquisition Strategies and Mitigates Nitrous Oxide Emissions

3.1 The P-limited Roots Strategies Following Struvite Application

The literature on struvite fertilisation performance (i.e. its capacity to increase plant yield and nutrient uptake) is very conflicting. In some cases, such as in Chapter 3, struvite fertilisation is much less effective in improving plant performance than conventional P fertilisers (Degryse et al., 2017; Nicksy et al., 2022). In some other cases, such as in Chapter 5, the performances of struvite and conventional P fertilisers were reported to be quite comparable (Bogdan et al., 2023; Uysal et al., 2014). That was the case in Chapter 5, where *L. perenne* fertilised with struvite showed the same P uptake and yield as when fertilised with TSP (Figure 5.2). Yet, when looking belowground, the roots of struvite-treated plants showed traces of P limitation as they position themselves between plants from the P limitation treatment and plants treated with TSP on the RES (Figure 5.5). A shift in root strategy has been observed before for lupine which had thinner roots with struvite fertilisation as compared to potassium phosphate (Robles-Aguilar et al., 2019). In Chapter 5, struvite-treated plants also had an AMF colonisation intensity almost 8-fold that of the plants fertilised with TSP, while having a similar P uptake (Figure 5.4). However, an experiment with tomatoes showed no difference in AMF colonisation rate between struvite and monoammonium phosphate-treated plants (Di Tomassi et al., 2021). This experiment differed in experimental length compared to the study shown in Chapter 5 (35 days in the greenhouse for Di Tomassi et al. vs 13 months in the field for Chapter 5) as well as in the way the AMF colonisation was measured (presence/absence for Di Tomassi et al. vs intensity for Chapter 5) (Di Tomassi et al., 2021). The difference in experimental conditions between the two studies might account for the different results, as might the different methods of AMF colonisation assessment. The study of Di Tomassi et al. (2021) did, however, observe a qualitative difference in the surface of struvite granules under tomato plants receptive of AMF vs. mutant plants with reduced colonisation. Together, these results suggest that plants, experiencing P limitation in soils with low-P status, might intensify their symbiosis with AMF, thereby mediating struvite dissolution.

3.2 The Roots of P Limitation

The hints of plant P limitation in the roots of struvite-treated plant as compared to plants treated with readily available P fertilisers (section 3.1) can be explained by the difference in the solubility of the mineral fertilisers. While TSP has a high solubility

and is readily available for uptake by plants, the solubility of struvite is relatively low, explaining why its dissolution in the soil is slower (Bogdan et al., 2021; Degryse et al., 2017). The difference in dissolution rate between struvite and conventional N and P fertilisers was observable in my two pot experiments in Chapters 3 and 4. In both cases, the 0.01 *M* $CaCl_2$ soil extraction method for soil samples taken from the struvite treatment at the end of the pot experiments led to an increase in phosphate ($P\text{-}PO_4$) and ammonium ($N\text{-}NH_4$) concentrations when prolonging the extraction time from 2 hours (Houba et al., 2000) up to 24 or 96 h (Chapter 3, section S3 and Chapter 4, section S4). This turned out to be the result of the dissolution of remaining struvite during the extraction period. A further detailed discussion regarding soil P tests and their use for struvite-amended soils is given in section 4.5 of this chapter.

3.3 The fruits of P Limitation

Although crop nutrient demand may not always be met due to the slow dissolution of struvite in soil, there is one benefit associated with the use of struvite as a fertiliser as it does reduce environmental risks. Both in the greenhouse and in the field, struvite did not increase N leaching compared to the unfertilised control (Leon et al., 2024; Liu et al., 2011). Phosphorus leaching is also less likely to occur as compared to conventional P fertiliser that are highly soluble (Talboys et al., 2016). In addition, since struvite dissolves slowly over time compared to conventional P fertilisers, the P it releases is more likely to be taken up by the plant before it gets bound to the soil solid phase (Talboys et al., 2016). In theory, this would result in a higher PUE for struvite compared to other conventional P fertilisers, but as struvite granules remain undissolved at the end of experiments, it is hard to measure. Indeed, the observable PUE in Chapter 3 and 4 were lower than that of diammonium phosphate and triple superphosphate combined with urea respectively (Table 3.2 and 4.S2). Furthermore, Liu et al. (2011) estimated N_2O emissions and concluded that, at a regular application rate, struvite would not lead to more N_2O emissions than unfertilized soil. In Chapter 4, I measured the N_2O emissions of pot grown with *L. perenne*. The application of struvite, even at very high rates, did not lead to more N_2O emissions than the unfertilized control on two different sandy soils of varying fertility. This was later confirmed to be true in field settings for water spinach cultivation (Wang et al., 2023).

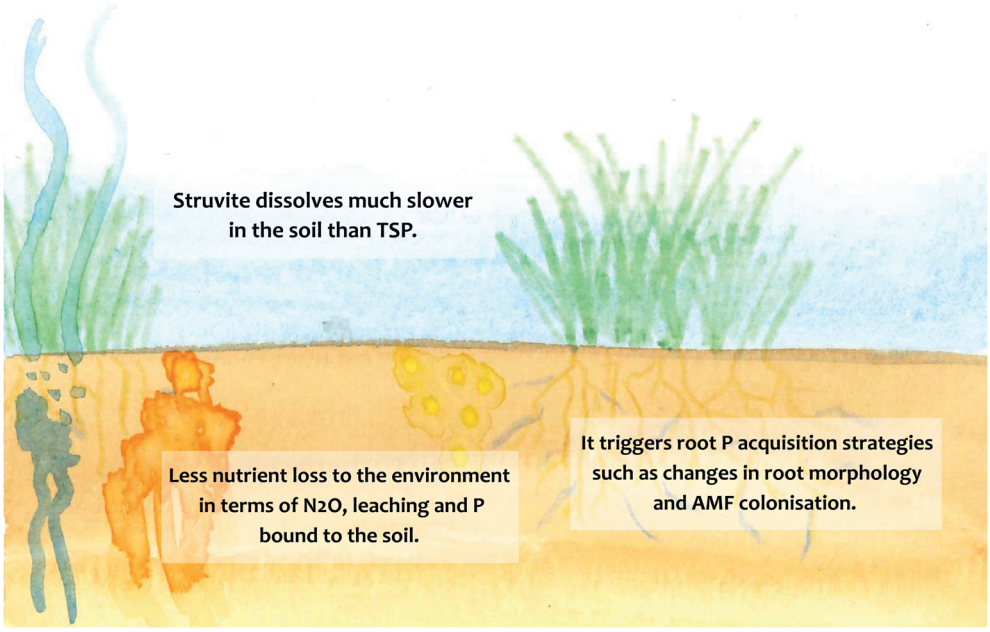

Figure 6.2 Highlights of Research Objective 2.

Struvite is a fertiliser with a slow dissolution rate. As such, it leads plants to put in place strategies that are usually seen in more nutrient-poor conditions. These strategies are sometimes enough to compensate for the slow dissolution, and sometimes not. Below, I will explore what this means for the agronomic application of struvite

4 *(RO3)* Struvite has a Place to Take in New Agroecosystems but Cannot Universally Replace Conventional Phosphorus Fertilisers

4.1 The Right Soil

In Chapters 3 and 4, I looked at the effect of struvite as a P fertiliser on plant yield and P uptake for different soils. I used two different soil types (an acidic sandy soil and a loamy soil) with both soils having a low agronomic soil P status in Chapter 3. In Chapter 4, I used two similar soil types (acidic sandy soil) but with different nutrient availability: a soil with a high soil P status and a suitable amount of readily available mineral N and a soil with a low P status and a relatively low amount of readily available mineral N. From the literature, we know that struvite dissolves better at lower pH and when the concentration of $P\text{-}PO_4$, $N\text{-}NH_4$ and Mg are

relatively low in the soil solution (Bhuiyan et al., 2007; Pérez-Piqueres et al., 2023; Talboys et al., 2016). Consequently, I would have expected struvite to dissolve to a higher extent and thus lead to a better agronomic performance in the sandy soil that had a lower pH (Chapter 3). I had the same expectation for the sandy soil with a low P status and a low mineral N content (Chapter 4). This expectation was confirmed for the soil with a low P status and low mineral N content as the nutrient use efficiencies were higher than for the soil with a high soil P status and a high mineral N content when treated with struvite granules (Figure 4.2, Table 4.S3). However, in the sandy soil used in Chapter 3, struvite led to a lower PUE than in the loamy soil (Table 3.2). When looking into the results of soil P extraction (Figures 3.3 and 3.4), it is likely that indeed more struvite had dissolved in the sandy soil as compared to the loamy soil. However, the P released by struvite dissolution then became less readily available for uptake by maize because of the higher soil P buffering capacity of the sandy soil compared to the loamy soil, which led to a faster absorption of the P released from struvite to the soil solid phase (Figure 3.4). The importance of the P buffering capacity to explain the performance of struvite as a fertiliser has been found in another study (Nongqwenga et al., 2017). Factors impacting the P buffering capacity are the soil pH, organic matter and clay content (Shirvani et al., 2005). Overall, struvite dissolution and its effectiveness are affected by pH and ion concentration of NH4, PO4 and Mg in the soil, as well as by the P buffering capacity (Bhuiyan et al., 2007; Ehlert et al., 2003; Helfenstein et al., 2018).

4.2 The Right Place?

There are several ways to apply fertiliser to a crop. Maybe the most common way is broadcast application. For species with good nutrient-scavenging abilities like grasses, this method is appropriate (Nkebiwe et al., 2016). However, some plants like maize are poor in taking up nutrients in the early growth stage and strongly benefit from placed fertilisation, for instance in a band close to a row of plants (Nkebiwe et al., 2016). Placement of fertiliser can lead to a yield benefit of up to 27% compared to broadcast application at the same rate, depending on the fertiliser; combinations of mineral N and P seem to benefit the most from this technique (Nkebiwe et al., 2016). Placed fertiliser application may also be beneficial for compacted soils or for soils with a high P buffering capacity (Meyer et al., 2023; Shierlaw and Alston, 1984). Although I did not test specifically the effect of placement on struvite fertiliser efficiency, I did use this placement technique in Chapter 3. Maize roots showed preferential growth toward the zone of struvite application (Figure 3.1B) and thus took up P from the placed struvite. Although the effect of struvite placement has not been extensively studied before, the literature tends to support this hypothesis for species that are sensitive to P placement (*i.e.*

spring wheat but not soybean) (Jama-Rodzeńska et al., 2023; Nkebiwe et al., 2016; Talboys et al., 2016). One counterargument could be the high density of struvite particles within the zone where struvite is banded as this may be detrimental to its dissolution. When particles are more closely packed in the zone where struvite is banded, the local concentrations of the ions that constitute the ion activity product of struvite will be relatively high, as they are not readily lowered by adsorption to surfaces of nearby reactive soil particles. As a consequence, the extent to which the soil solution is undersaturated with respect to struvite is less optimal than in a situation where struvite particles are mixed homogenously through a soil. (Bhuiyan et al., 2007; Talboys et al., 2016). Although the topic of struvite placement was touched upon in this thesis, more work is needed to evaluate the utilisation of a placement technique in the case of struvite.

4.3 The Right Crop

In Chapter 3, I showed that maize plants fertilised with placed struvite in the greenhouse were P deficient in comparison to the fertilisation with placed diammonium phosphate (Figures 3.1 and 3.2). However, using struvite to fertilise *L. perenne* in the field led to the same N or P supply as a conventional fertiliser (Chapter 5). This difference could be explained by conditions in the greenhouse *versus* the field, which will be further discussed in section 5.1 of this chapter. However, the plasticity of root traits with respect to P-limited growth conditions is species- and even ecotype-dependent (Niu et al., 2013). Moreover, maize and ryegrass have different nutrient requirements (Cadot et al., 2018; Duru and Ducrocq, 1996; Yu et al., 2022) and it is thus likely that they respond differently to struvite fertilisation as P is slowly released from struvite dissolution as described in section 3. Although the experimental conditions of agronomic trials in which struvite was tested as a P fertiliser are highly variable (Hertzberger et al., 2020), a meta-analysis has shown that on average cereal crops respond similarly to precipitated phosphate salts (i.e. crystallised out of liquid and liquefied waste streams in the form of phosphate salts, including but not limited to struvite) as to conventional mineral P fertilisers both in terms of yield and PUE (Huygens and Saveyn, 2018). Grasses, on the other hand, respond on average better to precipitated phosphate salts than to conventional mineral P fertilisers in terms of PUE (Huygens and Saveyn, 2018). What Huygens and Saveyn (2018) ascertain is that the response to struvite does vary per crop. Several factors are likely to have an impact on this. First, the time window during which a critical amount of P must be taken up by a crop to ensure a good crop growth differs. For instance, the yield of grain is determined by the P uptake in the early plant growth stage (El Mazlouzi et al., 2022). For winter wheat, this critical P uptake window is a rather longer window than for maize. Crops grown for their vegetative

parts such as leafy vegetables and grass do not have such a critical P window and can recover better from a P deficiency (Dietz, 1989; Oyarzabal and Oesterheld, 2009). Hence, these latter crops may be more successfully fertilised with struvite. Second, the P limitation alleviation strategy favoured by a plant (either mining or scavenging *sensu* Lambers et al. (2008) can help predict how well a species or even a genotype will respond to struvite fertilisation. As struvite dissolution is, among others, affected by pH (Bhuiyan et al., 2007), plants using mining strategies *sensu* Lambers et al. (2008), such as root exudation of low molecular weight organic acids which is often paired with a pH decrease, will be able to take up more nutrients from struvite (Talboys et al., 2016; Tuason and Arocena, 2009; Zhu et al., 2021). Scavenging plants, on the other hand, will have to rely more on P adsorbed to reactive soil particles that buffers the P concentration in soil solution (Lambers et al., 2008). Although this theory remains to be tested in a trial with multiple plant species of varying nutrient acquisition strategies, struvite might benefit the most plants that are not dependent on a timely P uptake and rely on mining strategies while facing nutrient limitations.

4.4 The Right Form

In Chapter 4, I applied struvite either as granules or as a powder. Struvite powder has been linked to a faster release of P in soil P diffusion tests (Degryse et al., 2017) and this is also what I observed in Chapter 4. Indeed, ryegrass treated with struvite powder took up more N and P than ryegrass treated with struvite granules, regardless of the soil P status (Figure 4.1). As discussed in sections 4.2 and 4.3 of this chapter, it is likely that my findings can be extended to grasses which generally respond well to struvite fertilisation and are not impacted by placement (Nkebiwe et al., 2016). A crop such as maize or wheat may perform better with struvite granules than with struvite powder as granular struvite could still be seen as a form of placement, although the density of struvite per volume of soil is much lower in the case of broadcasting as compared to actual fertiliser placement (Nkebiwe et al., 2016). Efforts have been made to produce pellets from pulverized struvite and a starch plasticiser, combining the practical advantage of pellets, which are easier to apply in practice for a farmer, with the agronomical advantages of struvite powder, but this resulted in a slower struvite dissolution than pure struvite in small granules (1 mm) (Valle et al., 2022a). It seems unlikely that farmers will be willing to apply struvite in another form than granules or pellets. Therefore, while in past agronomic studies, ground struvite was often used, scientists interested in the practical use of struvite should focus more on studying granular struvite in the future (Hertzberger et al., 2020).

4.5 The Right Soil Test?

In Chapter 3, I identified shortcomings in wet chemical extraction methods like 0.01 *M* $CaCl_2$ (Houba et al., 2000), a mixture of acetic acid and ammonium lactate (P-AL) (Egnér et al., 1960) and 0.2 *M* acid ammonium oxalate (Schwertmann, 1964), which are typically used to determine the size of various soil P pools (van Doorn et al., 2023), when they were applied to soils in which still struvite remnants were present. In Chapter 4, the 0.01 M $CaCl_2$ extraction method had the same shortcoming for N-NH_4 if struvite was present in soil. Gu at al. (2021) reported the shortcomings of five additional soil P test methods (Mehlich-3, Bray-1, Olsen, H3A-2 and resin methods). During a soil extraction, struvite remaining in soil either partly or fully dissolves, depending upon the extractant (i.e., water and dilute salt solutions versus acid solutions like the one used for P-AL (pH = 3.75) and 0.2 *M* acid ammonium oxalate (pH= 3.0)) and shaking time. For 0.01 M $CaCl_2$, leading to high values that represent neither the actual amount of P directly available for plants to take up, nor the potential amount of P available if all struvite would dissolve as extractions usually last two hours or less (Egnér et al., 1960; Gu et al., 2021; Houba et al., 2000; Schwertmann, 1964) while struvite has been shown to dissolves for at least four days in 0.01 *M* $CaCl_2$ (Figure 4.S1). When Dutch farmers broadly adopt struvite as a fertiliser, a suitable soil P test will need to be found to avoid putting them into delicate positions regarding the P status of their soil with subsequent consequences for the amount of P fertiliser they are allowed to apply in the future. Indeed, the amount of P that a farmer is allowed to apply on arable land and grassland depends upon the combination of 0.01 *M* $CaCl_2$-extractable P and P-AL (Commissie Bemesting Grasland en Voedergewassen, 2017). This struvite dissolution phenomenon will likely happen with any extraction-based soil P test (Bhuiyan et al., 2007). Although the method is not yet broadly adopted to measure soil P as its calibration is ongoing, near-infrared spectroscopy does not involve an extraction and might thus avoid the struvite dissolution problem (Maleki et al., 2006; Niederberger et al., 2015; Reijneveld et al., 2022; Sidorczuk et al., 2020). The method has been shown to reliably identify “labile”, “moderate labile” and “stable” P pools from the Hedley fractionation (Niederberger et al., 2015). Near-infrared spectroscopy has the potential to detect both the P from various pools in the soil as well as struvite. As such, it may be a promising way to evaluate the soil P status of soil containing struvite.

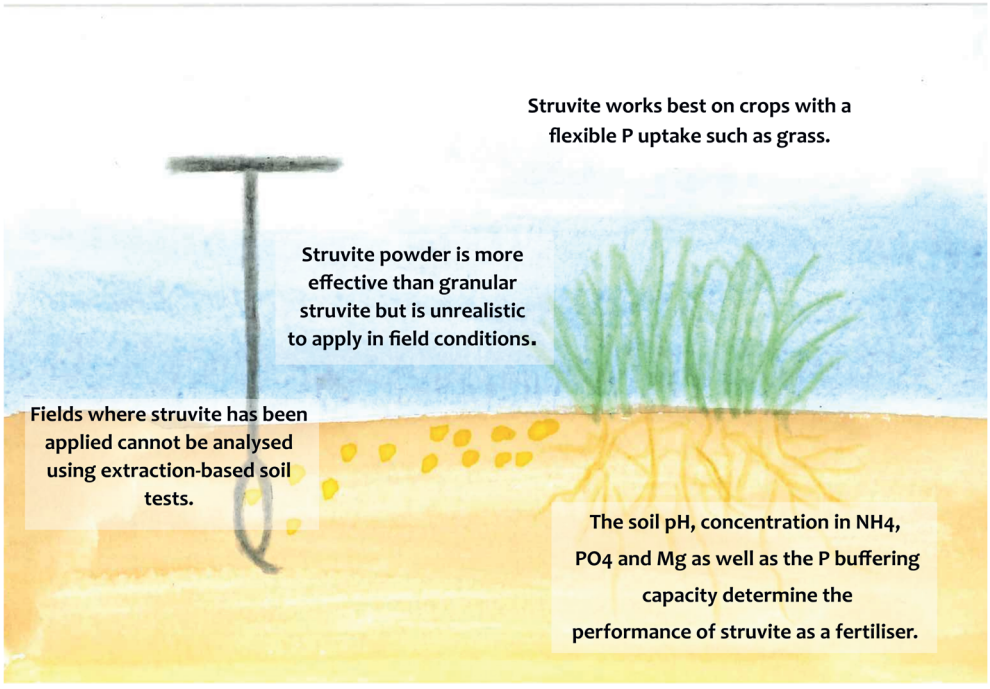

Figure 6.3 Highlights of Research Objective 3.

5 Methodological Considerations

5.1 A Greenhouse Bias?

Hertzberger et al. (2020) reviewed the performance of struvite as a fertiliser with respect to plant yield, P content and P uptake in a meta-analysis. They compiled 49 publications dating from 1962 until 2019, distinguishing between greenhouse and field experiments with struvite. My short systematic review of the literature using the same search terms and criteria in October 2023 led to the addition of another 36 publications. The combined results are presented in Figure 6.4. Most studies were carried out in pot experiments in the greenhouse. The first field experiment was only realised in 2009 and over the last 60 years, only 17% of agronomic struvite trials have been realised in the field versus 83% in the greenhouse. Although the proportion of field experiments has increased since 2020, almost half of the new field studies were performed in one location i.e., Arkansas, USA (Omidire et al., 2023, 2022b, 2022a; Omidire and Brye, 2022).

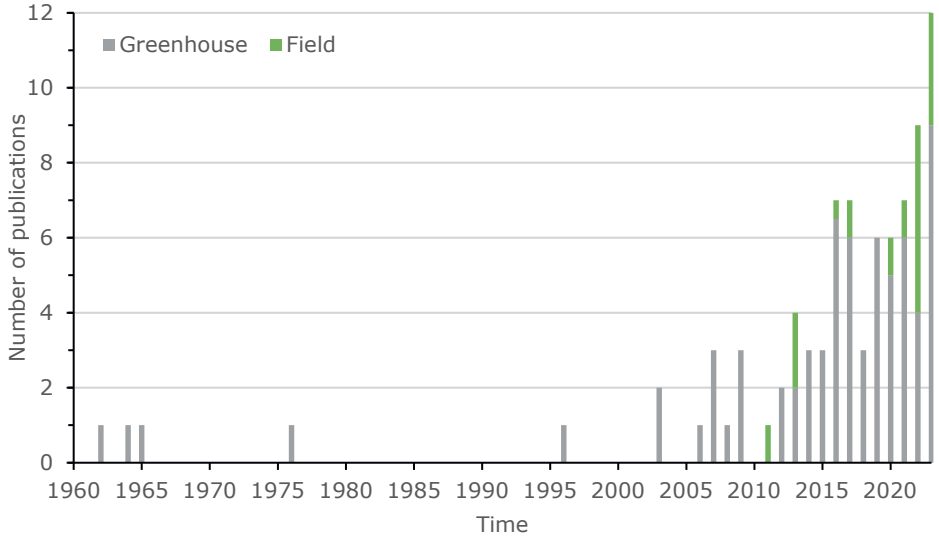

Figure 6.4 Number of agronomic publications assessing struvite either in the greenhouse or in the field. The data from 1960 until 2019 were collected from Hertzberger et al (2020) and hereafter the data answering to the same criteria were collected from Web of Science on the 25th of October 2023 by myself.

Studying struvite in the greenhouse may obfuscate the understanding of its performance in the field. Temperature plays a role in both struvite dissolution and plant growth. The fertiliser's dissolution rate is maximal at 35 °C, which is closer to the soil temperature in the greenhouse than in the field (Bhuiyan et al., 2007; Hogg and Moore, 1974; Jacobs et al., 2011; Jayasundara et al., 2017) Additionally, the dissolution of struvite is strongly impacted by the surface of contact between the soil and the fertiliser granules (Ariyanto et al., 2017; Degryse et al., 2017). From this knowledge, I would expect that although it occurs at different temperatures, the dissolution of struvite in a pot experiment in the greenhouse would not necessarily be different from the field as the surface of contact, would be the same. For plants, however, the optimal temperature for growth is important and varies per species (Went, 1953). The optimal temperature of common temperate crops is close to 20°C, with the optimal temperature for wheat and perennial ryegrass growth being between 17 and 23 °C (Porter and Gawith, 1999; Sato and Ito, 1969) and between 20 and 22 °C for maize (Waqas et al., 2021). In greenhouse conditions, plants are more likely to be at their optimal temperature, whereas in the field temperature would often be limiting in temperate regions. Moreover, greenhouse experiment are watered on a regular basis and growing conditions are generally more optimal for the plant because of added lighting and pest control for instance. Plants would

develop better and faster in the greenhouse than in the field and thus it is likely that the ability of struvite to meet the crop's nutrient demand over time will be more challenged in the greenhouse than in the field. In a meta-analysis, plants treated with precipitated phosphate salts in the greenhouse had on average a lower yield compared to plants treated with conventional mineral P fertilisers, while it was slightly above 1 for plants in field conditions (Huygens and Saveyn, 2018). Although this is just a trend, it underlines the need for further research the dissolution of struvite in the soil under various temperature conditions, as opposed to doing it in batch experiments in conditions replicating soil solution (Ariyanto et al., 2017; Bhuiyan et al., 2007). Additionally, since struvite has been approved for use as a fertiliser in Europe (European Parliament and Council of the European Union, 2019), also in organic farming (European Union Commission, 2023), the implementation of on-farm trials could lead to both a more realistic understanding of struvite's agronomic performances away from the potential greenhouse bias and an earlier adoption of the fertilisers by farmers.

5.2 Measuring Nutrient Limitation

Ecologists have strived to define ratios of chemical elements in the environment as a way to measure nutrient limitation or excess. In aquatic ecology, the Redfield ratio determines the ideal nutrient composition of plankton (Falkowski and Davis, 2004; Tyrrell, 2001). In the soil, microbes are defined by an equivalent C:N:P ratio (Cleveland and Liptzin, 2007). In the same line of thought, Koerselman and Meuleman (1996) tried to establish the critical N:P ratio of terrestrial plants communities to detect the nature of nutrient limitation. The N:P ratio and its thresholds indicating either N or P limitation were introduced as a tool to detect nutrient limitation at the plant community level in wetland ecosystems (Koerselman and Meuleman, 1996). The thresholds of N and P limitation were determined by three out of the 40 wetland plots for which there was a co-limitation of N and P. Two out of the 13 plots for which the study found a P limitation had a N:P ratio below 16 and two out of the 20 plots which were N limited had a N:P ratio above 16. The authors noted a difference in the ratios of different plant species at the same site, ranging for instance in site one from 8 for *Mentha aquatica* to 28 for *Phragmites australis*. From the data collected by Koerselman and Meuleman (1996), it is clear that the thresholds of 14 and 16 are not clearcuts and should be used with caution in the prescribed way: at the community level, in herbaceous freshwater wetlands, but not for single plant species. Moreover, although the concept of the N:P ratio is still applicable, the optimum ratio of other systems than wetlands may be lower (Tessier and Raynal, 2003). Yet, the tool of the N:P ratio and the thresholds of 14 and 16 are widely used (1507 published articles have referred to the original publication of

Koerselman and Meuleman (1996) as of 06/02/2024), including in agricultural soils, dryland systems, sometimes for single plant species (Baral et al., 2014; Ros et al., 2018). I also used the N:P ratio as a quick way to check for the nature of nutrient limitation in (Chapters 2, 3, 4 and 5). However, the optimal N:P ratio depends on the plant type, its age and even the region of the world it was grown in, with the natural fauna having evolved under N or P limitation (Güsewell, 2004). When conditions were not N-limiting, Ziadi et al (2007) found an N:P ratio close to 7 in a multi-site and multi-year study for maize, which would indicate N-limited growth conditions according to the threshold of 14 from Koerselman and Meuleman (1996). In a study in which crops were grouped (cereals, grain legumes and oilseeds), the optimum N:P ratio varies widely, from 2 to 20 for oilseeds for instance (Sadras, 2006). Despite these findings showing clear and sever limitations to the concept of N:P ratios, they are still routinely being used.

Other tools to detect either N or P limitations are the Nutrition Indexes (NNI and PNI) (Duru and Ducrocq, 1996; Lemaire and Gastal, 1997). I also used those in Chapter 2 of this thesis. The NNI is calculated from the plant's nitrogen content as well as the dry biomass and the PNI takes into consideration the P and N content of the plant. Other parameters are estimated per species. In theory, an index below 0.8 reflects nutrient limitation and a value above 1 indicates no limitation. However, in practice these thresholds may change per species as well (Gagnon et al., 2020). Moreover, the application of the method on a multispecies system is hazardous (Jouany et al., 2004).

On the one hand, therefore, the N:P ratio method is simplistic and ill-adapted to agricultural systems. On the other hand, the NNI and PNI methods are too complex, as they require different parameters per method and are poorly adapted to ecosystem assessment. A simple way forward would be the new definition of N:P ratio thresholds for common agricultural systems. The new thresholds would need to take into consideration the growth stage of the plant and the organ tested. For instance for maize, the N to P content of grain is very different from that of leaves. Leaves themselves might have a different optimum depending on the vegetative or reproductive stage (Bender et al., 2013). The thresholds should also adapt to multi-species systems such as grasslands. This work could be carried out using a combination of the vast amount of data that is now available open access and further measurement of N and P content on existing crops. This way, it will more thoroughly encompass the diversity of pedoclimatic conditions a plant might encounter. It could result in an open-access database referencing thresholds and their supporting literature.

5.3 Controlled Earthworm Field Experiments

In Chapter 5, I showed that earthworms had an impact on the P biogeochemical cycle in field conditions. In a meta-analysis evaluating the role of earthworms on plant growth (van Groenigen et al., 2014), 76% of the observations were collected from disturbed soil, most often from pot experiments in the greenhouse. The reason for this is the difficulty to control earthworm populations in the field, which comes with two different challenges. First, controlled experiments to assess the effect of earthworms traditionally require a control treatment without earthworms. While we have techniques to remove some (elektroshoking) (Bohlen et al., 1995) or all earthworms (gamma irradiation) (Nakamori et al., 2009), maintaining an earthworm-free treatment over time without further intervention can be challenging as seen in Chapter 5 and other studies (Frazão et al., 2019; Vos et al., 2022b). Second, the desired earthworm community needs to be maintained for the duration of the experiment in earthworm-treated plots. A solution used by several authors to maintain the desired community is to add earthworms at regular intervals (Bohlen et al., 1995; Vos et al., 2022b). However doing this too frequently may be a disguised way of introducing an imbalance in the nutrient inputs among the treatments as earthworms themselves contain nutrients that might be studied, which could be released to the soil in the event of earthworm dying (Sonntag et al., 2023; Vidal et al., 2023). One way around this would be to include dead earthworms in an additional control treatment. To keep treatments as intended, various barrier measures can be employed. In Chapter 5, I used a combination of methods. The mesocosms were made out of PET felt bags and insect mesh, with a band of Velcro (hook side) at the top of the mesocosm. The whole setup was installed into an environment of pure coarse sand to make the system inhospitable for earthworms so as to avoid individuals from the surrounding grassland to enter the mesocosms. Although these measures were mostly effective for endogeic and anecic earthworms, epigeic earthworms invaded all plots, whereas their numbers in treatments they were introduced to dropped drastically (Figure 5.2). Epigeic earthworms have an *r* strategy, meaning that they base their species' success on quantity rather than quality. They will produce a high number of offspring but also have a relatively short life span compared to other earthworms (Pianka, 1970). This makes them likely more challenging to maintain in controlled field experiments.

These considerations regarding the control of earthworms in field experiments originate from the traditional statistical approach, testing the effect of a parameter by adding or omitting it in a series of treatments repeated a number of times. This

type of experimental design will typically be analysed through so-called frequentist statistics such as ANOVAs. However, a different approach to statistics also exists: Bayesian statistics (Ellison, 1996). This approach relies not on fixed parameters but on random ones. For instance, in the case of an earthworm experiment, there would be no need to maintain strict treatments but rather ensure the diversity of earthworm communities among the experiment. Using the same technique of introduction and removal used in traditional experiments (Bohlen et al., 1995), this could easily be achieved and might result in a more straightforward hypothesis testing than planning a traditional frequentist experiment and having to change the initial statistical approach because earthworms have been crawling into the wrong plots (Chapter 5) (Vos et al., 2022b).

6 Outlook

Throughout this thesis, I have gained a better understanding of the functioning of struvite and its agronomic performance as a fertiliser. I showed that it comes with inherent slow-release properties, which the plant may or may not be able to overcome. For this reason, it is important to use struvite in the right context, which I tried to narrow down in this chapter with respect to optimal soil, crop, and granulation of the fertiliser. Some questions remain, especially concerning the placement of struvite and the agronomic soil P test to use after its utilisation. The inherent slow nutrient release of struvite results in important environmental benefits such as a drastic reduction of nitrous oxide emissions. The performance of a plant fertilised with struvite resides in its own biological abilities to overcome nutrient limitation, which could be helped further by increasing cultivated biodiversity and taking in consideration soil biota in agroecosystems.

This thesis aimed at testing options to increase the sustainability of agricultural systems with respect to the P cycle using biodiversity and circular fertilisers. I chose to study increased plant and earthworm diversity in grasslands as a way to use biodiversity and struvite as a circular, recycled P fertiliser. However, there are many other ways to increase biodiversity in agroecosystems: a few examples are cover crops (Hallama et al., 2019), strip cropping (Li, 2020) and agroforestry (Kuyah et al., 2019). Although struvite is a very promising circular P fertiliser, it is not the only one: attention should also be paid to other fertilisers that might be just as promising such as vivianite or hazenite (Huygens and Saveyn, 2018; Watson et al., 2020; Yang et al., 2022). Only with a diversity of resources, biota and approaches can we solve the sustainability crisis.

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Summaries

Summary

Agricultural intensified use of phosphorus (P) and nitrogen (N) fertilisers has led to unprecedented yield but also to unprecedented nutrient losses to the environment. The leaching of N and P from the field induces eutrophication of surface water and emissions of nitrogenous compounds are linked to N deposition in natural areas and soil acidification, as well as global warming. The large-scale use of P fertilizer, which is produced from phosphate rock reserves that are distributed very irregularly across the world, also causes geopolitical. Given these issues, it is clear that future agricultural systems need to be redesigned so that they (i) reduce the current leakiness of the P and N cycles; and (ii) reduce our dependency on phosphate rocks. In this thesis, I address two potential steps in this direction: (i) using targeted increases of plant and earthworm biodiversity to further close the P cycle in the field; and (ii) replacing conventional mineral P fertilisers with struvite, a circular fertiliser recycled from wastewater. My three research objectives were (RO1) to assess the potential of plant and earthworm biodiversity to further increase the Phosphorus Use Efficiency (PUE) in intensively managed grasslands; (RO2) to understand the plant and soil responses of agroecosystems fertilised with struvite; and (RO3) to determine the agronomic conditions for its successful use as a fertiliser.

In Chapter 2, I explore the potential of targeted increases in cultivated plant diversity to make better use of P in intensively managed grasslands. I carried out a field experiment in a sandy soil with a low P status. The experimental grass species diversity ranged from one to four species. Plant performance was measured during nine grass harvests over two years and root traits as well as microbial parameters were analysed at the end of the second growing season. In plots not fertilized with P, the combination of *Lolium perenne* L. and *Festuca arundinacea* Schreb. led to an over-yielding compared to the monocultures of these two species. This was related to a complementarity of root traits and strategies, resulting in a better utilisation of the soil P. The four-species combination did not yield higher results in terms of either biomass or P uptake. It did, however, show an increase in carboxylase activity compared to the monoculture of *L. perenne,* which suggests a higher turnover of organic carbon with increased species richness. I conclude that judiciously chosen pairs of grasses may help to maintain yield in grasslands with a low soil P status.

In Chapter 3, I studied the impact of placed struvite fertilisation close to the root zone of maize in comparison to placed conventional di-ammonium phosphate (DAP)

for two contrasting soil types with a low P status in a pot experiment. I measured the yield and P uptake of silage maize, as well as its root proliferation inside and outside of the fertiliser zone. Soil P-$CaCl_2$, -ammonium lactate and -ammonium oxalate tests were performed on soils from both the fertiliser zone and from the bulk soil. In both soils, placed struvite fertilisation led to lower maize performance in terms of yield and P uptake compared to placed DAP but significantly higher than the P unfertilised control. The results of the soil P tests performed on soil treated with struvite were surprising as they showed high soil P availability despite poor maize plant performance. This was explained by the dissolution of struvite during the P tests extraction, as confirmed by additional batch experiments. I concluded that soil P tests routinely used in the Netherlands were unsuitable for soil containing struvite.

Chapter 4 addresses N dynamics of struvite, in particular soil nitrous oxide (N_2O) emissions after struvite application. I compared its performance to that of urea, the most commonly used mineral N fertiliser globally. I performed a pot experiment where I applied fertilisation to ryegrass grown on two sandy soils of contrasting P status. Emissions of N_2O were measured during two growing cycles alongside soil and plant parameters. For the soil with low P status, emissions following any fertiliser application were very low and not significantly different from the unfertilised control. For the soil with high P status, however, struvite significantly mitigated N_2O emission: they were not different than the unfertilised control while emissions following urea application were on average five times higher. Powdering struvite led to an improvement in plant fertilisation without impacting the N_2O emissions. I concluded that struvite is a promising fertiliser to help mitigate N_2O emissions from agriculture.

In Chapter 5, I combined both approaches I explored in this thesis, testing the combined impact of earthworms and struvite on the performance of ryegrass in the field. The yield and P content of each of the five harvests over 13 months was measured to calculate the total P uptake. At the end of the experiment, the earthworm community of each mesocosm was retrieved and root traits were analysed. In the field, struvite proved to be just as effective a fertiliser as triple superphosphate with respect to grass yield and P uptake. The efficacy of struvite was not further helped by earthworms. However, all three earthworm species included in the experiment had small positive impacts on the grass yield and P uptake. Interestingly, although the shoots of ryegrass were identical under struvite and TSP fertilisation, the roots of plants treated with struvite showed signs of strategies seen

in roots of the P-limited treatment with respect to root morphology and AMF colonisation intensity. I conclude that struvite, which dissolves slowly in the soil, triggers root strategies that compensate for the slow provision of struvite P by making better use of the soil P.

In Chapter 6, I bring the results together and discuss them in relation to my three overall research objectives. I conclude that increased biodiversity can indeed help to further close the agricultural P cycle at the field scale, although this was clearer for grass species diversity than for earthworm diversity (RO1). The impact of plant and soil biota is especially important in conditions where P is not readily available such as those encountered with struvite fertilisation. Struvite, a slow-release fertiliser, triggers plant nutrient acquisition strategies. Overall, this could lead to less nutrients escaping from the agricultural systems to the environment, especially so in terms of N_2O (RO2). Because of its slow dissolution, struvite will not be efficient in all conditions, but in slightly acidic soils with a low P buffering capacity, it is likely to be an efficient fertiliser for plants that are flexible in the timing of their P uptake (RO3). In Chapter 6 I also address some methodological challenges I encountered during this thesis. These include a potential bias of studying struvite in the greenhouse compared to the field; the tools to assess N and P limitations in plants; as well as the statistical design of earthworm-related field experiments.

Samenvatting

Het intensievere gebruik van fosfor- (P) en stikstofhoudende (N) meststoffen in de landbouw heeft geleid tot zowel hogere opbrengsten als hogere nutriëntenverliezen naar het milieu. De uitspoeling van N en P uit landbouwgronden kan eutrofiëring van het oppervlaktewater veroorzaken. De atmosferische emissies van stikstofhoudende verbindingen kan daarnaast leiden N-depositie in natuurgebieden, met als gevolg verrijking van natuur van stikstof en bodemverzuring, evenals de opwarming van de aarde. Het intensieve en grootschalige gebruik van P-kunstmest, geproduceerd uit fosfaatreserves die zeer onregelmatig over de wereld verspreid zijn, zorgt voor geopolitieke problemen. Vanwege deze problemen dienen toekomstige landbouwsystemen opnieuw te worden ontworpen, zodat (i) de huidige lekkage van P en N uit de P- en N-kringlopen wordt verminderd en (ii) onze afhankelijkheid van fosfaatertsen voor de productie van P-kunstmest wordt verminderd. In dit proefschrift behandel ik twee mogelijke stappen in om dit te bewerkstelligen: (i) het doelgericht vergroten van de biodiversiteit van planten en regenwormen om de P-kringloop in landbouwgronden verder te sluiten; en (ii) het vervangen van conventionele minerale P-meststoffen door struviet, een circulaire meststof die wordt gerecycled uit afvalwater. Mijn drie onderzoeksdoelstellingen waren (RO1) het beoordelen van de potentie van de diversiteit in plantensoorten en regenwormensoorten om de fosforgebruiksefficiëntie (PUE) in intensief beheerde graslanden verder te verhogen; (RO2) het begrijpen van de plant- en bodemreacties van agro-ecosystemen bemest met struviet; en (RO3) om de agronomische omstandigheden voor een succesvol gebruik van struviet als meststof te bepalen.

In Hoofdstuk 2 onderzocht ik de potentie van gerichte verhogingen van de gecultiveerde plantendiversiteit om beter gebruik te maken van P in intensief beheerde graslanden. Ik heb een veldexperiment uitgevoerd in een zandgrond met een lage P-status. De diversiteit aan grassoorten in dit experiment varieerde van één tot vier per behandeling De groei van de planten werd gemeten door middel van negen grasoogsten gedurende een periode van twee jaar. Daarnaast werd aan het einde van het tweede groeiseizoen zowel worteleigenschappen als microbiële parameters geanalyseerd. In de behandelingen die niet bemest zijn met P leidde de combinatie van *Lolium perenne L.* en *Festuca arundinacea Schreb.* tot een overproductie vergeleken met de monoculturen van deze twee soorten. Dit hing samen met een complementariteit van worteleigenschappen en -strategieën, resulterend in een betere benutting van het in de bodem aanwezige P. De combinatie van vier soorten leverde geen hogere resultaten op in termen van biomassa of P-

opname. Het vertoonde echter wel een toename van de carboxylase-activiteit vergeleken met de monocultuur van *L. perenne*, wat duidt op een hogere omzet van organische koolstof met een grotere soortenrijkdom. Uit de data concludeer ik dat verstandig gekozen grasparen kunnen helpen om de opbrengst op peil te houden in graslanden waarvan de bodem een lage P-status heeft.

In Hoofdstuk 3 heb ik de effecten van struviet op de opbrengst van en P-opname door snijmaïs vergeleken met de effecten van diammoniumfosfaat (DAP) voor twee contrasterende grondsoorten met een lage P-status in een potexperiment. Zowel struviet als DAP werden dichtbij de wortelzone van maïs bemest, om het effect van rijenbemesting te simuleren. In aanvulling op de opbrengst van en P-opname door maïs heb ik de wortelproliferatie binnen en buiten de zone waarin het struviet en DAP waren aangebracht gemeten. Ik heb met een aantal verschillende extractiemethoden fosfaat geëxtraheerd uit grondmonsters die zowel buiten (bulkgrond) als uit de bemeste zone afkomstig waren, namelijk 0.01 M $CaCl_2$ (P-$CaCl_2$) en ammoniumlactaat (P-AL). In beide gronden leidde het plaatsen van struviet in de bemeste zone tot een lagere maïsprestatie in termen van opbrengst en P-opname vergeleken met het plaatsen van DAP in de bemeste zone, maar de effecten van struviet waren significant hoger dan die van de controlebehandeling zonder P-bemesting. De resultaten van P-$CaCl_2$ en P-AL van de met struviet behandelde grond waren verrassend omdat ze een hoge P-beschikbaarheid in de bodem lieten zien ondanks de slechte prestaties van de maïsplant. Dit is te verklaren door het oplossen van struviet, wat na afloop van het potexperiment nog in de grond aanwezig was, tijdens de extractie van de grond met 0.01 M $CaCl_2$ (P-$CaCl_2$) en ammoniumlactaat (P-AL). Voor P-$CaCl_2$ werd dit beeld bevestigd door middel van aanvullende batchexperimenten. Ik kwam tot de conclusie dat P-$CaCl_2$ en P-AL, die in Nederland routinematig worden gebruikt voor het vaststellen van de P-status van landbouwgronden en het geven van fosfaatbemestingsadviezen, niet geschikt zijn voor grond met struviet.

Hoofdstuk 4 behandelt de N-dynamiek van struviet, in het bijzonder de emissies van lachgas (N_2O) in de bodem na toepassing van struviet. Ik vergeleek de prestaties ervan met die van ureum, de meest gebruikte minerale N-meststof wereldwijd. Ik voerde een potexperiment uit waarbij ik bemesting toepaste op raaigras dat was geteeld op twee zandgronden met een contrasterende P-status. Tijdens twee groeicycli werd de uitstoot van N_2O gemeten naast bodem- en plantparameters. Voor de grond met een lage P-status waren de emissies na eventuele bemesting zeer laag

en niet significant verschillend van de onbemeste controle. Voor de bodems met een hoge P-status verminderde struviet de N_2O-emissie echter aanzienlijk: deze verschilden niet van de N2O-emissie van de onbemeste controle, terwijl de emissies na ureumtoepassing gemiddeld vijf keer hoger waren. Het verpoederen van struviet leidde tot een verbetering van de plantenbemesting, zonder dat dit gevolgen had voor de N_2O-uitstoot. Ik concludeerde dat struviet een veelbelovende meststof is om de N_2O-uitstoot door de landbouw te helpen verminderen.

In Hoofdstuk 5 combineerde ik beide benaderingen die ik in dit proefschrift heb onderzocht, waarbij ik de gecombineerde impact van regenwormen en struviet op de prestaties van raaigras in grasland testte. De opbrengst en het P-gehalte van de vijf oogsten over 13 maanden werden gemeten om de totale P-opname te berekenen. Aan het einde van het experiment werd de regenwormgemeenschap van elke mesocosm teruggevonden en werden de wortelkenmerken geanalyseerd. In akkers bleek struviet een even effectieve meststof als triple superfosfaat wat betreft grasopbrengst en P-opname. De werkzaamheid van struviet werd niet verder bevorderd door regenwormen. Alle drie de regenwormsoorten die in het experiment waren opgenomen, hadden echter een kleine positieve impact op de grasopbrengst en de P-opname. Interessant is dat, hoewel de bovengrondse biomassa van raaigras identiek waren onder struviet- en TSP-bemesting, de wortels van planten behandeld met struviet tekenen vertoonden van strategieën die te zien zijn in wortels van de P-gelimiteerde behandeling met betrekking tot wortelmorfologie en AMF-kolonisatie-intensiteit. Ik concludeer dat struviet, dat langzaam oplost in de bodem, wortelstrategieën in gang zet die de langzame aanvoer van P uit struviet compenseren door beter gebruik te maken van het bodem-P.

In hoofdstuk 6 breng ik de resultaten samen en bespreek ze in relatie tot mijn drie algemene onderzoeksdoelstellingen. Ik concludeer dat een grotere biodiversiteit inderdaad kan helpen om de agrarische P-cyclus op veldschaal verder te sluiten, hoewel dit duidelijker was voor de diversiteit van grassoorten dan voor de diversiteit van regenwormen (RO1). De impact van plant- en bodembiota is vooral belangrijk in omstandigheden waarin P niet direct beschikbaar is, zoals die voorkomen bij struviet bemesting. Struviet, een meststof met langzame afgifte, stimuleert strategieën voor het verwerven van voedingsstoffen voor planten. Over het geheel genomen zou dit ertoe kunnen leiden dat er minder nutriënten uit de landbouwsystemen naar het milieu ontsnappen, vooral in termen van N_2O (RO2). Vanwege het langzame oplossen zal struviet niet onder alle omstandigheden efficiënt

zijn, maar in lichtzure bodems met een laag P-bufferend vermogen is het waarschijnlijk een efficiënte meststof voor planten die flexibel zijn in de timing van hun P-opname (RO3). In Hoofdstuk 6 bespreek ik ook enkele methodologische uitdagingen die ik tijdens dit proefschrift tegenkwam. Deze omvatten een mogelijke bias bij het bestuderen van struviet in de kas vergeleken met het veld; de hulpmiddelen om N- en P-beperkingen in planten te beoordelen; evenals het statistische ontwerp van veldexperimenten met regenwormen.

Résumé

L'intensification de l'utilisation agricole d'engrais phosphorés (P) et azotés (N) a conduit à des rendements sans précédent, mais aussi à des pertes d'éléments nutritifs inégalées dans l'environnement. Le lessivage de l'N et du P dans les champs entraîne une eutrophisation des eaux de surface mais aussi des émissions de composés azotés, qui sont liées aux dépôts d'azote dans les zones naturelles, à l'acidification des sols et au réchauffement climatique. L'utilisation à grande échelle d'engrais phosphatés, produits à partir de réserves de roches phosphatées réparties de manière très irrégulière dans le monde, a également des conséquences géopolitiques. Compte tenu de ces problèmes, il est clair que les futurs systèmes agricoles doivent être repensés de manière à (i) réduire les fuites actuelles des cycles du P et de l'N et (ii) réduire notre dépendance à l'égard des roches phosphatées. Dans cette thèse, j'aborde deux étapes potentielles dans cette direction : (i) l'utilisation d'augmentations ciblées de la biodiversité des plantes et des vers de terre pour fermer davantage le cycle du P au champ ; et (ii) le remplacement des engrais minéraux phosphatés conventionnels par de la struvite, un engrais circulaire recyclé à partir d'eaux usées. Mes trois objectifs de recherche ont été les suivants : (RO1) évaluer le potentiel de la biodiversité des plantes et des vers de terre pour augmenter l'efficacité de l'utilisation du P (PUE) dans les prairies gérées de manière intensive ; (RO2) comprendre les réponses des plantes et du sol dans les agroécosystèmes fertilisés avec de la struvite ; et (RO3) déterminer les conditions agronomiques pour une utilisation réussie de la struvite en tant qu'engrais.

Dans le Chapitre 2, j'explore le potentiel d'augmentations ciblées de la diversité des plantes cultivées pour mieux utiliser le P dans les prairies gérées de manière intensive. J'ai réalisé une expérience sur le terrain dans un sol sablonneux à faible teneur en P. La diversité des espèces de graminées expérimentales variait d'une à quatre espèces. Les performances des plantes ont été mesurées au cours de neuf récoltes fourragères sur deux ans et les caractéristiques des racines ainsi que les paramètres microbiens du sol ont été analysés à la fin de la deuxième saison. Dans les parcelles non fertilisées avec du P, la combinaison de *Lolium perenne* L. et de *Festuca arundinacea* Schreb. a conduit à un rendement supérieur à ceux des monocultures de ces deux espèces. Cela s'explique par la complémentarité des caractéristiques et des stratégies racinaires, qui permettent une meilleure utilisation du P du sol. La combinaison de quatre espèces n'a pas donné de meilleurs résultats en termes de biomasse ou d'absorption de P. Elle a cependant montré une augmentation de l'utilisation du P du sol par une augmentation de l'activité

carboxylase par rapport à la monoculture de *L. perenne*, ce qui suggère un renouvellement plus important du carbone organique avec une plus grande richesse en espèces. J'en conclus que des paires de graminées judicieusement choisies peuvent contribuer à maintenir le rendement dans les prairies dont le sol est pauvre en P.

Dans le Chapitre 3, j'ai étudié l'impact de la fertilisation par de la struvite placée près de la zone racinaire du maïs en comparaison avec le conventionnel phosphate de di-ammonium (DAP) placé, pour deux types de sol contrastés avec un faible statut en P dans une expérience en pot. J'ai mesuré le rendement et l'absorption de P du maïs d'ensilage, ainsi que la prolifération de ses racines à l'intérieur et à l'extérieur de la zone de fertilisation. Des tests de P-$CaCl_2$, de lactate d'ammonium et d'oxalate d'ammonium ont été effectués sur des sols provenant de la zone de fertilisation et du sol principal. Dans les deux sols, la fertilisation à la struvite a entraîné une baisse des performances du maïs en termes de rendement et d'absorption de P par rapport à la fertilisation au DAP, mais elle a été significativement plus élevée que dans le cas du contrôle non fertilisé. Les résultats des tests de P du sol effectués sur le sol traité avec de la struvite ont été surprenants car ils ont montré une disponibilité élevée de P du sol malgré une performance médiocre du maïs. Cela s'explique par la dissolution de la struvite au cours de l'extraction des tests du sol, comme l'ont confirmé d'autres expériences chimiques. J'en ai conclu que les tests du sol couramment utilisés aux Pays-Bas n'étaient pas adaptés aux sols contenant de la struvite.

Le Chapitre 4 traite de la dynamique de l'N de la struvite, en particulier des émissions d'oxyde nitreux (N_2O) dans le sol après l'application de la struvite. J'ai comparé ses performances à celles de l'urée, l'engrais minéral azoté le plus couramment utilisé dans le monde. J'ai réalisé une expérience en pot au cours de laquelle j'ai appliqué différentes fertilisations à du ray-grass cultivé sur deux sols sablonneux dont la teneur en P était contrastée. Les émissions de N_2O ont été mesurées pendant deux cycles de croissance, ainsi que les paramètres du sol et de la plante. Pour le sol à faible teneur en P, les émissions après quelque application d'engrais étaient très faibles et ne différaient pas significativement du contrôle non fertilisé. Pour le sol à forte teneur en P cependant, la struvite a considérablement réduit les émissions de N_2O : elles n'étaient pas différentes de celles du témoin non fertilisé, tandis que les émissions ayant suivies l'application d'urée étaient en moyenne cinq fois plus élevées. La struvite en poudre a permis d'améliorer la fertilisation des plantes sans

avoir d'impact sur les émissions de N_2O. J'en ai conclu que la struvite est un engrais prometteur pour aider à réduire les émissions de N_2O provenant de l'agriculture.

Dans le Chapitre 5, j'ai combiné les deux approches que j'ai explorées dans cette thèse, en testant l'impact combiné des vers de terre et de la struvite sur la performance du ray-grass au champ. Le rendement et la teneur en P de chacune des cinq récoltes sur 13 mois ont été mesurés pour calculer l'absorption totale de P. A la fin de l'expérience, la communauté de vers de terre de chaque mésocosme a été récupérée et les caractéristiques des racines ont été analysées. Au champ, la struvite s'est avérée être un engrais aussi efficace que le superphosphate triple en ce qui concerne le rendement en herbe et l'absorption de P. L'efficacité de la struvite n'a pas été renforcée par les vers de terre. Cependant, les trois espèces de vers de terre incluses dans l'expérience ont eu un léger impact positif sur le rendement en herbe et l'assimilation du P. Il est intéressant de noter que, bien que les feuilles de ray-grass aient été identiques dans le cas d'une fertilisation à la struvite et au TSP, les racines des plantes traitées à la struvite ont montré des signes de stratégies observées dans les racines du traitement limité en P, en ce qui concerne la morphologie des racines et l'intensité de la colonisation par les AMF. J'en conclus que la struvite, qui se dissout lentement dans le sol, déclenche des stratégies racinaires qui compensent la lenteur de l'apport de P de la struvite en utilisant mieux le P du sol.

Dans le Chapitre 6, je rassemble les résultats et les discute par rapport à mes trois objectifs de recherche généraux. J'en conclus qu'une biodiversité accrue peut effectivement contribuer à fermer davantage le cycle du P agricole à l'échelle du champ, bien que cela soit plus clair pour la diversité des espèces de graminées que pour la diversité des vers de terre (RO1). L'impact de la biodiversité des plantes et du sol est particulièrement important dans les conditions où le P n'est pas facilement disponible, telles que celles rencontrées lors d'une fertilisation à la struvite. La struvite, un engrais à libération lente, déclenche des stratégies d'acquisition de nutriments par les plantes. Globalement, cela pourrait conduire à une diminution des éléments nutritifs s'échappant des systèmes agricoles vers l'environnement, notamment en termes de N_2O (RO2). En raison de sa dissolution lente, la struvite ne sera pas efficace dans toutes les conditions, mais dans les sols légèrement acides avec un faible pouvoir tampon du P, elle est susceptible d'être un engrais efficace pour les plantes qui sont flexibles dans le timing de leur absorption de P (RO3). Dans le Chapitre 6, j'aborde également certains défis méthodologiques que j'ai rencontrés au cours de cette thèse. Ceux-ci incluent un biais potentiel de l'étude de la struvite en

serre par rapport au champ, les outils pour évaluer les limitations de N et P dans les plantes, ainsi que la conception statistique des expériences au champ liées aux vers de terre.

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About the author

Laura Ferron has her roots in Bretagne (France) and her heart in Gelderland (the Netherlands). She grew up with a strong and equal attachment to food and nature. After completing a Plant Biology BSc, she chose to combine food and nature by following an MSc in Agronomy in Rennes, during which she actually spent nine months in Wageningen for two internships. She started her PhD in September 2018 at the Soil Biology group of Wageningen University within the European project "Circular Agronomics". Laura lives in Wageningen with her husband, their one-year-old daughter and their dog Charly.

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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/project proposal (6 ECTS)

Can increasing above- and belowground biodiversity in grasslands in combination with novel fertilizers contributes to a more circular economy

Post-graduate courses (3.9 ECTS)

Tidy data transformation and visualization with R; YEI (2018)

Soil ecology; PE&RC (2019)

Agricultural chemistry winter school; Italian Universities (2020)

Deficiency, refresh, brush-up courses (0.3 ECTS)

Lab skills course enzymatic assay; SBL/SOC (2019)

Invited review of journal manuscripts (3 ECTS)

Pedosphere: earthworm ecology and behaviour (2019)

Geoderma: earthworm and soil compaction (2020)

Journal of environmental quality: struvite and its environmental impact (2023)

Competence, skills and career-oriented activities (2.4 ECTS)

Start to supervise workshop; Education support center (2019)

Scientific writing; Into languages; WGS (2019)

PhD Workshop carousel; WGS (2022)

Scientific integrity/ethics in science activities (0.3 ECTS)

Ethics in plant and environmental sciences; WGS (2022)

PE&RC Annual meetings, seminars and PE&RC weekend (0.9 ECTS)

PE&RC First years weekend (2020)

Discussion groups/local seminars or scientific meetings (4.6 ECTS)

Celebration festschrift emeritus Professor Johan Bouma (2018)

Root symposium (2018)

Plant-soil interaction discussion group (2018-2019)

Circular agronomics meetings (2018-2022)

DISQ thesis ring (2018-2022)

Wageningen soil conference (2019)

Scientists4future online seminars (2021-2022)

Earthworms: the movers and shakers of the soil (2022)

International symposia, workshops and conferences (4.4 ECTS)

International congress soil zoology; Bolzano, Italy (2021)

International symposium earthworm ecology; Rennes, France (2022)

Societally relevant exposure (0.2 ECTS)

Organisation of the Wageningen worm charming championship (2019-2021)

Lecturing/supervision of practicals/tutorials (2.8 ECTS)

Soil plant interactions (2020)

Lab skills course (2020-2021)

Integration course SWA (2021)

Summer school soil ecology in the circular agroecology (2022)

BSc/MSc thesis supervision (6 ECTS)

Novel fertilizers and plant species can diminish nitrous oxide emissions from grasslands while maintaining biomass production

Studying nitrous oxide emissions after struvite application on two soils with different phosphorus status

Grass diversity and the pathways and quantity of phosphorus mining in a phosphorus deficient soil

Increasing the circularity of intensively managed grassland: can we find a match between plant species and novel nitrogen fertilizer

How do acquisition pathways and community composition affect phosphorus uptake in production grasslands

P for the future: can earthworm activity increase P uptake from struvite in intensively managed grassland

Plant species mixtures and circular fertilizers are promising ways of reducing nitrous oxide emissions in grasslands

Effects of earthworms on the availability of phosphorus from struvite

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