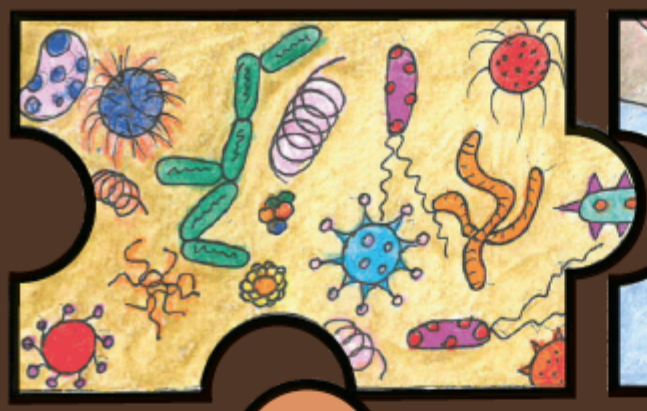
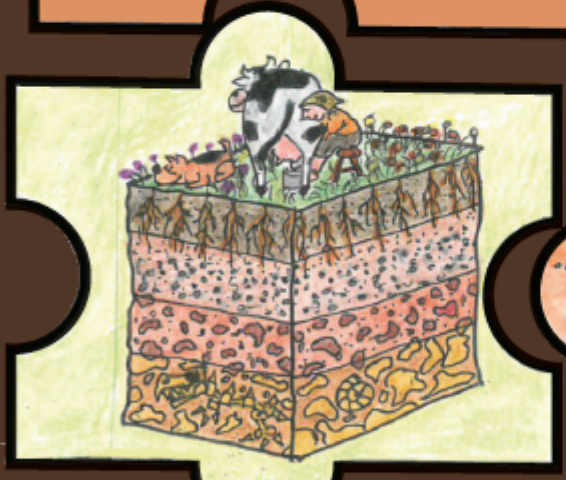


The role of soil microorganisms in enhancing phosphorus availability from dairy processing waste in grasslands

Ángel Velasco Sánchez



Propositions

- Transforming dairy processing waste into chars or ashes compromises phosphorus availability for plants.
  (this thesis)
- Managing plant and soil communities is pivotal in reducing dependency on mineral phosphorus fertilizers.
  (this thesis)
- The fear of AI in science is irrational.
- Statisticians are significant factors in statistical analyses.
- Scientific negativism contributes to societal scepticism.
- Social media is a global pandemic.

Propositions belonging to the thesis, entitled

The role of soil microorganisms in enhancing phosphorus availability from dairy processing waste in grasslands

Ángel Velasco Sánchez

Wageningen, 26 June 2024

The role of soil microorganisms in enhancing phosphorus availability from dairy processing waste in grasslands

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The role of soil microorganisms in enhancing phosphorus availability from dairy processing waste in grasslands

Ángel Velasco Sánchez

Thesis

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Dedicated to my grandfather,

Pedro Sánchez Malagón

Abstract

Phosphorus (P) is a crucial but finite agricultural resource. Excessive application of P in the past century and uneven global distribution of P reserves motivate a more efficient and circular use of P in agricultural systems. Dairy processing waste (DPW) is a P rich material that has been suggested as a potential replacement of mineral P fertilizers. In this thesis, I have studied the agronomic value of dairy processing waste, in particular the contribution of soil microorganisms and grassland management to improve P cycling in soils amended with DPW. I studied various aspects of this issue, taking into consideration the different chemical forms of P in dairy processing waste and soils; the contribution of soil microorganisms; and the implications of grass species selection in P cycling. My results confirm the agronomic potential of DPW as a replacement for conventional mineral P fertilizers. However, my results also show that a transformation of sludge into ash or (hydro)char may result in a decrease in P use efficiency of grasses. I also showed the potential of native soil microorganisms, in particular fungi, to improve P use and reduce the demand for P fertilization, as well as the potential for grass species management in doing so. Further research should test the role of soil microorganisms and grass species selection on DPW P use efficiency across a broader range of soils. Moreover, as my studies showed, further experiments should also aim to establish improved protocols to measure P fractions in soils and recycled P fertilizers, such as dairy processing waste.

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Normande dairy cows grazing at Brémontier-Merval, Pays de Bray, France

General Introduction

Á. Velasco-Sánchez

1. Phosphorus, from alchemy to circular economy

Phosphorus (P) was first isolated by the German alchemist Henning Brand in Hamburg in the second half of the 17th century (Weeks, 1933). Brand, in his quest to discover the *philosopher stone,* distilled large amounts of urine, finally purifying a substance that glowed in the dark. This substance, P, who Brand initially named *cold fire*, was later renamed *light-bearer,* Phosphorus (Φωσφόρος) in Greek. Many fellow chemists, such as the famous G.W. Leibniz and R. Boyle, improved Brand's formula and travelled widely to display this new mysterious substance to royal courts in many countries (Weeks, 1933). Today, P is less mysterious, but its importance to modern society, ranging from fertilizers to detergents, food additives and modern batteries, is immense.

The main use of P (encompassing over 90% of its mined P resources) is as fertilizer (Cordell et al., 2009; Ashley et al., 2011). Not long after the purification of P, it became clear that P played a major role in crop growth. For millennia, all P fertilization depended on organic depositions, such as manure or human excreta. During the 19th century, animal wastes, such as guano, became internationally traded P fertilizers (Kinsley, 2022). Similarly, bones were crushed and burned to be used as P fertilizers during the 19th century (Ashley et al., 2011). During this time, some of the very first experimental trials at the famous British research station of Rothamsted investigated the P fertilizing values of organic residues as well as bone-ash (Johnston and Poulton, 2019). It was not until the 20th century, during the green revolution, that phosphate rock became the primary source of P fertilizers multiplying its consumption during the 1950s to 2000s, reaching a consumption of 46 Tg of P fertilizers by agriculture in 2021 (Ashley et al., 2011; FAO, 2023).

The utilization of mineral P fertilizers in the recent decades has led, however, to undesired side-effects. In developed countries with intensive forms of agriculture, the low price and unrestricted application of mineral P fertilizers led to the accumulation of P in the soil, adsorbed into soil particles and not directly available for plants (Doydora et al., 2020). The excessive accumulation of P in soils also led to increased P leaching or runoff (Figure 1.1), contributing to the degradation of water bodies through increased eutrophication (Némery and Garnier, 2016). In addition, the large reliance on mineral P fertilizers is also threatening global food security.

Rock phosphate reserves are limited to a handful of countries (Morocco, China, USA, Jordan and South Africa) (Jasinski, 2012) and any disruption in trade or increase in costs in those areas would have negative global consequences in agricultural production as well as food prices. The fact that P fertilizer is produced from rock phosphate also means that P is a non-renewable resource. Some studies suggested that reserves may become depleted somewhere during the coming century, potentially posing severe food security threats (Van Vuuren et al., 2010). For this reason, the European Union, dependent on imports of P fertilizer, has listed P as a critical raw material (Bertrand et al., 2016).

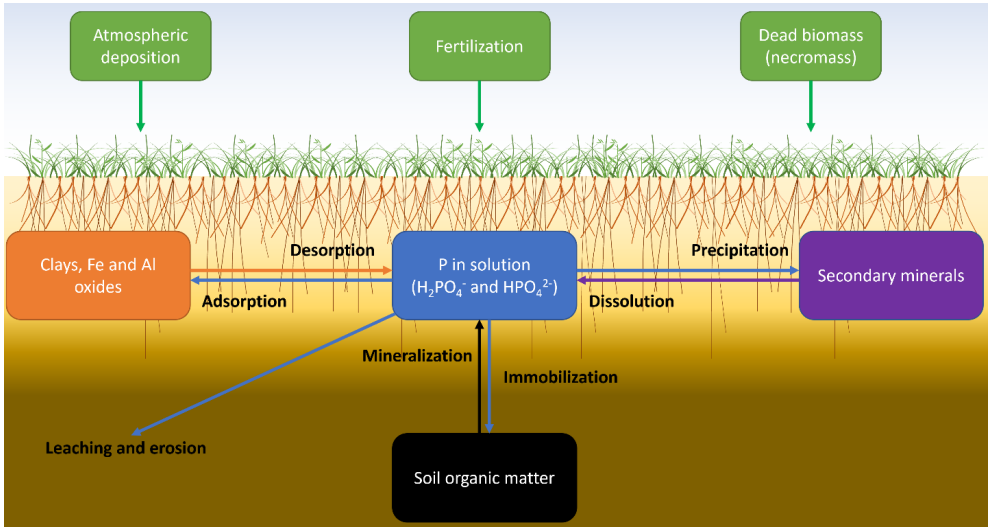

Figure 1.1. *A simplified overview of the phosphorus cycle in agricultural soils. Phosphorus may enter in the soil through 3 different pathways (green). Once in the soil, P can be found in 4 different fractions. Arrows represent the processes and transformations that P undergoes in the soil.*

To reduce the dependency on imports, a better management of P resources is required. Transition towards a more circular economy, e.g. through recycling P from waste, could be an opportunity to valorise waste while decreasing dependency on P imports (Chojnacka et al., 2020). It is considered that up to 71.1 Tg of P are generated as waste annually (Chen and Graedel, 2016). The recycling of P from waste materials has therefore become an important topic in environmental sciences. In an extensive report with guidelines and classifications, STRUBIAS (STRUbias, BIochar, AShes)

(Delgado Sancho et al., 2019) and the new fertilizer regulation (European Commission, 2019), the European Commission aimed to further advance the implementation of recycled P in the European market. At a global scale, transition towards circular economy has been included in the United Nation's sustainability goals (Belmonte-Ureña et al., 2021). Transitioning to a recycled P-based agriculture, such as before the 20th century, demands integrative new research, considering novel sources of P and implementing newer methods to both diminish the environmental damage of past excessive and uncontrolled fertilization, while at the same time reducing dependency on non-renewable P sources and maintaining high productivity.

2. Dairy processing waste as a source of P

One of the most promising systems to advance towards a circular use of P is the recovery of P from the dairy production sector. Dairy production is one of the largest agro-industries in Europe, Oceania and North-America. The global production of milk is expected to keep growing at a 1.7% annual rate up to 981 Mt by 2028 (Moscovici Joubran et al., 2021). The generation of dairy products is accompanied by the production of large amounts of wastewater and sludge with over 2.4 million tons of dairy processing waste (DPW) in Europe in 2020 (Hu et al., 2021). Almost 75% of the DPW of Europe is generated in Germany, France, Italy, Poland, Spain and The Netherlands (Stasinakis et al., 2022). DPW is considered a nutrient rich effluent that has been proposed as a potential fertilizer or as a source material for the production of other secondary fertilizers (Ashekuzzaman et al., 2021; Hu et al., 2021; Shi et al., 2022). Particularly, the high concentration in P, which can exceed in some cases 5% of its dry weight, makes DPW an interesting P fertilizer (Table 1.1).

Table 1.1. *Mean carbon and macronutrient content in dairy processing sludge across 10 dairy production plants in the north of France* (Velasco-Sánchez et al., 2023). *Results are shown in g* kg^{-1} *dry matter. C = Total Carbon, WSC = Water soluble Carbon, N = Total Nitrogen, P = Total Phosphorus,* $P\text{-}NaHCO_3$ *= Sodium bicarbonate extractable P, K = Total Potassium.*

	C	WSC	N	P	$P\text{-}NaHCO_3$	K
Mean	307.37	32.41	56.26	25.47	0.25	4.56
St. dev.	91.74	22.37	17.87	23.77	0.06	1.77
Median	305.17	27.19	52.01	14.39	0.26	4.86

One of the limitations of DPW for its widespread use is the heterogeneity of its composition. It has been shown that the nutrient concentration of DPW varies significantly among dairy plants (Ashekuzzaman et al., 2019). There are several reasons for this. First, the techniques used to precipitate P from wastewater or to stabilize the solid DPW are notably different between plants. For instance, some dairy plants add limestone to stabilize DPW or iron (Fe) and aluminium (Al) salts to precipitate P from their wastewater (Shi et al., 2021), considerably affecting its elemental composition (Tables 1.1 and 1.2). Moreover, the characteristics of DPW may change seasonally and are dependent on the end product of each plant (e.g. cheese, butter, yogurt, …) (Ashekuzzaman et al., 2019; Shi et al., 2021). Second, the characteristics of DPW may vary between countries as countries have different guidelines on how to treat and dispose wasteWater Researchidues (Kellis et al., 2013; Román-Sánchez et al., 2015; Collivignarelli et al., 2019). All these differences contribute to make DPW a highly chemically and physically heterogeneous material (Tables 1.1 and 1.2 and Figure 1.2).

Table 1.2. *Mean of micronutrients in DPW in the North of France* (Velasco-Sánchez et al., 2023). *Results are shown in g kg^{-1} dry weight. Plant number refer to different dairy wastewater treatment plants. Al = Aluminium, Ca = Calcium, Fe = Iron, Mg = Magnesium, Mn = Manganese, Na = Sodium, Zn = Zinc, EC = Electrical conductivity.*

Plant	Al	Ca	Fe	Mg	Mn	Na	Zn	pH	EC
1	0.08	142.32	0.22	2.34	0.01	6.75	0.09	8.15	3.38
2	1.84	64.04	170.42	2.11	0.27	11.40	0.12	8.47	8.73
3	0.23	147.97	0.53	4.86	0.04	7.23	0.12	7.91	4.51
4	0.25	35.18	0.91	2.30	0.01	10.07	0.14	8.54	4.20
5	0.27	64.21	96.73	2.12	0.14	12.06	0.25	8.49	2.84
6	0.63	300.23	1.12	3.08	0.03	8.40	0.19	12.33	7.61
7	23.77	10.48	0.23	2.40	0.01	6.01	0.08	6.77	5.10
8	0.28	174.15	17.38	3.64	0.10	6.91	0.41	12.39	11.28
9	0.16	111.94	33.38	2.42	0.07	12.41	0.20	8.53	7.31
10	0.21	50.39	216.90	2.02	0.28	7.14	0.17	7.85	3.95
Mean	2.77	110.09	53.78	2.73	0.10	8.84	0.18	8.94	5.89
St. dev.	7.40	85.67	80.24	0.90	0.10	2.43	0.10	1.88	2.73
Median	0.26	88.08	9.25	2.37	0.06	7.82	0.15	8.48	4.81

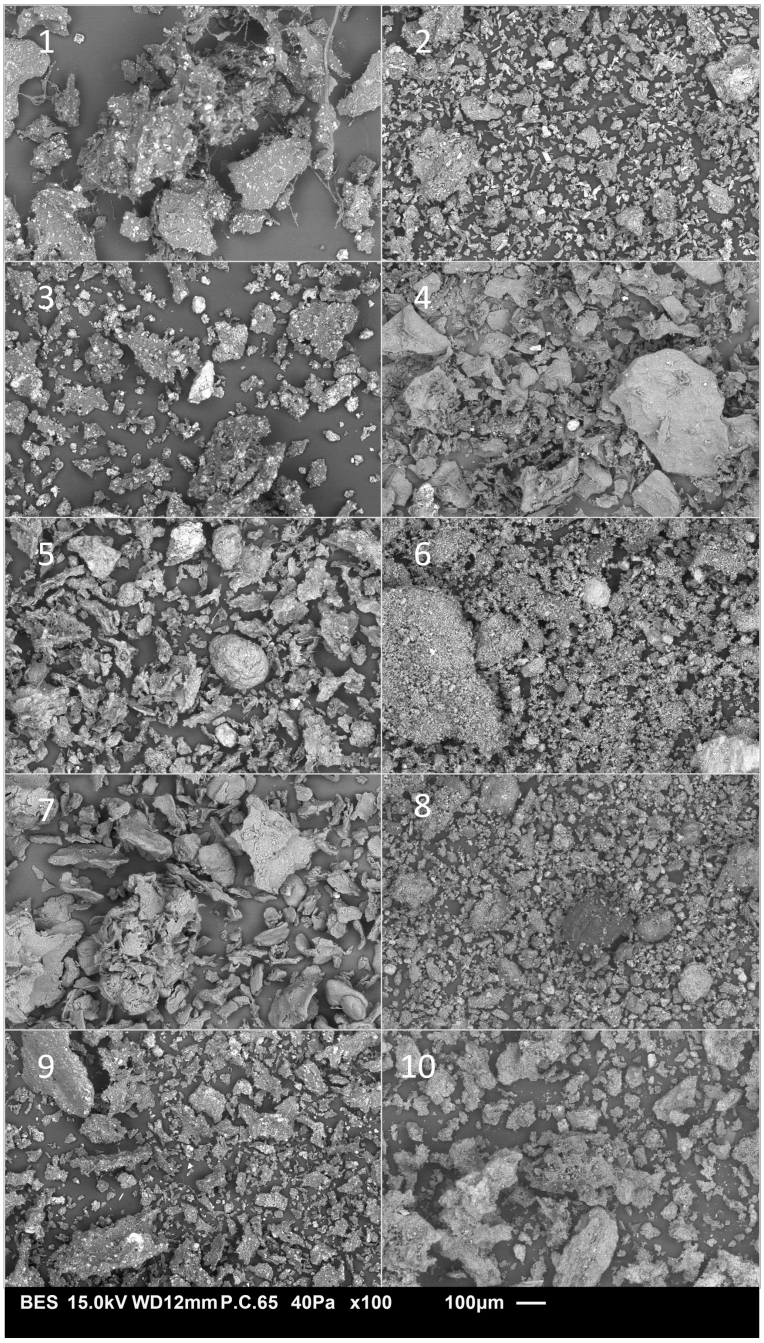

***Figure 1.2**. Images of freeze-dried DPW sludge taken with SEM microscope at x 100 magnification. Numbers 1-10 indicate samples from different dairy wastewater treatment plants in the North of France* (Velasco-Sánchez et al., 2023).

As industrial dairy production is very regulated in the European Union, concentrations of contaminants in DPW are expected to be lower than in other types of industrial waste, favouring its use in agriculture (Delgado Sancho et al., 2019). The concentration of heavy metals in Irish DPW observed by Ashekuzzaman et al. (2019), as well as those observed when characterising chemically DPWs from ten wastewater plants in the North of France are actually much lower than those found in mineral P fertilizers (Kratz et al 2016; Table 1.3).

Table 1.3. *Contaminants in Irish and French DPW and in mineral P fertilizers. Irish DPW was collected from 9 different dairy wastewater treatment plants, French was collected from 10 dairy wastewater treatment plants in the North of France and mineral fertilizer and mineral fertilizers are sold rock phosphates with a majority originated from sedimentary rocks. Numbers indicate average ± standard deviation of contaminants in mg kg^{-1} dry weight. See footnote for references.*

Material	Cd	Cr	Ni	Pb	Zn
Irish DPW [a]	< 0.15	9.5 ± 4.4	6.5 ± 5.4	1.6 ± 2.6	97 ± 66
French DPW [b]	1.5 ± 3.21	18.3 ± 14.3	15.6 ± 15	142 ± 196	176 ± 96
Mineral fertilizer [c]	20.5 ± 13	135 ± 61	33.8 ± 20.4	4.7 ± 3.8	354 ± 179

Agronomic trials have shown positive results on plant yield of soils amendment with DPW or secondary materials produced from DPW (Ashekuzzaman et al., 2021; Shi et al., 2022). However, Shi et al. (2022) noted that the exact chemical composition of DPW significantly affects its fertilizing value. They highlighted that Al treated DPW was superior than lime treated DPW, potentially because of a higher organic matter content. Studies on secondary materials (ash and hydrochar) have also shown that the chemical composition of DPW determines yield effects (Shi et al., 2022; Khomenko et al., 2023a). More research that considers the chemical composition of DPW and its secondary materials is required to fully assess their efficiency and thereby advance the utilization of DPW as a source of P in agricultural systems.

[a] = (Ashekuzzaman et al., 2019), [b] = (Velasco-Sánchez et al., 2023) *and* [c] = (Kratz et al., 2016).

3. The soil P cycle and the role of microorganisms

P is pivotal for life as it is part of nucleic acids, energy transfer molecules (ATP and ADP), cell membranes and in vertebrates bones (Filippelli, 2008). However, the processes by which organisms make use of P from soil are diverse and complex. Phosphorus enters in the soil through three different pathways (Figure 1.1). One of the sources is atmospheric deposition of dust, which can result in the deposition of up to 0.027 g P m^{-2} yearly (Tipping et al., 2014). The two other sources of P in soils are closely linked and involve anthropogenic additions of P (in the form of fertilizers or organic residues) and the necromass (dead biomass) of living organisms that grow on and in soil.

In soil, four different P pools are often distinguished (Figure 1.1) (Blume et al., 2016):

a) P in solution. This is typically the smallest form of P in soils with concentrations ranging from 0.001 to 5 mg P L^{-1}. It is dominated by the $H_2PO_4^-$ and HPO_4^{2-} ions, which are the only forms of P that plants can take up. Other forms of P such as very soluble calcium (Ca) Ca-P molecules or dissolved organic P (DOP) might also be found in soil solution at lower concentrations.
b) P Adsorbed to Fe, Al oxides and clays. The sorption of P depends strongly on the sorption capacity of the soil as well on the soil pH, decreasing its sorption with increasing pH.
c) P in secondary minerals. P, depending on the soil pH can precipitate into Ca-P forms at alkaline pH or into Fe-P and Al-P minerals in acidic soils.
d) P in organic matter. This fraction can represent up to 35% of the total P content of a soil. P in organic matter is typically found in the organic molecules of plant and microbial necromass. Organic P can also be introduced in the soil by the application of sludge, manures or other organic materials. The molecules of organic P are very heterogeneous, ranging from easily mineralizable forms to more complex molecules such as phytate.

Typically, when a mineral P fertilizer, such as triple superphosphate ($Ca(H_2PO_4)_2 \cdot H_2O$), (with most of its P in a readily soluble form) enters the soil it is rapidly adsorbed to clay minerals or Fe or Al oxides or it precipitates into secondary

minerals, depending on soil mineralogy and pH. Crops rarely take up more than 25% of the added P from fertilizers within the year of application (Roberts and Johnston, 2015). An even lower percentage of P is expected to be taken up by plants from waste materials, such as DPW, as the forms of P in these products are highly heterogeneous and therefore less soluble (Römer and Steingrobe, 2018; Shi et al., 2022). Plants invest large amounts of energy in desorbing and/or dissolving P into the soil solution. The desorption and/or dissolution of P is normally driven by mass flow and mostly diffusion forces (Blume et al., 2016).

Soil microorganisms, often considered secondary actors in P cycling compared to chemical processes, can be important in increasing plant available P (Richardson and Simpson, 2011). Fungi, archaea and bacteria have mechanisms to increase the availability of P from many forms and species of P (Figure 1.3). Soil microorganisms are key players in the mineralization of P from organic P molecules. Enzymes of particular importance are phosphatases, which hydrolyse ester bonds resulting in the release of P (Nannipieri et al., 2011). The production of extracellular enzymes is normally triggered when P availability for soil microorganisms is limited (Allison et al., 2011).

Soil microorganisms are also able to desorb P from Fe, Al and clay minerals, as well as dissolve P from secondary minerals (Figure 1.3). They do this by producing a wide variety of chelates that result in the release of P into the soil solution. A common chelating agent produced by soil microorganisms are siderophores, which are a chemically diverse group of molecules that selectively bind to Fe ions, resulting in the indirect solubilization of P (Cui et al., 2022). Similarly, soil microorganisms produce a wide variety of complex organic substances such as organic acids that can result in the alteration of soil pH and the solubilization of P (Menezes-Blackburn et al., 2016). The most common organic acids are citric and oxalic acids (Menezes-Blackburn et al., 2016). Finally, soil microorganisms can also release protons that result in the direct change of soil pH (Jones and Oburger, 2011).

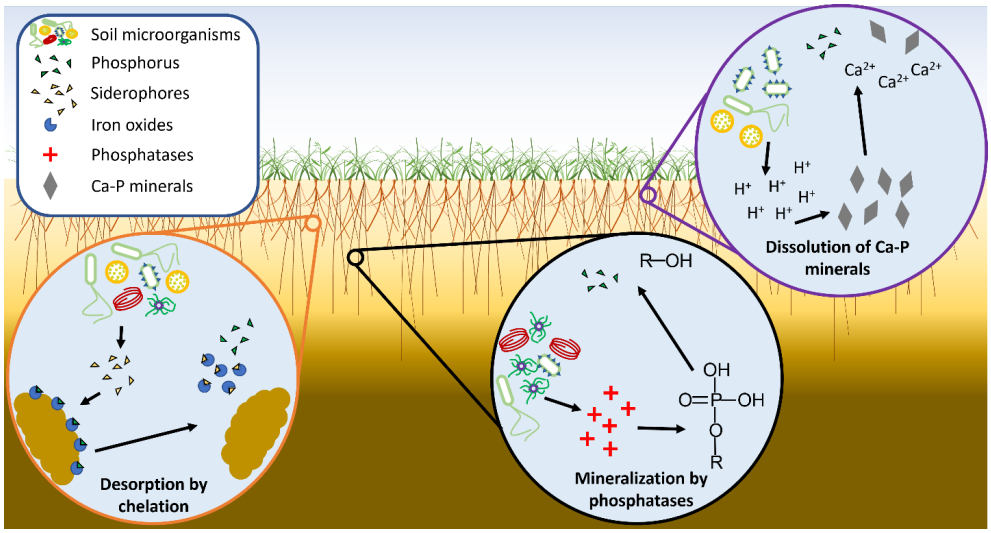

Figure 1.3. *Pathways through which soil microorganisms increase phosphorus availability. In the orange panel the chelation of Fe and the consequent release of P by siderophores is depicted. In the black panel the hydrolysis of ester bonds by phosphatases and the mineralization of P is shown. In the purple panel the dissolution of Ca-P minerals by the microbial production of protons is represented.*

Release of P into the soil solution, either by mineralization, desorption or solubilization can be performed by many different microorganisms, normally called phosphorus solubilizing microorganisms (PSM). Many bacterial groups have been identified as PSM. Bacterial groups of interest are: *Pseudomonas, Enterobacter, Bacillus, Serratia, Pantoea, Rhizobium, Arthrobacter, Burkholderia, Rahnella* or *Leclercia* (Rawat et al., 2021). Fungi are also involved in the solubilization of P, and important fungal groups are *Penicillium, Aspergillus, Acremonium, Hymenella,* or *Neosartorya* (Rawat et al., 2021). Potentially more groups of soil microorganisms are involved in the solubilization of P as the characterization of microbial groups in soil remains challenging (Lemanceau et al., 2015). Moreover, most of the experiments that screen PSM are conducted in *in vitro* conditions, which might portray a very different representation compared to the actual microbial communities in natural soils (O'Callaghan et al., 2022).

Shifting agricultural practice to P fertilization with recycled materials, with presumably a lower concentration of available P, will therefore increase the

importance of optimizing solubilization of P from other pools of P (organic matter, secondary materials and Fe and Al oxides). This might result in a more important role of microorganisms in the P cycle of agricultural systems than hitherto considered.

4. Root traits to improve P nutrition in grasslands

The production of dairy products is associated with a specific agricultural system, grasslands, which provide the fodder and grazing resources for ruminants (van den Pol-van Dasselaar et al., 2020). Grasslands also provide a wide variety of ecosystem services including soil erosion prevention, landscape conservation and storage of one third of terrestrial carbon, among others (Francksen et al., 2022; Lucie et al., 2023). These ecosystems have different levels of intervention and can be classified into: i) natural grasslands, with very little anthropogenic intervention; ii) permanent grasslands in which human intervention is limited to sporadic interventions; and iii), intensively managed grasslands in which fertilizers are applied, with regular re-seeding and several harvests throughout the year (Ros, 2019). In Europe, grasslands represent more than one third of the total agricultural land use (Schils et al., 2022). This value is however highly variable, for instance in Ireland 75% of the agricultural use is dominated by permanent grasslands, whereas in Finland this value is only 2% (Eurostat, 2023). In France, permanent grasslands occupy 9.3 million hectares of land, almost 20% of the European permanent grasslands (Eurostat, 2023). In The Netherlands, around 50% of the agricultural land and 25% of the country's surface is used permanently by grasslands (Schils et al., 2007; Eurostat, 2023). In The Netherlands, grasses are also introduced in rotations with arable crops generally after maize cultivation (Schils et al., 2007).

Grasslands are agricultural ecosystems dominated by species of the *Poaceae* family (grasses). However, depending on the degree of human intervention, they can also comprise plant species from many other families, such as leguminous plants. Natural grasslands can host up to 100 herbaceous species per square meter (Petermann and Buzhdygan, 2021). Also in agricultural systems, diversification of species in grasslands can result in better agronomic performance, for example by increasing N

availability by the inclusion of legumes or by providing a higher resilience to climatic events such as drought (Isselstein, 2005; Oelmann et al., 2015). Increasing plant biodiversity in grasslands results also in a higher below-ground diversity that can improve P nutrition under limiting conditions (Oelmann et al., 2021). Different grass species have different root traits that could be of importance to improve P nutrition (Ros et al., 2018) (Figure 1.4). Such traits include root biomass, root length and root diameter. Generally, greater root biomass and root length, thinner roots and higher specific root length are associated with higher P acquisition (Jackson et al., 1997; Faucon et al., 2017; Ros et al., 2018). Similarly, the spatial distribution of roots (e.g. deep *vs* shallow rooting system) can result in access to different pools of P, thus improving P nutrition, this mechanism is normally referred as niche complementarity (Oram et al., 2018) (Figure 1.4).

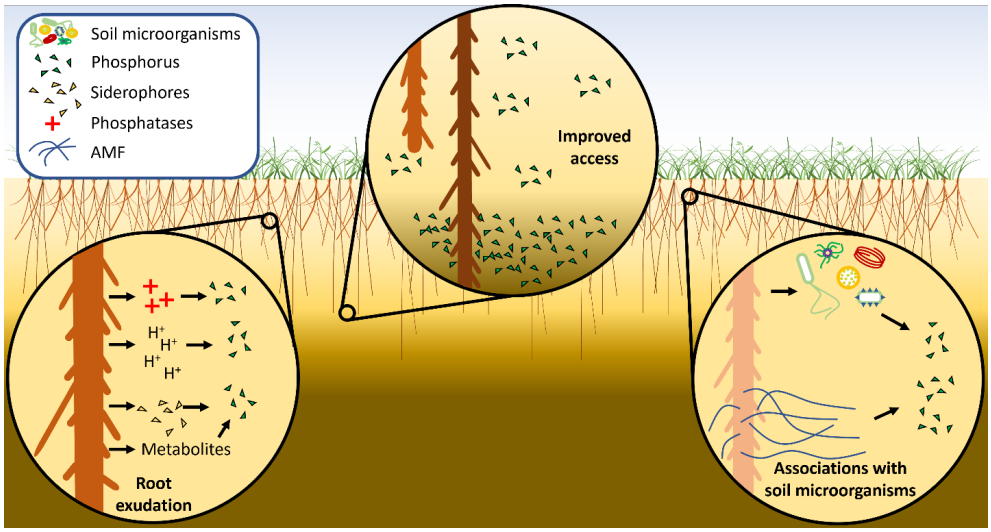

Figure 1.4. *Root mechanisms to enhance phosphorus availability in soils. In the left panel, root exudation of metabolites and production of phosphatases, siderophores result in the increase of P availability. In the central panel, improved access (niche complementarity) to P rich layer is shown. In the right panel, the root associations with microorganisms and AMF result in an improved P availability. AMF = Arbuscular mycorrhiza fungi.*

Diversity in roots can also improve P nutrition through the exudation of chemical compounds. Grasses, like soil microorganisms, can produce different organic acids

and sugars or produce extracellular enzymes that can result in an increase in P availability in the rhizosphere (Hinsinger et al., 2011; Richardson and Simpson, 2011) (Figure 1.4). Root exudates can also indirectly improve P availability by promoting PSMs through their exudates (Amy et al., 2022; Pantigoso et al., 2023). Finally, roots can establish symbiotic relationships with arbuscular mycorrhiza fungi (AMF) that provide improved access to nutrients such as P (Plassard et al., 2019; Fornara et al., 2020). Hyphae from AMF increase considerably the absorbing surface area of plants and can access smaller soil pores than root hairs (Figure 1.4) (Bennett and Groten, 2022). AMF can provide up to 90% of the P requirements of plants while AMF receive up to a 20% the plant's photo-assimilates (lipids and carbohydrates) (Bennett and Groten, 2022).

Efficient P fertilization with DPW or its secondary materials in grasslands would therefore also require selection of combinations of grass species that can make optimal use of the more complex forms of P. Moreover, selection of species that promote microbial communities or symbiotic relationships, such as AMF, would become more important under conditions of lower P availability.

5. Objectives and outline of this thesis

The main overarching objective of this thesis is to **study the fertilizing effects of DPW in grasses and the contribution of soil microorganisms to improve their efficiency**. I have studied this objective considering the chemical characteristics of P in DPW and its secondary materials, as well as the role of soil microorganisms with different analytical techniques, and finally the role of different grass species with different growth strategies and root traits. I have performed experiments with an increasing level of environmental complexity, ranging from laboratory incubations and a greenhouse study to a field experiment. The partial research questions of my thesis are:

I. What are the forms of P present in DPW materials?
II. What is the plant availability of P from DPW materials?
III. Can soil microorganisms increase the plant availability of P from DPW materials?

IV. Can the selection of grass species contribute to increase the P use efficiency from DPW materials?

Chapter 2 deals with the research objectives I and II. In this chapter, I present the results of an experiment aimed at characterising DPW samples and their secondary materials using the common SMT characterization procedure. I attempted to modify the protocol by adding an additional step in which water soluble P is first measured. This chapter highlights the challenges to correctly assign P to discrete fractions.

In chapter 3, I study the research objectives II and III. To do so, I carried out an incubation experiment with different forms of poorly soluble P that may be present in DPW. I studied changes in P availability and associated such changes with changes in microbial biomass, composition and activity.

In chapter 4, I focus on research objectives II, III and IV. To investigate these objectives, I performed a greenhouse experiment with common cultivated grasses but with contrasting growth strategies (*Lollium perenne* and *Dactylis glomerata*) under the fertilization of DPW sludge, ash and hydrochar. I studied the role of soil microorganisms in P uptake by measuring their enzymatic activities, biomass and composition.

In chapter 5, I study the research objectives III and IV. I performed a field experiment with *Lollium perenne* and *Festuca arundinacea* in monocultures, in combination and in a four species combination with other grasses with high palatability indices. I also studied microbial contribution towards P cycling by measuring enzymatic activities and their biomass.

In chapter 6, I discuss the main findings of chapters 2, 3, 4 and 5. I also provide a discussion on challenges related to DPW utilization in agriculture and provide recommendations for further research.

Dairy wastewater treatment plant in Livarot, Pays d'Auge, France

Phosphorus fractionation of recycled fertilizers reveals inadequacy of the SMT protocol

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Abstract

The Standards, Measurements, and Testing protocol (SMT) is widely use to fractionate phosphorus (P), however it lacks the determination of soluble forms of P. We tested the addition of a separate pre-wash step with H_2O as a first step added to the SMT protocol. Results were compared to a control unmodified SMT experiment. The differences in P-fractions were analysed to determine the origin of the readily available P. Six different P recycled fertilizers from dairy processing waste were investigated in the form of sludge, hydrochar and ash. Water soluble P (WSP) was correlated with weakly bound calcium (Ca), aluminium (Al) and iron (Fe). However, the SMT protocol failed to correctly identify the different pools of P as unexpected correlations were found between P and Ca, Al and Fe. For instance, large amounts of organic P were found in ashes (> 10 mg P g^{-1}). The organic P fraction included substantial amounts of Fe that correlated highly with P (R^2 = 0.84). Significant correlations ($p < 0.05$) were found between Al and Fe in the apatite P (Ca-P) fraction. We could not associate WSP with any of the pools of P defined by SMT, with the exception of total P and inorganic P. We conclude that SMT erroneously classifies P into different discrete fractions across various recycled P fertilizers. We recommend critical re-evaluation of the SMT protocol. In particular, we suggest to abandon categorising P into discrete pools and to switch to chemicals used in each extraction.

1. Introduction

The recovery of phosphorus (P) from industrial waste streams has been gaining increasing attention for both environmental and agronomic reasons. On one side, offloading phosphorus into marine and terrestrial ecosystems imposes severe environmental concerns over potential eutrophication and water poisoning in the absence of proper management (Liu et al., 2019). On the other side, an increasing scarcity of phosphate rocks threatens agricultural production for lack of access to P, labelled consequently as critical raw material by the European Union (EU) (Bertrand et al., 2016). The significant concentration of P in industrial waste streams presents a potential secondary source for P and an opportunity to address both access to, and environmental losses of, P. Studies have shown that recovering phosphorus from sewage sludge can provide 12 - 15% of the total P demand (Cordell et al., 2009). Recovering P aims at providing this essential nutrient either directly through novel fertilizers or indirectly as additives used to enrich fertilizers. Recently, several techniques have been developed to recover P from different sources, including raw and processed industrial waste streams (Delgado Sancho et al., 2019; Hu et al., 2021; Jupp et al., 2021).

Transitioning towards the use of recycled P fertilizers however raises, an array of challenges. Recycled fertilizers are more diverse and chemically complex than those derived from phosphate-rock (Delgado Sancho et al., 2019; Liu et al., 2019). P in recycled fertilizers is generally classified into different inorganic P-molecules (such as calcium (Ca), aluminium (Al) or iron (Fe) phosphates) or organic P forms (Ghanim et al., 2018). However, these groups of P are also very heterogeneous. For instance, the solubility of P from hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is lower than P from triple super phosphate ($Ca(H_2PO_4)_2 \cdot H_2O$, TSP), even though both are considered Ca-P molecules (Zwetsloot et al., 2015; Xiong et al., 2018). The solubility of strengite ($FePO_4 \cdot 2H_2O$) is lower than vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$) (Lundager Madsen and Bruun Hansen, 2014) and the solubility of aluminum phosphate ($AlPO_4$) is lower than aluminum hydroxide phosphate ($AlHO_5P^{-4}$) (Berthon and Daydé, 2013). It has also been demonstrated that organic P forms such as phospholipids are easily decomposable (Veum et al., 2019) in contrast to other organic molecules like phytate (Liu et al., 2022). These contrasts have strong

implications for the use of recycled P as fertilizers since crops can only benefit from the more soluble forms of P.

It is also relevant to identify the forms of P present in the novel recycled P fertilizers from a legislation perspective. The European Fertilizer Regulation 1069/2019 stipulates in Article 2 that producers of recycled fertilizers must report amounts of water soluble P (Delgado Sancho et al., 2019). Part II of Annex III in the same regulation declares that it is essential to report the quantity of water-soluble P in products qualifying as organo-mineral fertilizers (European Commission, 2019). It is likely that future regulations will also cover the chemical fractions of P as their fertilizer equivalent value are possibly different from each other (Shi et al., 2022; Khomenko et al., 2023a).

The most common ways to characterize the different pools of P involve sequential fractionations or extractions with different acids or bases that extract operationally defined pools of P. There are many different fractionation techniques such as Jackson & Chang (1957), Hedley's (1982) or Tiessen & Moir (2008) (Condron and Newman, 2011). The majority of these sequential fractionation schemes include early steps in which the more soluble forms of P, commonly associated with crop uptake, are first quantified. For instance, Jackson & Chang's uses ammonium chloride (NH_4Cl) and the Hedley's and Tiessen & Moir's make use of resin strips and sodium bicarbonate ($NaHCO_3$).

For the characterization of P in wastes, a universal, non-sequential method was proposed by the European Commission under the framework "Standards, Measurements, and Testing" (Ruban et al., 1999). This procedure is also known as the SMT protocol and is based on the Williams P fractionation for soils and sediments (Williams et al., 1980). Both protocols, Ruban et al. (1999) and Williams et al. (1980), have received 254 and 139 citations respectively (Clarivate Web of Science; May 2023). The SMT protocol has several advantages, including its simplicity and universal application. Since it is not a sequential fractionation, its application is easier than other techniques such as Hedley's P fractionation (Hedley et al., 1982), which involve many steps with a higher probability of introducing errors (Cao et al., 2022). SMT divides P into 5 operationally defined pools; total P (TP), organic P (OP), inorganic P (IP), apatite P (AP) and non-apatite inorganic P (NAIP).

However, one of the drawbacks of the SMT protocol is the lack of identification of the most soluble P-forms, which is the major pool of interest for the production of bio-based fertilizers (García-Albacete et al., 2012). Some authors suggested that the most available forms of P are the NAIP and OP, since they are easily mobilizable and weakly bound to sample matrices (García-Albacete et al., 2012; Ghanim et al., 2018; Cristina et al., 2022). Other studies suggested that AP has a higher bioavailability for plants compared to the less available NAIP (Takahashi et al., 2001; Li et al., 2015).

Here, we modified the SMT protocol by adding a preliminary step to determine water soluble P (WSP) from different recycled P fertilisers from dairy processing waste. Furthermore, we aimed to assess the contribution of different P pools to the newly added WSP pool by quantifying the different SMT P pools before and after water extraction. We hypothesized that by comparing samples that were washed with water with samples unwashed, we would be able to trace the origin of WSP.

2. Materials and Methods

2.1. Modified SMT protocol

We conducted a modified version of the SMT protocol in which water soluble P (WSP) is first extracted on a separate batch of samples after an extraction with water for 16 h (washed) and, in parallel, we compared this to a standard fractionation following the SMT protocol (control) (Figure 2.1) (Pardo et al., 2003; Wang et al., 2020). The experiment was setup as a completely randomized design with three replicates.

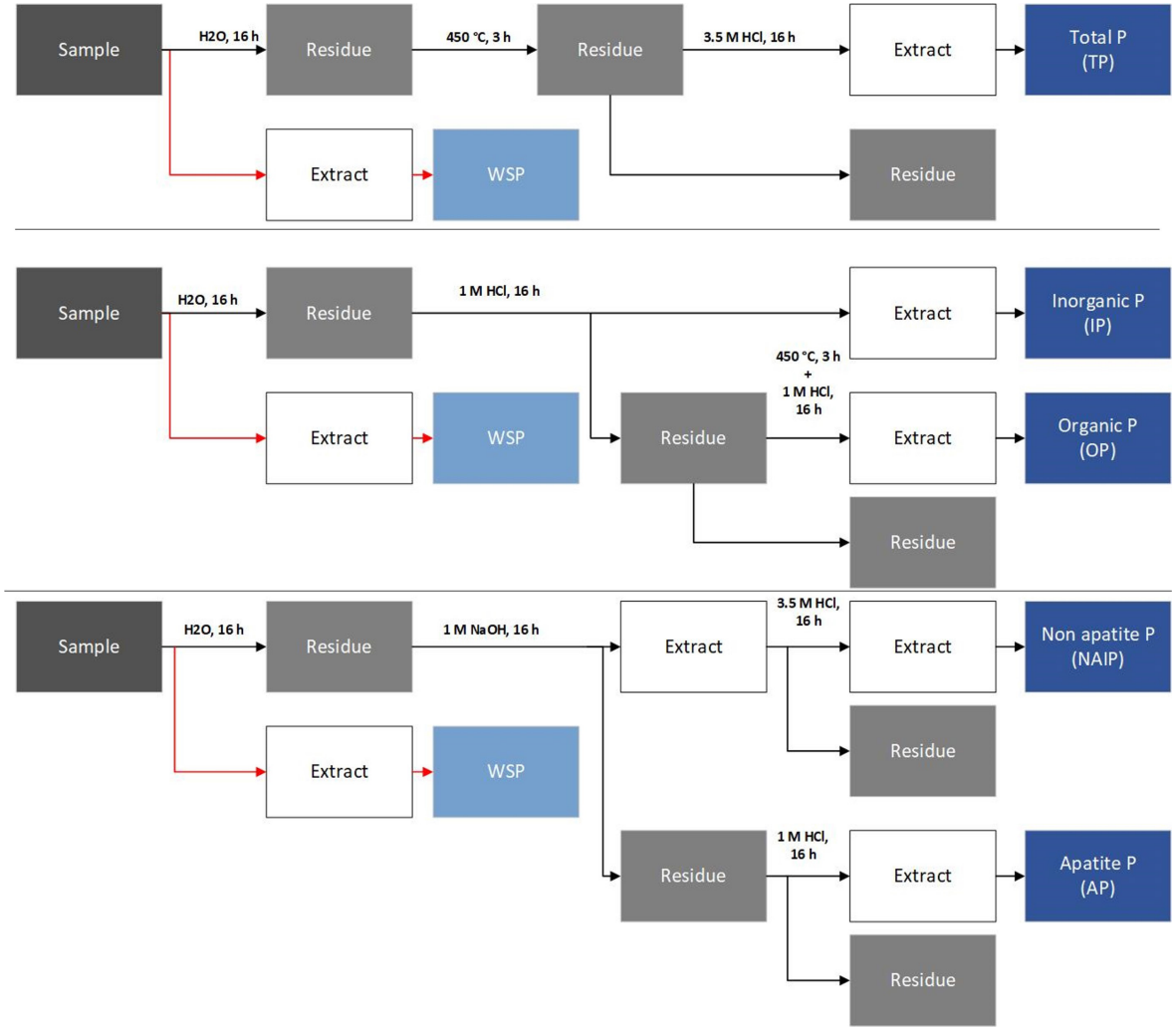

Figure 2.1. *Flowchart of the modified fractionation protocol. Each of the three steps is conducted with (modified SMT protocol) and without (original SMT protocol) an initial step to determine water soluble P (WSP, red arrows and light blue boxes) by shaking samples with H_2O for 16 h, referred to as the washing treatment. The share of SMT in each of the original SMT P pools (blue boxes) can thus be determined by comparison of the P content in each fraction with and without washing. Each extraction (each step with and without washing) was conducted on separate samples for 6 different recycled P fertilisers (Supplementary material, Table 2.S1) in triplicates.*

2.2. Products analysed

We characterized 6 different recycled P fertilizers (Supplementary material, Table 2.S1). The set of materials includes two sludges (SL) from dairy processing waste and the subsequent products derived from them: hydrochar (HC) and ash (AS). SL1 comes from a dairy factory in Denmark and SL2 comes from a dairy plant in Ireland. Both SL1 and SL2 received chemical treatments with Fe to precipitate P at their respective wastewater treatment plants. AS1 and AS2 were produced after pyrolysis of SL1 and SL2, respectively, at 250 ℃ for 2 h followed by 3 h at 550 ℃. HC1 was produced by adjusting the DM content of SL1 to 15%, adjusting the pH to 5 and carbonizing at 225 ℃ for 1 h. Meanwhile, HC2 was produced from SL2 by adjusting the DM content to 19%, the pH to 8.3 and temperature to 180℃ for a residence time of 2 h.

2.3. SMT measurements

For each fertilizer, 3 samples were tested total P (TP), inorganic P (IP), organic P (OP), non-apatite inorganic P (NAIP) and apatite P (AP) for both washed and unwashed versions of the SMT protocol. For WSP, 9 samples were analysed for each fertilizer. In total, 108 samples were measured in this experiment. All solid samples were dried at 105 °C for 24 h before the start of the experiment.

For the determination of WSP, 0.2 g of sample was placed into shaking tubes with 20 mL of demineralized H2O. After 16 h of shaking in an orbital shaker, samples were centrifuged for 5 min at 5000 rpm. The duration of shaking was fixed to 16 h of extraction time in order to ensure consistency with the rest of extractions in the unmodified SMT protocol. The supernatant was collected for analysis and the residue was used to continue with the measurements of the SMT protocol. For the samples which followed the standard SMT protocol, this first wash with H_2O was not performed.

To determine total P (TP), the samples were placed in porcelain crucibles and calcinated at 450 °C in a muffle oven for 3 h. Afterwards, the residue from the sample was placed into shaking tubes with 20 mL of 3.5 M HCl. After 16 h of shaking at room temperature in an orbital shaker, the samples were centrifugated at 5000 rpm for 5 minutes. The extract was collected for TP measurement.

For determination of inorganic (IP) and organic P (OP) the samples were shaken with 20 mL of 1 M HCl for 16 h and then centrifugated at 5000 rpm for 5 minutes. The supernatant was collected separately to assess the IP fraction. To quantify OP, the residue from the 1 M HCl extraction was initially calcinated at 450 °C and then extracted with 20 mL of 1 M HCl for 16 h. After centrifugation, the extract was separated from the residue for follow-up measurements.

Finally, for the measurement of non-apatite inorganic P (NAIP) and apatite P (AP), samples were first shaken for 16 h with 20 mL of 1 M NaOH. After centrifugation, 10 mL of extract were collected and 4 mL of 3.5 M HCl are added to each sample. The acidified supernatant was incubated at room temperature for 16 h for NAIP measurement. To obtain the AP fraction, the residue from the 1 M NaOH extraction was collected and underwent shaking with 20 mL of 1 M HCl. After centrifugation, the supernatant was collected for AP determination.

P, Ca, Fe and Al were determined using ICP-OES in all collected extracts: WSP extract, TP extract, IP extract, OP extract, NAIP extract and AP extract.

2.4. Statistical analyses

The effect of the washing treatment and product type was assessed by a two-factor analysis of variance (ANOVA). Model residuals were inspected for normality, homogeneity and heteroscedasticity assumptions. Correlations were performed to show which cation or anion dominated the P binding forms (P-Ca, P-Fe or P-Al) in each extract. When ANOVAs indicated significant effects ($p < 0.05$), differences between products were tested using Tukey's post-hoc test.

All statistical analyses and figures were made using the software RStudio v1.4.1717 (R Core Team, 2022). The following packages were used: ggplot2, psych, emmeans, multcompView, ggpubr, grid and PerformanceAnalytics.

Original dataset is available at ZENODO online repository (Velasco-Sánchez et al., 2023b).

3. Results

3.1. Differences between products and washing treatment

The different products analysed did not show statistical differences in water soluble P (WSP) ($p = 0.09$) (Figure 2.2, panel A). Ashes showed higher values of WSP (5.3 ± 1.8 mg P g^{-1} for AS1 and 4.7 ± 3.1 WSP for AS2), but their high variation led to no statistical differences. AS1 and AS2 showed the highest TP, IP and OP values (Figure 2.2, panels B, C and D). NAIP was highest in AS2, and AP was highest in AS1 (Figure 2.2, panels E and F). SL2 and HC2 showed higher amounts of TP, IP and NAIP than their SL1 and HC1 counterparts. No differences were found in OP across sludges and hydrochars. Hydrochars showed a higher amount of AP than sludges. The recovery of P in the unwashed samples was complete for IP + OP and NAIP + AP for the ashes and hydrochar (Supplementary material, Table 2.S2). The recovery of P for the sludges was below 90% for the NAIP + AP fractions (Supplementary material, Table 2.S2).

A significant effect of the washing treatment was only found in the TP and IP fractions, showing a reduction in extracted P in the washed samples (Figure 2.2, panels B and C). For the remaining pools of P (OP, NAIP and AP) no statistically significant effect was observed after washing with water ($p > 0.05$).

Water soluble Ca was highest in AS1, AS2 and HC2 with 23.4 ± 4.3, 14.4 ± 14.2 and 8.2 ± 0.3 mg Ca g^{-1} respectively. No differences in water soluble Fe were found among the products with an averaged extracted of 3.2 ± 2.3 mg Fe g^{-1}. With respect to water soluble Al, no differences across products were found. Negligible amounts of water soluble Al were detected in any of the products with the exception of AS1 in which a small amount was detected, 0.25 ± 0.17 mg Al g^{-1}.

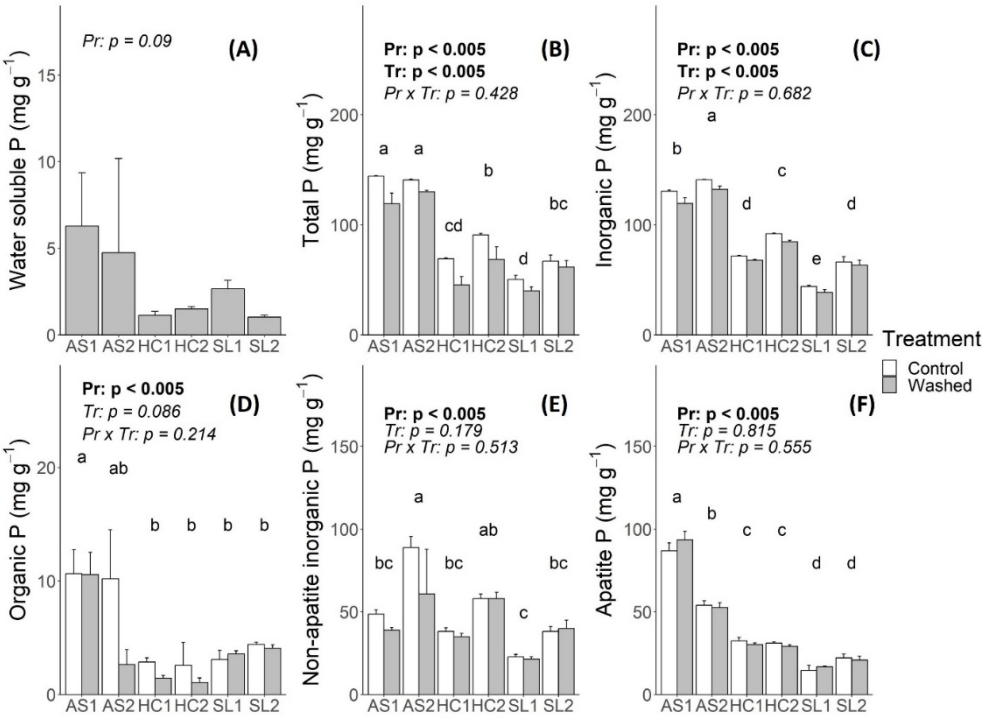

Figure 2.2. *P content of the different SMT P fractions with (washed, grey bars) and without (control, white bars) the additional water washing step. Values are expressed in mg P by g of dry weight. Whiskers represent the standard error of the mean (n = 9 for panel A and n = 3 for panels B to F). Pr = Product, Tr = Washing treatment. Letters show statistical differences between products. Bars indicate standard error, n = 3. Bold characters are used to highlight significant effects.*

3.2. Correlations of P with Ca, Al and Fe

TP was significantly correlated with total Ca and Fe and to a lower extent Al, showing adjusted R^2's of 0.92, 0.88 and 0.51 respectively (Figure 2.3). Fe was predominant in the TP extract with values ranging roughly from 50 to 450 mg Fe g^{-1}. Ca was the second most abundant with values in the range between 50 to 250 mg Ca g^{-1} in the TP extract. Values of Al ranged roughly between 0 to 20 mg Al g^{-1}.

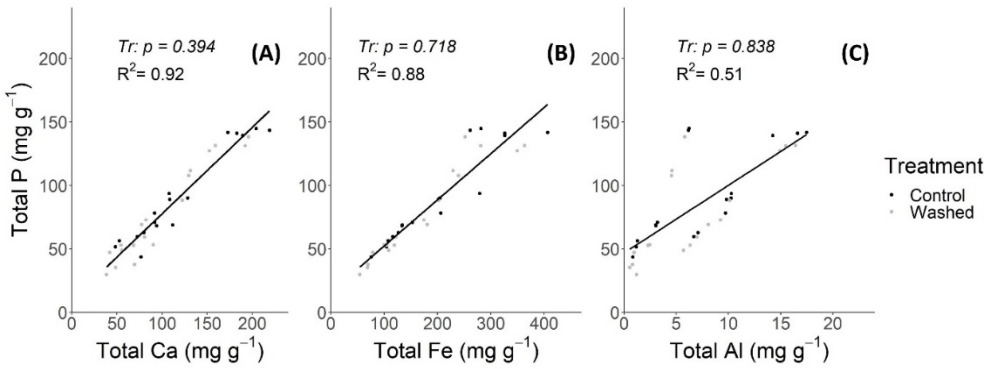

Figure 2.3. *Correlations between total P and total Ca (A), Fe (B) and Al (C). Solid lines indicate significant correlations, p < 0.05. Tr = Treatment effect. Values are expressed in mg P by g of dry weight. Solid black colour stands for unmodified SMT protocol and grey symbols represent the modified one.*

WSP was found to be positively correlated ($p < 0.05$) with water soluble Ca and Fe and to a lower extent Al (Figure 2.4). Ca was the most soluble element with values recovered in the water extract ranging roughly from 0 to 50 mg Ca g^{-1}. Fe in the water extract ranged approximately from 0 to 15 mg Fe g^{-1}, yet most of the samples had values of water soluble Fe below 5 mg Fe g^{-1}. Al was poorly soluble in water as the highest value found was less than 1 mg Al g^{-1}.

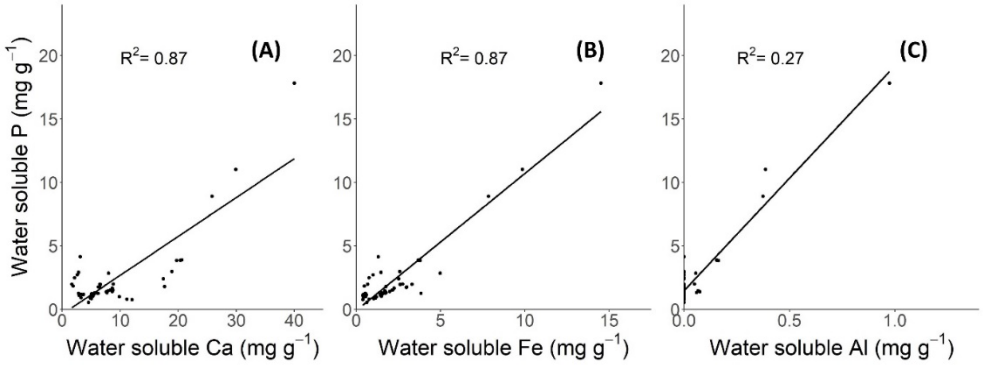

Figure 2.4. *Correlations between water soluble P and water soluble Ca (A), Fe (B) and Al (C). Solid lines indicate significant correlations, p < 0.05. Values are expressed in mg P by g of dry weight.*

IP was strongly correlated with Ca in the IP extract (Inorganic Ca); to a lesser extend with inorganic Al; and only marginally with Fe (Figure 2.5). Very slight amounts of Fe were extracted in the IP extract (< 3 mg Fe g^{-1}). The values of Al and Ca extracted in the IP extract were in the same range as those from the TP extract (Figure 2.3). For the correlations with OP, both Fe and Al in the organic P extract correlated significantly with OP. The adjusted R^2's were 0.84 and 0.4 respectively. No significant correlation between OP and Ca in the OP extract was observed. Large amounts of Ca and Fe were found in the OP extract, roughly ranging from 0 to 30 mg g^{-1}.

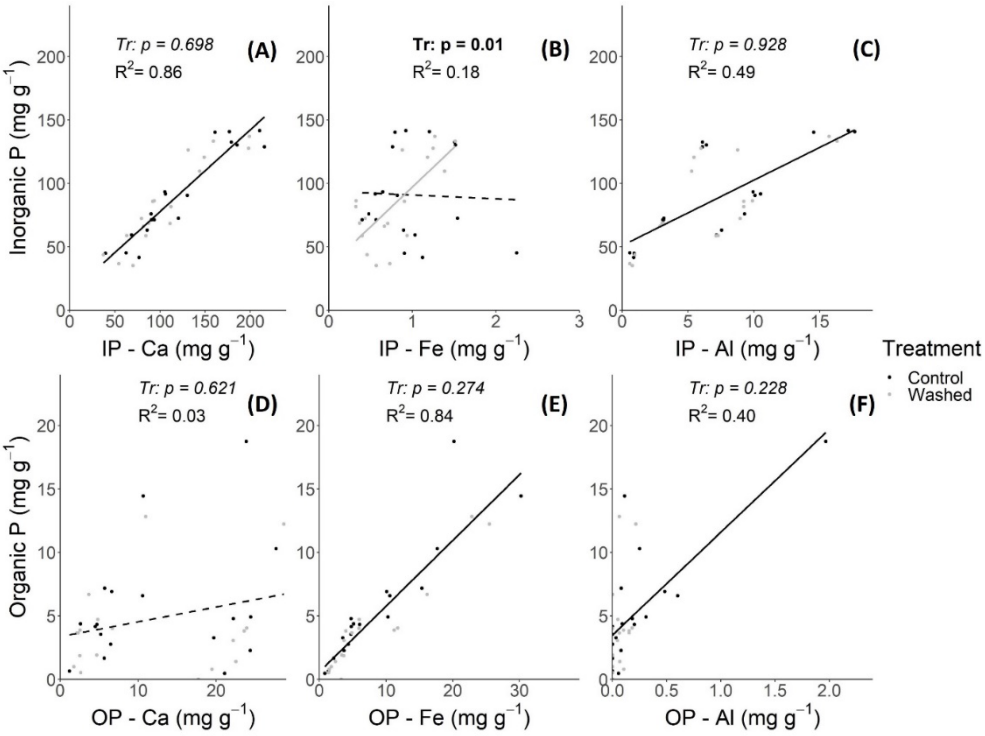

Figure 2.5. *Correlations between P and Ca, Fe and Al for in the inorganic P (IP) and organic P (OP) extracts. Values on the x axis show the concentration of Ca, Fe and Al in the IP (upper row) and OP (lower row) extracts. Solid lines indicate significant correlations, p < 0.05. Dashed lines indicate non-significant correlations. Tr = Treatment effect. Values are expressed in mg P by g of dry weight. Solid black colour represents the unmodified SMT protocol and grey the modified one.*

NAIP was only correlated with Al in the NAIP extract, R^2 = 0.93 (Figure 2.6, panel C). The NAIP extract contained relatively large amounts of Al, being in the same range as total Al. The correlation between AP and Ca in the AP fraction was significant and showed a high adjusted R^2. Al in the AP extract was also positively correlated with AP. Fe in the AP extract was not correlated with AP.

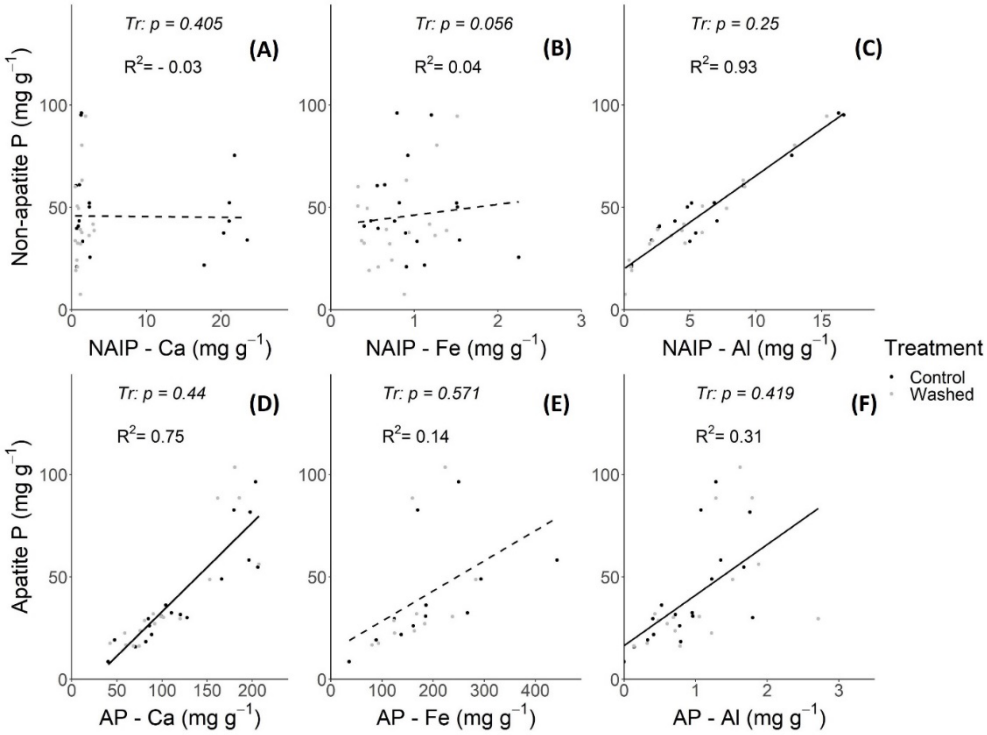

Figure 2.6. *Correlations between P and Ca, Fe and Al for each fraction in the non-apatite (NAIP) and apatite (AP) extracts. Values on the x axis show the concentration of Ca, Fe and Al in the NAIP (upper row) and AP (lower row) extracts. Solid lines indicate significant correlations, p < 0.05. Dashed lines indicate non-significant correlations. Tr = Treatment effect. Values are expressed in mg P by g of dry weight. Solid black colour represent the unmodified SMT protocol and grey the modified protocol.*

4. Discussion

4.1. Water soluble P

We aimed to modify the SMT protocol by adding a step in which WSP is assessed and to investigate which forms of P contributed most to the WSP pool. We observed that the levels of WSP were similar across all the products studied (Figure 2.2, panel A). Ashes showed higher levels of WSP compared to the other products, but due to their increased variation no statistical differences were found. Neither the ashing nor the hydrothermal carbonization reduced the levels of readily soluble P compared to the sludge. This contrasts with earlier findings of lower solubility from ash and hydrochar compared to their raw original material (Delgado Sancho et al., 2019; Lemming et al., 2020; Khomenko et al., 2023a).

To assess which forms of P contributed more to the WSP pool, we reasoned that washing samples with water as a first step would result in the removal of all the WSP forms and, therefore, we would observe a reduction of P across the different pools (as compared to the traditional protocol). This would reveal the pools of P that contributed most to WSP. WSP correlated with water-soluble Ca, Fe and Al (Figure 2.4). In particular, it correlated strongly with Ca and Fe and poorly with Al, as very low amounts of Al were found. This would suggest that WSP comes from loosely bound forms of Ca and Fe. This is not uncommon, as Fe-P and Ca-P are among the more common forms of P across recycled fertilizers (Delgado Sancho et al., 2019). However, we only observed a reduction of P in the TP and IP pools after the washing step (Figure 2.2, panels B and C). No reduction was observed for the OP, NAIP or AP fractions, and in some cases even more P was extracted after the wash (Figure 2.2, panels D to F and Supplementary material, Table 2.S2). This is surprising as AP and NAIP can be comprised of a wide range of soluble P-Ca and P-Fe molecules (Zwetsloot et al., 2015; Wu et al., 2019a; Hertzberger et al., 2020). We propose that the lack of reduction in the OP, NAIP and AP fraction is linked to the inadequacy of the SMT protocol to allocate P into different pools of P.

4.2. SMT protocol inadequacy and labelling misconceptions

The SMT protocol lead to mismatches between observations and the purported meaning of the SMT fractions. For example, almost no Fe was extracted in the IP

pool (Figure 2.5, panel B) despite Fe-P being an important constituent of ashes, hydrochars and dairy processing sludge (Hu et al., 2021; Belibagli et al., 2022; Yu et al., 2022), but large amounts of Fe were found in the OP extract that correlated strongly with the P contained in this fraction (Figure 2.5, panel E). In the NAIP extract, very small amounts of Fe were detected, even though Fe is more abundant then Al in the samples studied (Figures 2.3, panels B and C, and Figure 2.6, panel B). In contrast, large amounts of Fe were detected in the AP extract (Figure 2.6, panel E). We extracted significant amounts of OP from ashes (Figure 2, panel D), which was unexpected as ashes contain little to no organic molecules after pyrolysis at 550 °C (Vassilev et al., 2013).

These inconsistencies can be attributed to erroneous assumptions adopted by the SMT protocol and its interpretation. First, the protocol assumes that no organic P is extracted after 16 h of extraction in 1 M HCl (Figure 1). This is highly unlikely as 1 M HCl is strong enough to dissolve large amounts of carbon (Silveira et al., 2008; Worsfold et al., 2008), which could result in the solubilization of significant amounts of OP. The most readily soluble forms of OP may therefore have been ascribed as IP. Similarly, after calcination and extraction with HCl, SMT assumes that only OP is extracted. We demonstrated that this is not true as significant amounts of Ca and Fe were measured in this extract, which could be associated with Ca-P and Fe-P that were not dissolved in the previous step (Figure 2.5). The strong correlation between OP and Fe in the OP fraction could imply that also non soluble Fe-P salts were being extracted (Figure 2.5, panel E).

The assumption of the SMT protocol is that NaOH selectively extracts P from cations such as Fe and Al. However, it is not clear that most insoluble forms of NAIP are extracted with 1 M NaOH and, more importantly, there is no reason why it would not extract P from soluble organic or from Ca-P forms (Sano et al., 2012). If not all the Fe-P and Al-P forms were extracted by NaOH, they could potentially be extracted later in the 1 M HCl extraction as shown by the large amounts of Fe found in this extract (Figure 2.6, panel E). Other authors have already demonstrated that acids such as HCl or HNO_3 can successfully extract significant amounts of Fe-P (Sano et al., 2012; Elomaa et al., 2019).

Ultimately, one of the main reasons for the confusion caused by the SMT fractionation is the compartmentalization of P into different pools and not into groups related to the chemicals used. For example, the SMT scheme refers to 1 M HCl extractable P as IP or the 1 M NaOH extractable as NAIP (Figure 2.1) (Pardo et al., 2003; García-Albacete et al., 2012; Wang et al., 2020). As reflected by the results of this study, such correlation between the nomenclature and the actual nature of the extracted fractions is found to be inaccurate, as it is unlikely for the extraction to solely account for the corresponding P-fractions specified in the protocol. Using a operationally defined nomenclature in which the extractant is linked to the pool of P could be more accurate for assessing the extracted fractions (Gu and Margenot, 2021). This is already the case in other fractionation schemes, such as Hedley's (1982) in which P is allocated into resin-P, NaOH-P, 1 M HCl-P etc. (Hedley et al., 1982) or in the earlier versions of the SMT protocol (Ruban et al., 1999).

4.3. P fractionation schemes limitations

The erroneous attribution of groups of P to various extracts or fractions has also been shown in other fractionation schemes in soils and sediments (Gu et al., 2020; Barrow et al., 2021). In a noteworthy paper, Barrow et al. (2021), analysed hydroxyapatite, Al oxide and goethite using the Chang and Jackson (1957) and Zhang and Kovar P (2009) fractionation procedures (Chang and Jackson, 1957; Zhang and Kovar, 2009). Large amounts of Ca-P were detected in both goethite and Al oxides regardless of the scheme followed. Similarly, Fe-P was found in Al oxides and Al-P was found in hydroxyapatite and goethite. The false detection of OP in ashes revealed in the present study (Figure 2.2, panel D) is also in line with the results of Barrow et al. (2021).

The main hypothesis of our study was that washing samples with water would remove all WSP from our samples causing a reduction in the measured pools of P. The lack of responses for AP, NAIP or OP found in this study could also be associated with the misconceptions of the precipitate-particulate theory (Barrow et al., 2021). In this sense, P would be present in our materials as a continuum and not as a set of discrete fractions (Barrow, 2021). Barrow et al. (2021) and Barrow (2021) extensively describe the impossibility of allocating P into different discrete groups using P fractionations schemes, suggesting that P is controlled by adsorption-diffusion and

not by a precipitation-particulate phenomenon (Barrow, 1999, 2021; Barrow et al., 2021). The washing step could have then caused P to be re-distributed across the reactive surfaces in the materials, not allowing us to conveniently trace back soluble P across the discrete fractions. The lack of differences between the modified and unmodified SMT samples in most fractions supports this suggestion.

5. Conclusion

This paper demonstrated that the conventional SMT fractionation method is inconsistent in describing the different pools of P in recycled fertilizers. Our results suggest: (i) that the assumptions of the selective extractions of P in the SMT protocol are erroneous; (ii) that allocating P into discrete pools might be non-realistic; and (iii) that naming P extracts as representing functional, discrete pools is potentially inaccurate and could be misleading.

The necessity to achieve environmentally viable P-recycled fertilizers demands a higher degree of precision and standardization in identifying P-groups. Therefore, we recommend adjusting the nomenclature of the SMT protocol and developing new methods that can characterize agronomically relevant pools of P in line with the EU fertilizer regulation. Adjusting the extraction time, temperature and extractants used to mimic plant absorption would be essential for the correct assessment of the fertilizer value of recycled P fertilizers.

6. Acknowledgements

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Supplementary material

Table 2.S1. *Recycled P fertilizers used. SL = Sludge, AS = Ash, HC = Hydrochar, HTC = hydrothermal carbonization, T = Temperature, DM = Dry matter content.*

Sample	Origin	Treatment	Conditions
SL1	Dairy Processing Waste		
SL2	Dairy Processing Waste		
AS1	SL1	Ashing	T = 250 °C, 2 h then T = 550 °C, 3 h
AS2	SL2	Ashing	T = 250 °C, 2 h then T = 550 °C, 3 h
HC1	SL1	HTC	DM = 15%, pH = 5, T = 225 °C,1 h
HC2	SL2	HTC	DM = 19%, pH=8.3, T = 180 °C, 2 h

Table 2.S2. *Relative amounts of P, Ca, Fe and Al with respect to their respective total concentrations in unmodified SMT protocol. Values indicate mean ± standard error, n = 3. Values are expressed in% over their total concentrations. IP + OP and NAIP + AP were added to observe the nutrient recovery for each of the two branches of the SMT protocol for P, Ca, Fe and Al.*

	Extract (%)	AS1	AS2	HC1	HC2	SL1	SL2
P	IP	90.6 ± 0.74	100.14 ± 0.24	103.64 ± 0.6	101.11 ± 0.85	87.09 ± 2.22	98.88 ± 7.48
	OP	7.38 ± 1.46	7.24 ± 3.07	4.13 ± 0.54	2.82 ± 2.22	6.13 ± 1.56	6.61 ± 0.28
	IP + OP	97.98 ± 1.64	107.38 ± 3.08	107.76 ± 0.81	103.94 ± 2.37	93.22 ± 2.72	105.5 ± 7.49
	NAIP	33.69 ± 1.88	63.13 ± 4.78	55.12 ± 3.05	63.81 ± 3.16	45.16 ± 2.91	56.93 ± 4.35
	AP	60.3 ± 3.3	38.35 ± 1.93	46.88 ± 2.84	34.25 ± 0.76	28.81 ± 6.18	33.03 ± 3.39
	AP + NAIP	93.99 ± 3.8	101.48 ± 5.16	102.01 ± 4.17	98.06 ± 3.25	73.97 ± 6.83	89.97 ± 5.52
Ca	IP	91.32 ± 5.33	100.6 ± 8.01	102.23 ± 9.5	99.13 ± 7.14	100.56 ± 18.29	99.97 ± 8.1
	OP	6.93 ± 3.14	10.07 ± 3.22	12.1 ± 6.23	9.52 ± 5	15.72 ± 8.87	12.85 ± 7.2
	IP + OP	98.25 ± 6.19	110.67 ± 8.64	114.32 ± 11.36	108.65 ± 8.72	116.28 ± 20.33	112.81 ± 10.84
	NAIP	4.06 ± 2.95	4.46 ± 3.76	8.4 ± 7.63	6.58 ± 5.89	11.71 ± 9.11	9.33 ± 7.83
	AP	91.45 ± 3.41	104.3 ± 6.68	103.84 ± 10.34	97.78 ± 7.21	89.08 ± 15.36	104.96 ± 2.33
	AP + NAIP	95.51 ± 4.51	108.76 ± 7.66	112.24 ± 12.85	104.36 ± 9.31	100.8 ± 17.86	114.28 ± 8.17
Fe	IP	0.47 ± 0.09	0.28 ± 0.03	0.6 ± 0.25	0.29 ± 0.03	1.49 ± 0.44	0.54 ± 0.11
	OP	7.78 ± 1.7	3.83 ± 0.94	3.05 ± 0.22	1.86 ± 1.38	3.78 ± 0.89	3.48 ± 0.28
	IP + OP	8.25 ± 1.7	4.11 ± 0.94	3.64 ± 0.33	2.16 ± 1.38	5.27 ± 1	4.01 ± 0.3
	NAIP	0.47 ± 0.09	0.28 ± 0.03	0.6 ± 0.25	0.29 ± 0.03	1.49 ± 0.44	0.54 ± 0.11
	AP	77.37 ± 12.09	104.2 ± 17.19	110.64 ± 17.97	98.96 ± 14.66	65.14 ± 22.59	99.81 ± 6.48
	AP + NAIP	77.84 ± 12.09	104.47 ± 17.19	11.23 ± 17.97	99.25 ± 14.66	66.63 ± 22.6	100.34 ± 6.48
Al	IP	99.9 ± 1.62	102.05 ± 6	101.43 ± 0.78	100.57 ± 1.69	72.5 ± 9.35	102.08 ± 8.35
	OP	2.38 ± 0.84	5.71 ± 3.27	0.86 ± 0.86	2.17 ± 1.9	3.75 ± 2.43	1.64 ± 0.82
	IP + OP	102.37 ± 1.82	107.76 ± 6.83	102.29 ± 1.16	102.74 ± 2.54	76.26 ± 9.66	103.72 ± 8.39
	NAIP	73.99 ± 6.11	94.47 ± 7.8	78.97 ± 5.97	82.22 ± 7.29	52.18 ± 1.45	74.27 ± 7.96
	AP	22.11 ± 3.26	8.79 ± 0.83	17.69 ± 2.96	12.22 ± 2.77	14.18 ± 8.66	8.44 ± 1.57
	AP + NAIP	96.1 ± 6.92	103.25 ± 7.84	96.66 ± 6.66	94.44 ± 7.8	66.36 ± 8.78	82.72 ± 8.11

Measurement of enzymatic activities at Unilasalle, Mont-Saint-Aignan, France

Soil microorganisms increase Olsen phosphorus from poorly soluble organic phosphate: a soil incubation study

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Abstract

The potential shortage of mineral phosphorus (P) sources and the shift towards a circular economy motivates the introduction of new forms of P fertilizers in agriculture. However, the solubility of P in new fertilizers as well as their availability to plants may be low. In this experiment, we incubated an agricultural soil poor in P (28 mg P_2O_5 kg^{-1}) for 63 days in the presence of a range of organic and inorganic poorly soluble P forms commonly found in new fertilisers: hydroxyapatite (P-Ca), iron phosphate (P-Fe), phytic acid (P-Org) and a combination of P-Ca and P-Org (P-Mix). Cellulose and potassium nitrate (KNO_3) were added to stimulate microbial activity at the beginning of the incubation. We included a positive control with triple superphosphate (TSP) and negative controls with no P application (with and without cellulose and KNO_3). We assessed the fate of the different poorly soluble P forms in $NaHCO_3$ extracts (Olsen P) over time as a proxy for plant available P. Soil microbial biomass, fungal to bacterial ratio, soil respiration, enzymatic activities (β-glucosidase, arylamidase and acid and alkaline phosphatase), N mineralisation and soil pH were also monitored. At the beginning of the incubation, TSP showed the highest Olsen P across all treatments and P-Fe showed higher levels of Olsen P than the other poorly soluble P forms ($p < 0.05$). During the incubation, the levels of Olsen P decreased over time for TSP (positive control). Contrastingly, Olsen P increased significantly over time for all the poorly soluble P forms and the negative controls, indicating an increase in plant available P. Particularly, levels of Olsen P for the P-Org treatment roughly doubled (shifting from 16.5 mg kg^{-1} to 32.9 mg kg^{-1}) over the whole incubation period. The rate of increase in Olsen P was positively correlated with microbial biomass C: P ratio ($p < 0.01$) for all poorly soluble treatments. The higher levels of Olsen P for the P-Org treatment were also explained by a positive correlation with fungal biomass. Our results show that poorly soluble forms of P may be made available to plants under the influence of the microbial community, with a stronger effect for organic P forms.

1. Introduction

Phosphorus (P) fertilization is among the most common practices in agriculture worldwide. The utilization of mineral P fertilizers is accompanied by a series of issues which vary across the globe. From an economic perspective, the production of mineral P fertilizers is restricted to a handful of countries, posing a substantial dependency on imports (Schoumans et al., 2015). Moreover, P resources are limited and are currently listed as a critical raw material in the European Union (Van Vuuren et al., 2010; Bertrand et al., 2016). From an environmental perspective, P fertilization can incidentally cause P enrichment of water bodies leading to water eutrophication (Mekonnen and Hoekstra, 2018; Ortiz-Reyes and Anex, 2018), known to cause severe damage in water ecosystems (Dorgham, 2014). In addition, large areas of the world's soils contain an excess of previously applied P fertilizer that cannot used by crops (MacDonald et al., 2011; Zhu et al., 2018). All these economic and environmental challenges motivate a more efficient utilization of P resources by using new forms of P fertilizers and more effective P solubilization and mineralization from P bound to soil particles (Faucon et al., 2015).

One solution to tackle P scarcity is the utilization of recycled P from different waste sources (Delgado Sancho et al., 2019; Hu et al., 2021). However, most of the recycled P fertilizers share a lower P solubility compared to mineral fertilizers (e.g. triple superphosphate) (Römer and Steingrobe, 2018). In recycled fertilizers, a large proportion of the P can be strongly chelated by iron (Fe), aluminium (Al) or calcium (Ca) cations or organic molecules, thus reducing P availability to plants. Large efforts are made during the production phase to reduce the formation of insoluble P molecules in chars (Mercl et al., 2020; Han et al., 2022; Khalaf et al., 2022), ashes (Kumpiene et al., 2016; Ahmed et al., 2021) or during the formation of P-salt precipitates such as struvite (Numviyimana et al., 2020) or vivianite (Prot et al., 2021). Unfortunately, it remains poorly understood how the solubilities of poorly soluble Fe-P, Al-P, Ca-P or organic-P compare to one another.

Similarly, when P fertilizers are applied to soil, most of their soluble forms can be adsorbed into the soil mineral phase or immobilized into soil organic matter. Organic forms of P can represent up to 65% of the total P in soils (Blume et al., 2016). In particular, monoesters forms such as inositol or phytic acid account for the majority

of the organic P pool (up to 70%) and are considered relatively unavailable (Turrión et al., 2001; Schneider et al., 2016). Inorganic P forms are also numerous and their abundance depends strongly on soil physico-chemical properties. For example, P is precipitated by Ca cations at alkaline pH, and adsorbed to Fe or Al (hydro-)oxides in more acidic conditions (Penn and Camberato, 2019).

Soil microorganisms play a crucial role in the release of orthophosphate from the organic and inorganic P pools (Richardson and Simpson, 2011; Amy et al., 2022). Doing so, soil microbes can improve plant growth by acting as an intermediary between unavailable P forms and plant uptake (Richardson and Simpson, 2011). Organic P is mineralized by the excretion of extracellular enzymes – phosphatases – that are able to hydrolyse organic molecules and release orthophosphate in the soil solution (Richardson and Simpson, 2011; Margalef et al., 2017). Solubilisation of inorganic P can also be facilitated by soil microorganisms. By the excretion of low molecular weight organic acids (LMWOAs) such as citrate, oxalate, gluconate, malate or lactate (Richardson and Simpson, 2011; Mihoub et al., 2017) P can be released from Ca-P bonds (Richardson and Simpson, 2011; Mihoub et al., 2017; Brucker et al., 2020) and from Al-P and Fe-P molecules (Zhuo et al., 2009; Wang et al., 2021). The main pathways of LMWOAs to solubilize P are the direct dissolution of minerals, shifts in soil pH, alteration in the surface characteristics of soil minerals and the formation of complexes with Ca, Fe and Al (Wang et al., 2016).

The role of soil microbes in the release of poorly soluble P sources as plant-available P has been widely studied in the past. However, most of the experiments were conducted on culturable microorganisms or on soil extracts. For instance, in culture medias, P was shown to be solubilised from apatite (Kim et al., 1997; Monroy Miguel et al., 2020; Amy et al., 2022), from phytate (organic P) (Shulse et al., 2019; Amy et al., 2022) and from Fe phosphates (He et al., 2007; Jha et al., 2013). Similarly, tests were conducted on soil extracts in which the solubilisation from apatite (Efthymiou et al., 2018; Brucker et al., 2020; Pastore et al., 2020b) and Fe phosphates (Efthymiou et al., 2018; Pastore et al., 2020a) was assessed. Unfortunately, the percentage of soil microorganisms that thrive in such laboratory conditions is far from the actual numbers that can be found in nature (Roesch et al., 2007). Studies

that make use of direct soil incubations or pot experiments are few and use mostly radioactive ^{31}P isotope (Pistocchi et al., 2018; Chen et al., 2021).

The availability to plants of P from poorly soluble sources is highly dependent on the availability of carbon (C) and to a lower extent nitrogen (N) (Demoling et al., 2007; Spohn and Kuzyakov, 2013; Pistocchi et al., 2018; Brucker et al., 2020; Pastore et al., 2020b, 2020a). Indeed, in the soil, solubilisation of P might be affected to meet stoichiometric homeostasis of the microbial biomass (Heuck et al., 2015; Spohn, 2016). Similarly, stoichiometry can also reveal P limitation of soil microorganisms when looking at extracellular enzymes (Moorhead et al., 2016). It is expected that soil microorganisms will invest relatively more in producing enzymes that mediate the mineralization of the nutrients by which they are most limited (Allison et al., 2010).

Here, we aimed to (i) assess the release of P as sodium bicarbonate ($NaHCO_3$)-extractable P (Olsen P) over time from various forms of poorly soluble P often found in recycled P fertilizers, and (ii) study the role of indigenous soil microorganisms in the change of Olsen P over time. To do this, we incubated a cropland soil with different organic and inorganic sources of poorly soluble P (hydroxyapatite, iron phosphate (III) and phytate) and measured at different dates Olsen P (a proxy for plant available P) and microbial biomass and activity. To our knowledge, this is the first time these forms of P are compared to each other and studied directly in soil.

2. Materials and methods

2.1. Soil sampling

We collected soil from the top 20 cm of a cropland in Haudricourt, Normandy, France (49°42'16.6"N 1°41'10.9"E) on 26/04/2021. This soil is classified as a Cambisol (WRB) with a loamy texture (8.5% clay, 71% silt and 20.5% sand). The soil was characterized before the start of the experiment, showing a pH-H_2O of 7.1, plant available P (Olsen P) of 28 mg P_2O_5 kg^{-1}, potassium oxide (K_2O) 177 mg kg^{-1}, magnesium oxide (MgO) 95 mg kg^{-1}, calcium oxide (CaO) 2198 mg kg^{-1}, K_2O:MgO 1.86, cation exchange capacity (CEC) 9.15 meq 100 g^{-1} and a soil organic matter content of 3%. We selected this soil because of its low P content and its neutral pH

(COMIFER, 2019). Soil was homogenized, sieved at 5 mm and kept at 4 °C before the start of the incubation experiments for no longer than 7 days.

2.2. Incubation conditions and P treatments

Soil moisture was adjusted to 60% water holding capacity (15% gravimetric moisture content) and placed into Microbox©, Sac 0_2 boxes (540 mL), which contain a specific filter that allows air exchange while preventing dehydration. Water content was monitored on a weekly basis and water was added when necessary. Water holding capacity was determined by placing dry soil in a steel cylinder. The cylinder was then placed in a container where water was added to rewet the soil by capillarity and immersion. Then, the cylinder with wet soil was placed over a dry sand bed where soil was allowed to drain for 2 hours. Afterwards, the gravimetric moisture content was measured, this value stands for 100% water holding capacity.

Different forms of P were added to the soil at a rate of 100 mg of P kg^{-1} of dry soil: iron phosphate (III) (P-Fe) (CAS registry number: 13463-10-0), hydroxyapatite (P-Ca) (CAS: 7758-87-4), phytic acid (P-Org) (CAS: 14306-25-3) and a 50/50 mixture of P-Ca and P-Org (P-Mix). The total amounts added were: 75.83 mg for P-Fe, 62.9 mg for P-Ca and 44.67 mg for P-Org. The high rate of P was selected because of the potentially low solubility of the P forms. We also included a positive control with a regular P fertilizer, triple super-phosphate (TSP), and a negative control with no P application. The different forms of P were mixed thoroughly in powdered form (< 0.5 mm) with 170 g of fresh soil. The boxes were placed in an air-ventilated incubator at 25 °C during 63 days. Subsets of boxes were destructively sampled 6 times in total during the 63 days: immediately after the application of P and 7, 14, 21, 35 and 63 days after the application of the different P forms. We included 4 replicates per treatment and per sampling date in a completely randomized design, for a total of 168 incubation boxes.

In a previous incubation experiment, the same soil was sampled 4 months earlier (January) and amended with the same P forms. The selection of a soil with a low P content (< 30 mg P_2O_5 kg^{-1}) was aiming at inducing conditions of P limitation, in which P fertilisation would be most useful in an agricultural setting (COMIFER, 2019). In those P limiting conditions, we hypothesised that the addition of poorly

soluble P sources would stimulate P acquisition strategies from soil microbes, thus allowing us to compare the effect of those strategies on the different P forms. Yet, we observed no differences between the P treatments in terms of changes in Olsen P over time or in terms of microbial response (the results are briefly presented in the Supplementary material). Therefore, in the present experiment, to alleviate C and N limitations and increase microbial demand for P, soil was amended with cellulose (α-cellulose, CAS: 9004-34-6) and potassium nitrate (KNO_3) at a rate of 2 g C kg^{-1} and 35 mg N kg^{-1} respectively. Another control treatment (Amend) was made in which KNO_3 and cellulose were added without any P application. We used these rates to ensure no C or N limitation while limiting effects on the soil microbial community composition.

2.3. Soil biological analyses

2.3.1. Soil microbial respiration

The activity of soil microorganisms was derived from measurements of soil carbon dioxide (CO_2) emissions (R_s) (AFNOR XP U44-163). In parallel to the microboxes, separate closed containers were used for these measurements. Fresh soil samples (equivalent of 25 g of dry soil) were mixed with the P forms and amendment to reproduce the different treatments described above and placed together with a flask with 10 mL of 0.5 M sodium hydroxide (NaOH) solution, and another flask filled with water to maintain the moisture content. The soil, water and NaOH solution flasks were placed in a 1 L closed container. The bottles followed the same experimental conditions as the main incubation and were sampled at the same dates. We included 4 repetitions per treatment, and the NaOH flasks were sampled destructively at each measurement date and replaced with freshly made NaOH solution. The containers were kept in the same incubator as the soil samples, in total randomization. A container with no soil was also included as a blank to account for the background CO_2 content. Containers were opened for analysis twice a week in the first two weeks and then weekly for the rest of the experiment, ensuring air ventilation. Carbon dioxide (CO_2) efflux was calculated by measuring the electrical conductivity (EC) in the sodium hydroxide (NaOH) solution. EC was measured at day 3, 7, 10, 14, 21, 28, 35, 42, 49, 56 and 63 after the start of the incubation. Results are expressed as μg CO_2-C g^{-1} dry soil.

2.3.2. Microbial biomass

Microbial biomass was quantified in two ways: by the chloroform fumigation-extraction method and by qPCR (total microbial DNA and genes of 16S and 18S rRNA subunits separately for bacterial and fungal biomass, respectively).

Carbon, N and P in the microbial biomass were calculated following the chloroform-fumigation protocol of (Jenkinson and Powlson, 1976) and the standardized protocol AFNOR ISO 14240. Briefly, 30 grams of fresh soil were placed in a beaker inside a desiccator under vacuum conditions. Inside the desiccator a beaker with chloroform is also placed. Samples are left in the dark for 24 hours and then C and N is extracted with 0.05 M potassium sulphate (K_2SO_4). The measurement was then performed in a TOC analyser (Shimadzu). Microbial C and N biomass (Microbial C and Microbial N) is then calculated. A blank of each sample, unfumigated, was also included. The values of Microbial C and Microbial N are then calculated by subtracting the blank values from the fumigated ones. Results are expressed in mg kg^{-1} of dry soil. No correction values were used. For the determination of microbial P biomass (Microbial P), we also fumigated soil samples with chloroform (Brookes et al., 1982). This protocol is very similar as the one previously described. Yet, in this case, only 5 g of fresh soil was fumigated. Phosphorus was subsequently extracted with 0.5 M $NaHCO_3$ (Olsen P). Results are also expressed in mg kg^{-1} of dry soil. No correction factor was applied. Different ratios of nutrients in microbial biomass were calculated, including C: P, C: N, N: P and C: N: P.

Total nucleic acids were extracted from 0.5 g of fresh soil using a FastDNA SPIN Kit (MP-Biomedicals, Santa Ana, CA, USA). The soil used was stored at – 80 °C before analysis. Total DNA was quantified by fluorimetry using the Fluorescent DNA quantitation Kit Hoechst 33258 (Biorad, Hercules, CA, USA). The results were expressed in mg kg^{-1} dry soil.

Fungal and bacterial biomass were quantified by performing 18S and 16S rDNA amplification respectively. Real time qPCR was performed in a total volume of 25 µL. Briefly, 18S primers (FU18S1 5′-GGAAACTCACCAGGTCCAGA-3′ and Nu-SSU-1536 5′-ATTGCAATGCYCTATCCCCA-3′) or 16S primers (63f 5′-CAGGCCTAACACATGCAAGTC-3′ and BU16S4 5′-CTGCTGCCTCCCGTAGG-3′)

were mixed with 5 ng of soil microbial DNA, 0.5 µM of 25 µL of LightCycler1 480 DNA SYBR Green I Master mix (Roche, Basel, Switzerland) and 0.25 mg mL^{-1} BSA (GeneON Bioscience, Ludwigshafen, Germany). Standard curves were obtained using serial dilutions of linearized plasmids containing the cloned 18S rRNA gene of *Fusarium graminearum* or 16S rRNA genes from the *Pseudomonas aeruginosa*. The amplification consisted of 40 cycles of PCR, 20 s at 95 °C, 30 s at 62 °C and 30 s at 72 °C. It was performed using LightCycler 480 real-time PCR system (Roche, Basel, Switzerland). The results are expressed as 18S or 16S rDNA gene copy number per gram of dry soil.

2.3.3. Potential enzymatic activities

Four different enzymes from the C, N and P cycle were analysed in this experiment. β-Glucosidase (E.C. 3.2.1.21) (Bglu), arylamidase (E.C. 3.4.11.2) (ARYLN) and acid and alkaline phosphatase (E.C. 3.1.4.1) (PAC) and (PAK) respectively.

Potential soil enzymatic activities were measured colorimetrically following a microplate technique (ISO 20130:2018) (Cheviron et al., 2021). Briefly, for Bglu and ARYLN activities, 4 g of fresh soil were homogenized with 25 mL of ultrapure water during 10 minutes at 250 rpm. For PAC and PAK a solution of Trizma buffer (50 mM) adjusted to pH 5.5 and 11 respectively was used. 125 µL were pipetted into 96-well microplates and mixed with their respective substrate solutions. 4-nitrophenyl β-D-glucopyranoside (CAS N°: 2492-87-7) for Bglu, L-leucine β-naphthylamide hydrochloride (CAS N°: 893-36-7) for ARYLN and 4-nitro-phenylphosphate disodium salt hexahydrate (CAS N°: 333338-18-4) for PAC and PAK. The final concentrations of the substrate solutions were 0.05, 0.008 and 0.05 mol L^{-1} respectively. Microplates were incubated at 37 °C for 1 hour for Bglu, 2 hours for ARYLN and 30 minutes for PAC and PAK. The reaction was then stopped by the addition of 25 µl 0.5 M calcium chloride ($CaCl_2$) and 100 mM 100 µl Trizma at pH 12 for Bglu, PAC and PAK. Then plates are centrifuged during 5 minutes at 1500 g. For ARYLN, the reaction was stopped by the addition of ethanol 96% and the coloration revealed after the addition of 100 µl of acidified ethanol and 100 µl of DMCA. ARYLN's plates were incubated in the dark for 20 minutes at room temperature before measurement. The measurements were performed in a Varioskan Flash-Thermo microplate reader. Absorbance was measured at 405 nm for Bglu, PAC and

PAK and at 540 nm for ARYLN. Results of potential enzymatic activities were expressed in nmol PNP (paranitrophenol) min^{-1} g^{-1} of dry soil for Bglu, PAC and PAK activities and in β-naphthylamine min^{-1} g^{-1} of dry soil for ARYLN activities.

Stoichiometric ratios of enzymes were also calculated based on the vectorial approach proposed by Moorhead et al., (2016). Vector length and angle were calculated as in equation [1] and [2]:

$$\text{Vector length} = \sqrt{(\text{Bglu/PAC})^2 + (\text{Bglu/ARYLN})^2} \quad [1]$$

$$\text{Vector angle} = \text{DEGREES}\,(\text{ATAN2}(\text{Bglu/PAC}), (\text{Bglu/ARYLN}))) \quad [2]$$

In general terms, the steeper angles are associated with higher P limitation of soil microorganisms and the longer vectors are associated with a more important C limitation. Yet, the interpretations from these approaches should be carefully considered as recent papers have raised debate on their utilization (Rosinger et al., 2019; Mori et al., 2023).

2.4. Soil chemical analyses

We used the Olsen method (ISO 11263) (extraction in 0.5 M sodium bicarbonate ($NaHCO_3$)) as a proxy for plant available P. In brief, 5 g of fresh soil were mixed with 100 mL of 0.5 M $NaHCO_3$ at pH 8.5 for 30 minutes. One g of P-free and pH 7 active charcoal was added to the mix. This was done to reduce the interference of soil organic matter in the colour development. After the extraction, the solution was filtered with a P-free 5 µm paper filter. Two mL of extract were mixed with 8 mL of sulfomolibdic reagent and was incubated for 60 minutes. After, coloration was revealed after heating in a water bath for 10 minutes at 90 °C. P was measured in the 825 nm wavelength using a spectrophotometer (Varian Cary 50 Scan UV Visible Spectrophotometer).

Changes in Olsen P were investigated in absolute and relative values. The relative values of Olsen P were calculated by dividing each value of Olsen P by the mean of the initial value (beginning of incubation) of the corresponding treatment (Equation 3), where P_a is the content of P at a given time and treatment, P_{a1} is the level of P at

the beginning of the experiment for that particular treatment. This was done to assess the changes in Olsen P over time with respect to their initial values.

$$\text{Relative P} = P_a/P_{a1} \quad [3]$$

Soil pH was measured in 1:5 v:w in water extracts using a glass-electrode (Mettler Toledo). Soluble C and N were determined in a 0.05 M potassium sulphate (K_2SO_4) extract performed on fresh soil (Makarov et al., 2013). Carbon and N were extracted from 30 g of soil in 100 mL 0.05 M K_2SO_4 solution during 2 hours. Extract was centrifugated at 5000 rpm for 5 minutes to remove solid particles. Soil extracts were frozen at -20 °C before measurement. The measurement was performed in a TOC device (TOC-V CSH and TNM-1, Shimadzu). Nitrate (NO_3) and ammonium (NH_4) were also extracted from 25 g of soil in 75 mL 1 M potassium chloride (KCl) solution for one hour. Extract was centrifugated at 5000 rpm for 5 minutes to remove solid particles. Soil extracts were also preserved at -20 °C before performing the measurement. NO_3 and NH_4 were determined colorimetrically in a Gallery device (Thermo Fisher Scientific).

2.5. Statistical analyses

All the statistical analyses were done using RStudio software (v4.0.2).

For each measured variable, differences between the P form treatments were estimated performing an analysis of variance (ANOVA). The assumptions of homogeneity, normality and heteroscedasticity were inspected by analysing the residuals of ANOVA models. Homogeneity was breached due to higher variance in the TSP treatment. Therefore, when ANOVA results showed significant differences, pairwise comparison between treatments were performed using Games Howell's post-hoc test, which accounts for heterogeneous variances between treatments (Hilton and Armstrong, 2006).

Differences over time in absolute and relative changes in Olsen P between the different P form treatments were analysed by performing an analysis of covariance (ANCOVA). ANCOVA was performed using the function "lm" in RStudio (R Core Team, 2022). The assumptions of homogeneity, normality and heteroscedasticity were inspected by analysing the residuals. Similarly as for the ANOVA analyses,

higher variability in the TSP treatment led to heterogeneity of the residuals. It was therefore decided to study the changes in absolute and relative Olsen P over time for TSP separately from the other treatments. Model assumptions were checked again on those separate models.

To assess changes in microbial activity over time cumulative soil respiration (Rs) was analysed using an asymptotic exponential function of time (Equation 4).

$$y = a * (1 - \exp(-b * \text{Time})) \quad [4]$$

Where parameter a describes the asymptote of the curve (the maximum value of Rs) and parameter b describes the shape of the curve (how quickly Rs reaches its asymptotic value).

To assess the differences in microbial activity over time between treatments, models fitting different parameters to the different treatments were compared (using AIC) with models in which parameters were constrained to be the same for all treatments using the R "gnls" function (Generalized Least Squares) (Pinheiro et al., 2022).

To test for the effects of soil chemical and biological properties on the changes in Olsen P, a multiple regression analysis was conducted following the procedure used in Moinet et al. (2016). A candidate set of models was established to identify which of the soil properties best explained the changes in Olsen P (using relative values of Olsen P). The set of models was created using different combinations of potential explanatory variables with and without accounting for the interaction with the different P form treatments. In total, over 160 models were evaluated. The models were ranked using the AICc to determine the Kullback-Leibler (KL) best model (Burnham and Anderson, 2002). The AICc identifies the model(s) most strongly supported by the data and is based on bias-corrected, maximized log-likelihood (LogLik) of the fitted model with a penalty for the number of parameters used. The model with the smallest AICc ($AICc_{min}$) is the most strongly supported. The ΔAICc value is calculated for each model i as $\Delta AICc = AICc_i - AICc_{min}$. Following convention, models with $\Delta AICc < 2$ are substantially supported by the data; whereas models with $\Delta AICc > 2$ indicate considerably less or no support (Anderson, 2007). A measure of the strength of support for either model is described by the model probability (Akaike weights, A_{wi}). This is the probability that model i is the KL best

model, given the data and candidate set of models (Anderson, 2007). The sum of A_{wi} of the models in a candidate set equates to 1.

All data files are present in the ZENODO online repository (Velasco-Sánchez et al., 2022).

3. Results

3.1. Soil biological activities

The addition of cellulose and KNO_3 increased largely soil respiration, yet the different P forms had a limited effect on soil respiration (Figure 3.1). TSP and P-Fe treatments, showed slightly different soil respiration curves. Following Equation 4, P-Fe showed different ($p < 0.05$) a and b parameters, and TSP showed a significantly different b parameter compared to the rest of treatments showing slightly increased activities at the beginning of the incubation. However, these differences were very small and no difference was observed on total C respired between the P treatments ($p > 0.05$) (Figure 3.1).

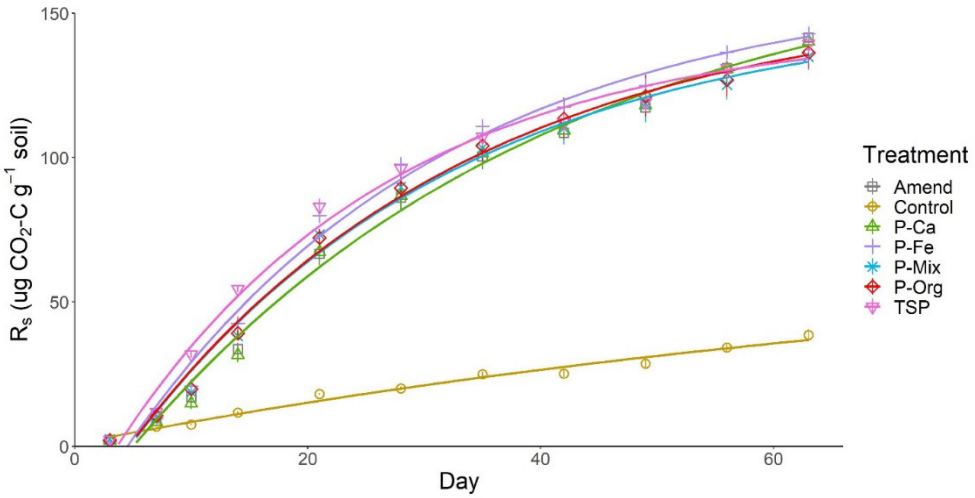

Figure 3.1. *Cumulative soil respiration (R_s). P-Fe showed significantly higher growth and asymptote and TSP showed higher growth rate than the rest of amended treatments. No effect was observed on cumulative respiration across the amended treatments*

Microbial biomass C and N increased over time over the incubation but no differences across treatments were found (Table 3.1). Microbial P showed no difference across treatments and sampling dates. The different ratios of microbial biomass nutrient concentration showed slight differences between treatments, only P-Ca showed statistically different intercepts for microbial C: N and microbial C: N: P ratios. Over time, only C: P showed a positive slope, the other ratios studied showed no change over time.

Total DNA in the soil showed no difference across treatments or over time (Table 1). Similarly, 16S gene copies (bacterial biomass) did not reveal differences between treatments nor time. Fungal biomass (18S gene copies), showed no difference across treatments but it significantly increased over time. The intercept of bacterial to fungal biomass ratio (16S: 18S) was significantly higher for the unamended control (175.27 ± 10.2, $p < 0.01$) compared to the amended treatments.

Enzymatic activities were partially affected by the addition of cellulose and nitrogen but not by the application of different poorly soluble P forms (Table 1). The addition of cellulose and KNO_3 slightly increased the intercept of Bglu activities compared to the unamended control (23.37 ± 0.89 PNP min^{-1} g^{-1}). The intercept of PAC activity was decreased by the addition of TSP (46.71 ± 1.54 PNP min^{-1} g^{-1}) compared to the rest of treatments. No treatment effect was found in PAK and ARYLN activities. The activities of Bglu, PAK and ARYLN did not increase nor decrease over time. PAC activities slightly decreased over time for all treatments. Regarding enzymatic vectors (length and angle) no treatment effect was observed. Both length and angle showed no significant slope for the amended treatments.

Table 3.1. *Analysis of covariance (ANCOVA) on the different soil biological variables studied. Values represent the estimates for intercepts (I) and slopes (S) of all treatments. Letters in bold indicate significant differences between treatments ($p < 0.05$). When I or S estimates are not significantly different from 0, ns (non-significant) is indicated.*

Variable		Control	Amend	TSP	P-Fe	P-Ca	P-Org	P-Mix
Microbial biomass C (mg C kg^{-1})	I	54.25	38.75	52.97	64.21	56.21	53.11	46.88
	S	1	2.04	1.68	1.54	1.86	2.16	2.64
Microbial biomass N (mg N kg^{-1})	I	23.29	21.11	24.24	26.72	28	28.62	26.07
	S	0.46	0.67	0.83	0.55	0.61	0.62	0.68
Microbial biomass P (mg P kg^{-1})	I	7.75	8.67	9.02	8.15	9.11	9.16	10.21
	S	ns	ns	ns	ns	ns	ns	ns
Microbial C: N	I	3.67 **(b)**	3.24 **(b)**	2.43 **(b)**	3.01 **(b)**	7.99 **(a)**	2.89 **(b)**	3.15 **(b)**
	S	ns **(a)**	ns **(a)**	ns **(a)**	ns **(a)**	-0.13 **(b)**	ns **(a)**	ns **(a)**
Microbial C: P	I	8.05	5.1	4.48	9.49	8.68	9.74	6.14
	S	0.09	0.22	0.13	0.07	0.1	0.14	0.22
Microbial N: P	I	2.86	3.04	3.82	3.74	3	3.9	2.79
	S	ns	ns	ns	ns	ns	ns	ns
Microbial C: N: P	I	0.57	0.58	0.14	0.47	1.34	0.55	0.48
	S	ns **(a)**	ns **(a)**	ns **(a)**	ns **(a)**	-0.03 **(b)**	ns **(a)**	ns **(a)**
Total DNA (mg DNA kg^{-1})	I	120.02	122.94	126.95	122.94	126.93	126.73	128.03
	S	ns	ns	ns	ns	ns	ns	ns
Fungal biomass (18S gene copies)	I	1.81 $x10^7$	8.95 $x10^7$	8.66 $x10^7$	7.7 $x10^7$	13.84 $x10^7$	9.5 $x10^7$	9.6 $x10^7$
	S	0.13 $x10^6$	2.76 $x10^6$	2.23 $x10^6$	3 $x10^6$	2.4 $x10^6$	4.48 $x10^6$	4.55 $x10^6$
Bacterial biomass (16S gene copies)	I	3.2 $x10^9$	3.35 $x10^9$	3.12 $x10^9$	3.18 $x10^9$	3.41 $x10^9$	3.45 $x10^9$	3.81 $x10^9$
	S	ns	ns	ns	ns	ns	ns	ns
Bacterial: Fungal biomass (16S: 18S)	I	175.3 **(a)**	50.68 **(b)**	44.44 **(b)**	69.02 **(b)**	47.84 **(b)**	62.68 **(b)**	58.85 **(b)**
	S	-0.58	-0.78	-0.64	-1.24	-0.72	-1.05	-1.03

Table 3.1 continues on next page

(Table 3.1 continued)

Variable		Control	Amend	TSP	P-Fe	P-Ca	P-Org	P-Mix
β-Glucosidase (PNP min^{-1} g^{-1})	I	23.37 **(b)**	26.33 **(a)**	26.49 **(a)**	26.32 **(a)**	26.38 **(a)**	26.4 **(a)**	24.11 **(a)**
	S	ns	ns	ns	ns	ns	ns	ns
Acid phosphatase (PNP min^{-1} g^{-1})	I	48.99 **(a)**	50.77 **(a)**	46.71 **(b)**	51.2 **(a)**	49.84 **(a)**	50.66 **(a)**	50.14 **(a)**
	S	-0.11	-0.1	-0.08	-0.1	-0.09	-0.12	-0.05
Alkaline phosphatase (PNP min^{-1} g^{-1})	I	45.51	44.71	42.58	45.25	44.27	45.76	44.8
	S	ns	ns	ns	ns	ns	ns	ns
Arylamidase (β-naph min^{-1} g^{-1})	I	2.39	2.59	2.66	2.57	2.52	2.64	2.47
	S	ns	ns	ns	ns	ns	ns	ns
Enzymatic Vector Length	I	9.38	10.26	10.12	9.85	10.6	9.73	9.87
	S	ns	ns	ns	ns	ns	ns	ns
Enzymatic Vector Angle	I	86.61	87.04	86.7	87.14	87.09	87	87.17
	S	0.02 **(a)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**

3.2. Soil chemical changes

Application of the different forms of poorly soluble P had a small impact on the soil chemical variables studied (Table 3.2). Carbon in K_2SO_4 extracts increased over time for all treatments but showed no statistical difference between treatments. NO_3 and N in K_2SO_4 extracts increased over time for the unamended control (2.58 and 0.24 respectively) but showed no significant slope for the rest of treatments. Intercepts for NO_3 and N in K_2SO_4 extracts were also significantly lower for the unamended control (40.17 and 11.46 mg N kg^{-1} respectively) (Table 3.2). C: N ratio in K_2SO_4 extracts presented a different intercept and slope for the unamended control. No NH_4 was detected over the course of the incubation. Regarding soil pH, we found no significant change over time. TSP intercept had a significantly different intercept compared to the poorly soluble P forms and negative controls (7.35 ± 0.05). The average pH of the soil for all treatments was 7.5 ± 0.02.

***Table 3.2**. Analysis of covariance (ANCOVA) on the different soil chemical variables studied. Values represent the estimates for intercepts (I) and slopes (S) of all treatments. Letters in bold indicate significant differences between treatments ($p < 0.05$). When I or S estimates are not significantly different from 0, ns (non-significant) is indicated.*

Variable		Control	Amend	TSP	P-Fe	P-Ca	P-Org	P-Mix
pH	I	7.47 **(a)**	7.5 **(a)**	7.35 **(b)**	7.53 **(a)**	7.44 **(a)**	7.53 **(a)**	7.53 **(a)**
	S	ns	ns	ns	ns	ns	ns	ns
Olsen P	I	9.58 **(c)**	8.47 **(c)**	63.93 **(a)**	14.66 **(b)**	9.31 **(c)**	12.14 **(b)**	8.73 **(c)**
(mg P kg^{-1})	S	ns **(b)**	ns **(b)**	-0.32 **(c)**	ns **(b)**	ns **(b)**	0.19 **(a)**	ns **(b)**
Relative Olsen P	I	0.88 **(b)**	0.75 **(b)**	0.82 **(b)**	0.86 **(b)**	0.76 **(b)**	1.08 **(a)**	0.82 **(b)**
	S	0.008 **(b)**	0.006 **(b)**	-0.004 **(c)**	0.003 **(b)**	0.006 **(b)**	0.014 **(a)**	0.013 **(a)**
K2SO4-extractable	I	19.07	21.34	19.43	18.2	18.88	20.83	22.75
C (mg C kg-1)	S	0.24	0.28	0.4	0.39	0.34	0.33	0.28
K_2SO_4-extractable N	I	11.46 **(b)**	42.12 **(a)**	38.01 **(a)**	31.93 **(a)**	40.55 **(a)**	40.3 **(a)**	37.39 **(a)**
(mg N kg^{-1})	S	0.87 **(a)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**
NO_3	I	40.17 **(b)**	145.51 **(a)**	142.03 **(a)**	134.26 **(a)**	149.2 **(a)**	156.08 **(a)**	152.2 **(a)**
(mg NO_3 kg^{-1})	S	2.58 **(a)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**
Soil C: N (K_2SO_4-extractable)	I	2.49 **(a)**	0.49 **(b)**	0.89 **(b)**	0.95 **(b)**	0.5 **(b)**	0.66 **(b)**	0.74 **(b)**
	S	-0.04 **(b)**	ns **(a)**	ns **(a)**	ns **(a)**	ns **(a)**	ns **(a)**	ns **(a)**

3.3. Differences in Olsen P

Initial Olsen P at the first measurement date differed significantly between treatments (Figure 3.2 & Table 3.2). TSP was the form of P with the highest Olsen P. Across the poorly soluble P forms, P-Fe showed a significantly higher Olsen P. P-Ca, P-Org and P-Mix showed the same levels of Olsen P as the control treatments (Control and Amend, which were not significantly different from each other).

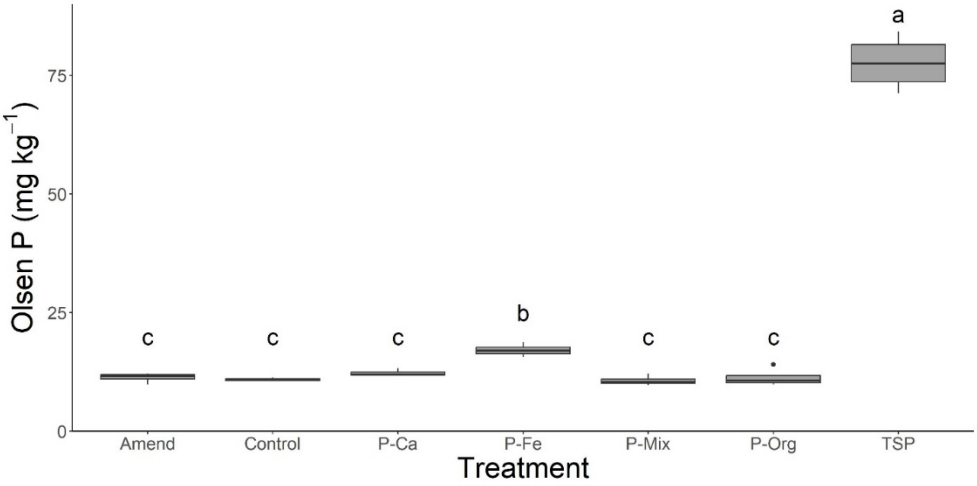

Figure 3.2. *Boxplot of Olsen P at the first measurement date. Boxes represent the interquartile range of the data, line inside the box indicates the median value, whiskers show maximum and minimum values and dots indicate potential outliers. Control = no additions, Amend = addition of cellulose and KNO_3, P-Ca = Hydroxyapatite, P-Org = Phytic acid, P-Fe = Iron phosphate (III), P-Mix = 50/50 mixture of P-Ca and P-Org, TSP = Triple Super Phosphate. Letters indicate statistically different groups based on Games-Howell post-hoc test.*

Changes in Olsen P over time were markedly different between treatments (Table 3.2 & Figure 3.3). Olsen P in the TSP treatment decreased over time exponentially showing an asymptote of 49.56 mg kg^{-1} (Figure 3.3). However, P levels significantly increased linearly over time in the P-Org treatment (P-Org slope 0.19 ± 0.056, $p = 0.027$) (Figure 3.3). The rest of the treatments, including the controls with no P addition showed no significant change in Olsen P over time ($p > 0.05$). The intercepts of TSP ($p < 0.005$) and P-Fe ($p < 0.005$) were greater than to that of the control treatment, indicating higher initial Olsen P. The intercept of P-Org appeared marginally lower than that of the control ($p = 0.043$). No differences were found between Control and Amend.

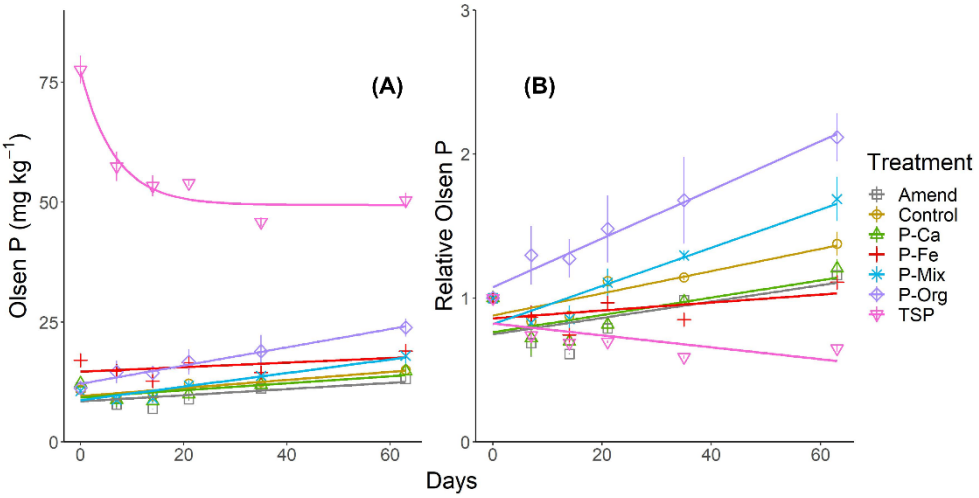

Figure 3.3. *Change over time for absolute (A) and relative Olsen P (B). Relative Olsen P refers to changes in Olsen P with respect to their correspondent initial values. Control = no additions, Amend = addition of cellulose and KNO_3, P-Ca = Hydroxyapatite, P-Org = Phytic acid, P-Fe = Iron phosphate (III), P-Mix = 50/50 mixture of P-Ca and P-Org, TSP = Triple Super Phosphate.*

The relative change in Olsen P, calculated by normalizing the values of Olsen P for each treatment, increased for all poorly soluble P treatments and the controls with no P addition (slope 0.006 ± 0.001, p = 0.004) (Figure 3.3 and Table 3.2). Moreover, relative Olsen P increased more for the treatments that received organic P, P-Org and P-Mix, than for the other treatments (P-Org slope 0.017 ± 0.003, p < 0.001, and P-Mix slope 0.013 ± 0.003, p = 0.006). Relative change in Olsen P was negative for TSP (TSP slope -0.004 ± 0.003, p < 0.001).

3.4. Multiple regression analysis of relative change in Olsen P

Different linear models in multiple combinations were ranked to determine the best explanatory model for the changes in relative Olsen P (Table 3.3). Models were ranked based on the their Akaike´s information criterion (AICc) (Moinet et al., 2016).

Table 3.3. *Summary of the first 5 top ranked models to explain the change in the relative change in Olsen P. AICc = Akaike's information criterion. ΔAICc = AICc – $AICc_{min}$ for best model. Aw = Akaike's weight. LogLik = Log likelihood ratio.*

Rank	Model	AICc	ΔAICc	Aw	Cumulative Aw	LogLik
1	Microbial C: P x Treatment + 18S x Treatment	- 35.34	0	0.94	0.94	43.39
2	Microbial P x Treatment + Microbial C x Treatment + 18S x Treatment	- 29.92	5.42	0.06	1	50.76
3	Microbial C: P x Treatment	- 10	25.34	0	1	21.63
4	Microbial C x Treatment + Microbial P x Treatment + Microbial N x Treatment	- 4.92	30.42	0	1	39.99
5	Microbial C x Treatment + Microbial P x Treatment	- 2.69	32.65	0	1	26.99

The first model in Table 3.3 (Microbial C: P x Treatment + 18S x Treatment) is the best at explaining the change in relative Olsen P. The ΔAICc with the second model was 5.42, showing a considerably better explanatory power of the first model. The Aw of the first model was 0.94, which means a 94% chance of being the most robust model. Model 1 contained biological variables (C: P in microbial biomass and 18S gene copies) as well as their interaction with the P forms treatments. Models 2 to 5 were also related to biological values. The adjusted R^2 of the first model was 0.693.

In the first ranked model, C: P in microbial biomass was positively correlated with relative change in Olsen P for all treatments ($p = 0.0015$) and no statistical difference was found in slopes among treatments nor intercepts (Figure 3.4, panel A). The model relating changes in relative Olsen P to microbial C:P biomass and treatment explained a substantial amount of the variation in the data, with an adjusted R^2 of 0.61, indicating that 61% of the variability in the changes in relative Olsen P was accounted by the model. Similarly, in the first ranked model, fungal biomass (18S gene copies) was only positively correlated with the relative change in Olsen P for the treatments that received organic P, P-Org treatment ($p < 0.001$) and P-Mix

treatment with marginal significance ($p = 0.051$) (Figure 3.4, panel B). No significant correlation was found for 18S gene copies for the other treatments. The adjusted R^2 of this model was 0.5.

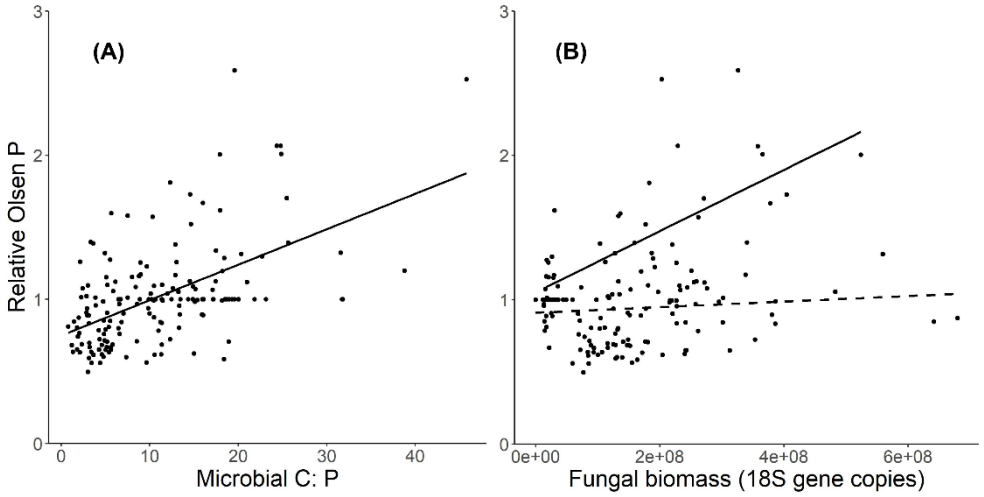

Figure 3.4. *Correlation between relative change in Olsen P and microbial C: P (panel A), and fungal biomass (18S gene copies) (panel B) as explained by the first ranked model: Relative Olsen P = Microbial C: P x Treatment + 18S x Treatment. In panel A, the plain line represents the average fit of the positive correlation for all treatments ($p < 0.05$). In panel B, the plain line represents the fit of the significant positive correlation for the P-Org treatment only and the dashed line represents the average fit for the rest of the treatments ($p > 0.05$). The adjusted R^2 were 0.69, 0.61 and 0.5 for the first ranked model, panel A and panel B respectively.*

4. Discussion

4.1. Olsen P from different poorly soluble P forms

Olsen P in the TSP positive control was found to be the highest across all treatments (Figure 3.2 & 3.3). As expected, Olsen P levels in the poorly soluble P forms were much lower than that of TSP. Olsen P in the TSP treatment decreased over time, indicating a fixation of the most readily soluble P molecules onto soil particles and soil organic matter.

Soils amended with P-Fe, however, showed a slightly higher Olsen P than the other non-soluble treatments at the beginning of the incubation (Figure 3.2). It has been

previously shown that the solubility of poorly soluble P-Fe minerals increases with alkaline pH (Lindsay and De Ment, 1961; Penn and Camberato, 2019), which could explain the larger difference in Olsen P between P-Fe and the other treatments. Iron phosphates can supply significant amounts of P for crops. The use of soluble sources of P from iron phosphates, such as vivianite, is of great potential in agriculture (Wu et al., 2019b). In our study, we selected a crystalline form of Fe-P that may be formed during the production of biofertilizers from wastewaters which have been treated with Fe salts. Studies which have tested and compared the solubility from crystalline Fe sources are scarce. Strengite, a crystalline P-Fe mineral, has been compared with fluorapatite (a poorly soluble calcium phosphate) showing slightly higher P solubility values in alkaline pH (Lindsay and De Ment, 1961). Strengite has also been shown to be used by plants in greenhouse studies (Armstrong et al., 1993). P from strengite can also be solubilized by soil microorganisms (Bolan et al., 1987). However, in other field and greenhouse studies in which biosolids with different concentrations of P-Fe and P-Ca forms, no large differences were found and their low plant availability was confirmed (Maguire et al., 2001; Ashekuzzaman et al., 2021). It should be noted that different degrees of crystallinity in the poorly soluble forms that were used in this study can also affect largely their extractability in $NaHCO_3$. For example, different types of P-Fe salts (Lindsay and De Ment, 1961; Bolan et al., 1987), P-Ca (Tosun et al., 2021), and organic P forms (Amadou et al., 2021) are known to lead to different levels of available P.

The estimated Olsen P of the studied forms is also affected by the extraction method used. In our study, we chose extraction in $NaHCO_3$ (Olsen) because of its widespread use as an indicator of potentially plant available P. Olsen P is also suitable for this specific soil as its pH is slightly alkaline. Yet, other methods are used for other types of soils and may show different levels of P (Wuenscher et al., 2015). For instance, Ashekuzzaman et al., (2021) reported that P-Ca forms were overestimated when using the Morgan's P test. In our study, we observed also an overestimation of soluble P when using ammonium oxalate (Joret-Hébert) as a P extractant (data not shown).

4.2. Change in Olsen P: the role of soil microbes

The absolute change in Olsen P depends on the initial levels of Olsen P, as the P values in the TSP treatments were considerably higher than in the poorly soluble ones. We, therefore, normalised the values of Olsen P and analysed the relative change over time.

Relative Olsen P was found to significantly increase over time for all the poorly soluble treatments including the controls (Figure 3.3, panel B). Soil microorganisms have been found to solubilize P from a wide range of P forms and different soils (Pistocchi et al., 2018; Houben et al., 2019; Brucker et al., 2020; Pastore et al., 2020a, 2020b). As shown by the model in Table 3.3, the changes in Olsen P in our incubation were correlated with microbial C: P and, in the case of the P-Org treatment, fungal biomass (18S gene copies). These results can be interpreted as a dependence of microbes on C to access P. Microbes might start mobilizing poorly soluble P when their C: P is over their homeostatic value (Spohn, 2016). The correlation between microbial C:P and changes in Olsen P were observed also in our control treatment in which no cellulose nor KNO_3 was added. This could mean that the change in Olsen P does not depend uniquely on the amount of C in the soil, but also on the ability of soil microorganisms to incorporate C into their biomass. In a previous experiment (Supplementary material), we did not observe microbial-induced changes in Olsen P. Nevertheless, in the rhizosphere, the constant release of different C sources by roots or the decomposition of crop residues could improve the acquisition of C by soil microorganisms (Shahbaz et al., 2017; Zhang et al., 2019). We attempted to replicate this higher exchange of C and N by the addition of cellulose and nitrogen.

Relative Olsen P increased to a greater extent in the organic P treatments (P-Org and P-Mix) (Figure 3.3) compared to the rest of poorly soluble P treatments and controls. Concurrently, the number of 18S gene copies was a significant factor in the best explanatory model for P-Org and (marginally) for P-Mix (Figure 4). This would suggest that fungi were particularly adapted to access P from organic recalcitrant sources such as phytate. Organic sources of P have been shown to be solubilised by fungi such as *Aspergillus* and *Penicillium* genera (Gaind and Nain, 2015; Efthymiou et al., 2018). Moreover, fungi have been shown to solubilize P from phytate at a

greater extent than bacteria by their greater production of phytase (Gaind and Nain, 2015; Singh and Satyanarayana, 2015). In our experiment, the addition of cellulose significantly reduced 16S: 18S ratio and, on average, increased fungal biomass (yet non-significant) (Table 3.1). This higher proportion of fungal biomass might have contributed to a higher production of phytase that ultimately could have increased Olsen P in the P-Org treatment.

On the other hand, the lack of differences in relative Olsen P between the controls with no P application and the P-Ca and P-Fe treatments would suggest that the newly soluble P does not necessarily come from the added P (hydroxyapatite or iron phosphate (III)), but potentially from the native soil P pool. These results suggest that P-Ca and P-Fe were very recalcitrant for the soil microorganisms under our experimental conditions. This contrasts with other studies in which soil microorganisms were able to solubilise P from apatite (Brucker et al., 2020) and goethite (Fe-phosphate) (Pastore et al., 2020a).

For the TSP positive control, relative Olsen P decreased over time (Figure 3.3). In this case, levels of soluble P likely exceeded the requirements of soil microorganisms. The application of large concentrations of soluble P had also a negligible impact on soil microbial activities (Figure 3.1 and Tables 3.1 & 3.2), P microbial biomass and the relative abundances of bacteria and fungi (16S: 18S). These results contrast with literature in which phosphatase activities decreased (Mori, 2022), soil respiration increased (Ozlu and Kumar, 2018), P microbial biomass increased (Fontana et al., 2021) and microbial community composition was altered (Wang and Huang, 2021) after P application. We assume that these effects are dependent on long-term and repeated P fertilization practices and not observable in shorter experiments like ours and that the effect of P fertilizer is generally small on soil microorganisms (Bünemann et al., 2004).

Strategies to solubilise P by soil microorganisms are numerous. In our study, we could not associate any strategy with changes in Olsen P as we did not detect a significant difference among treatments for pH (as a proxy of soil acidification by LMWOAs production) or in phosphatase activities. Nevertheless, the production of LMWOAs is expected to be small and could not directly change soil pH (Evangelou et al., 2008; Shi et al., 2011). Likewise, enzymatic activity results indicate potential

activity and not the actual enzymatic activity. Therefore, our results would indicate that there is no significant difference in the potential of soil microorganisms in producing phosphatase (Nannipieri et al., 2018). Solubilization of P-Org though, might be more related with the activity of other P-related enzymes such as phytase (Amy et al., 2022).

5. **Conclusion**

In this study, we compared Olsen P of different poorly soluble P forms and assessed the role of microbes in the changes found over time in the top horizon of a Cambisol. To our knowledge, this is the first time these forms are compared to one another in a soil incubation. We found that Olsen P values were dependent on the interaction between the nature of the P form applied and the soil microbial community. Our findings show that activity of soil micro-organisms has potential to alter the solubility of P from different common recalcitrant P molecules in recycled fertilizers.

Our results suggest that rather than a static pool, soil microbes have the potential to mobilise poorly soluble organic sources such as phytate. This is supported by our most strongly supported model, which involved both the Microbial C: P and Fungal biomass as explanatory variables and which, combined, explained 69% of the variation in the temporal dynamics of Olsen P. Our findings suggest that improving fungal biomass and available C in soils could result in increasing the amount of plant available P. This may have an impact on the valorisation of new recycled biofertilizers that contain organic P forms (e.g. hydrochars, biochars, digestates, etc.), as these forms might become available for crops by the action of soil microorganisms.

Further research should investigate which chemical and biological soil factors trigger the different dynamics of P when it comes to compare poorly soluble forms of P. Different soil properties (e.g. pH, soil organic matter, texture, microbial composition, …) may lead to different trends. It remains unknown how soil microorganisms would contribute to the release of P from different P molecules in soils with contrasting pH. Similarly, different initial ratios of bacteria to fungi or land uses could also result in the solubilization of different poorly soluble forms of P.

6. **Acknowledgments**

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Supplementary material

Note: The figures here displayed represent a preliminary incubation conducted with the same soil and conditions as the ones presented for the incubation in the manuscript. In this preliminary incubation no C nor N were added. The sampling date for this preliminary incubation was earlier in the season, January 2021.

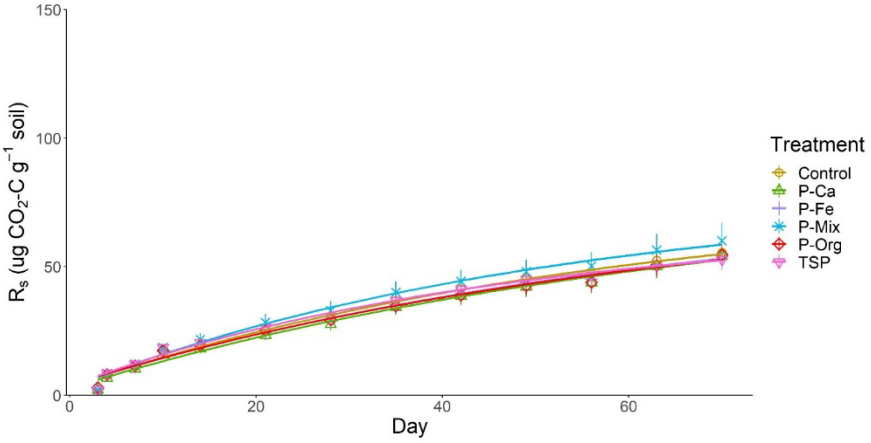


Figure 3.S1. *Cumulative C respiration (R_s) of preliminary incubation. Regression lines are exponential equations. Colour bands indicate 95% confidence interval.*

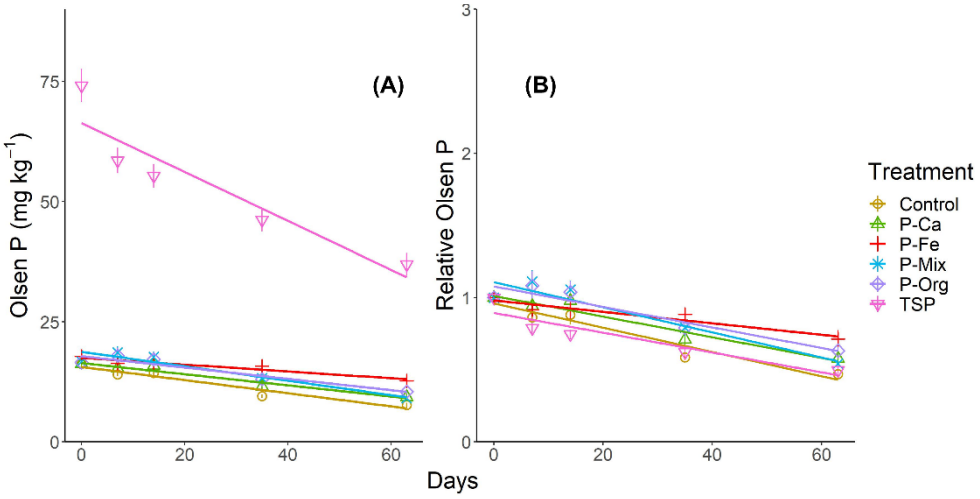


Figure 3.S2. *Change over time for absolute (panel A) and relative Olsen P (panel B) of preliminary incubation. Control = no additions, P-Ca = Hydroxyapatite, P-Org = Phytic acid, P-Fe = Iron phosphate (III), P-Mix = 50/50 mixture of P-Ca and P-Org, TSP = Triple Super Phosphate. Coloured bands indicate 95% confidence interval.*

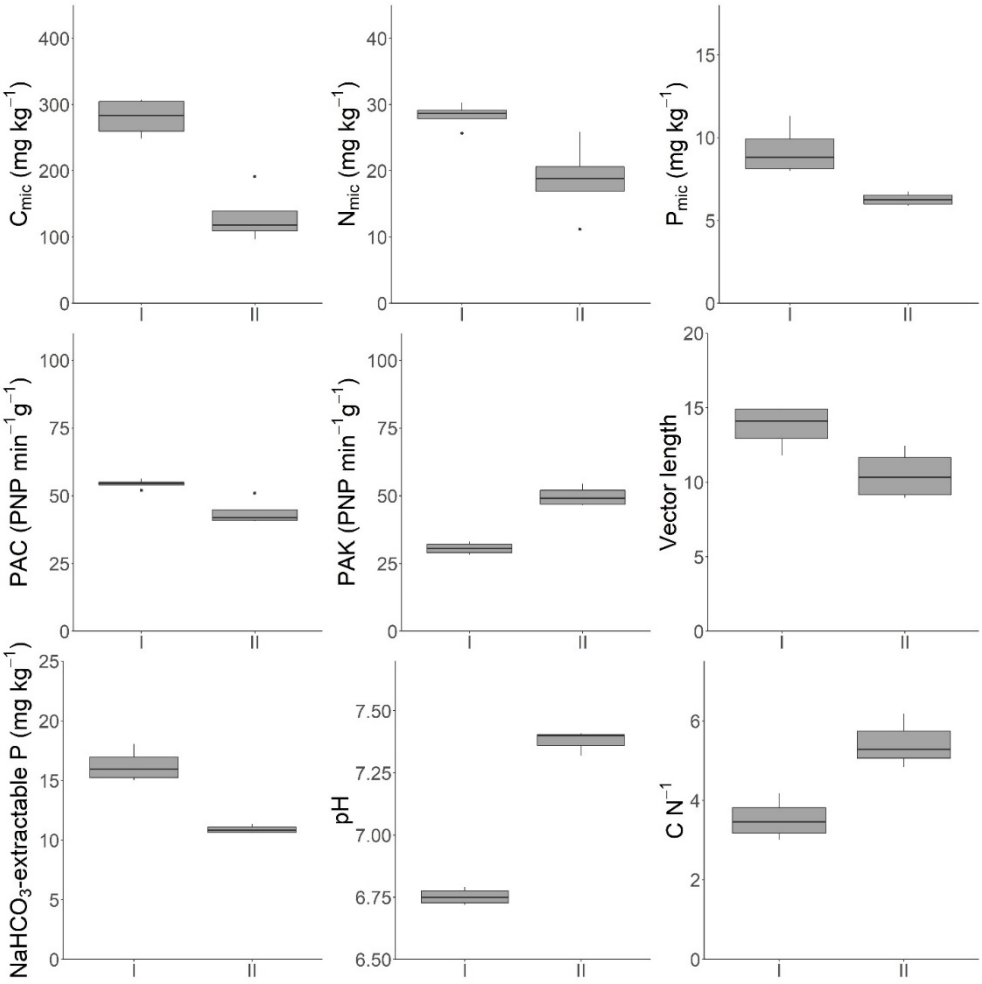
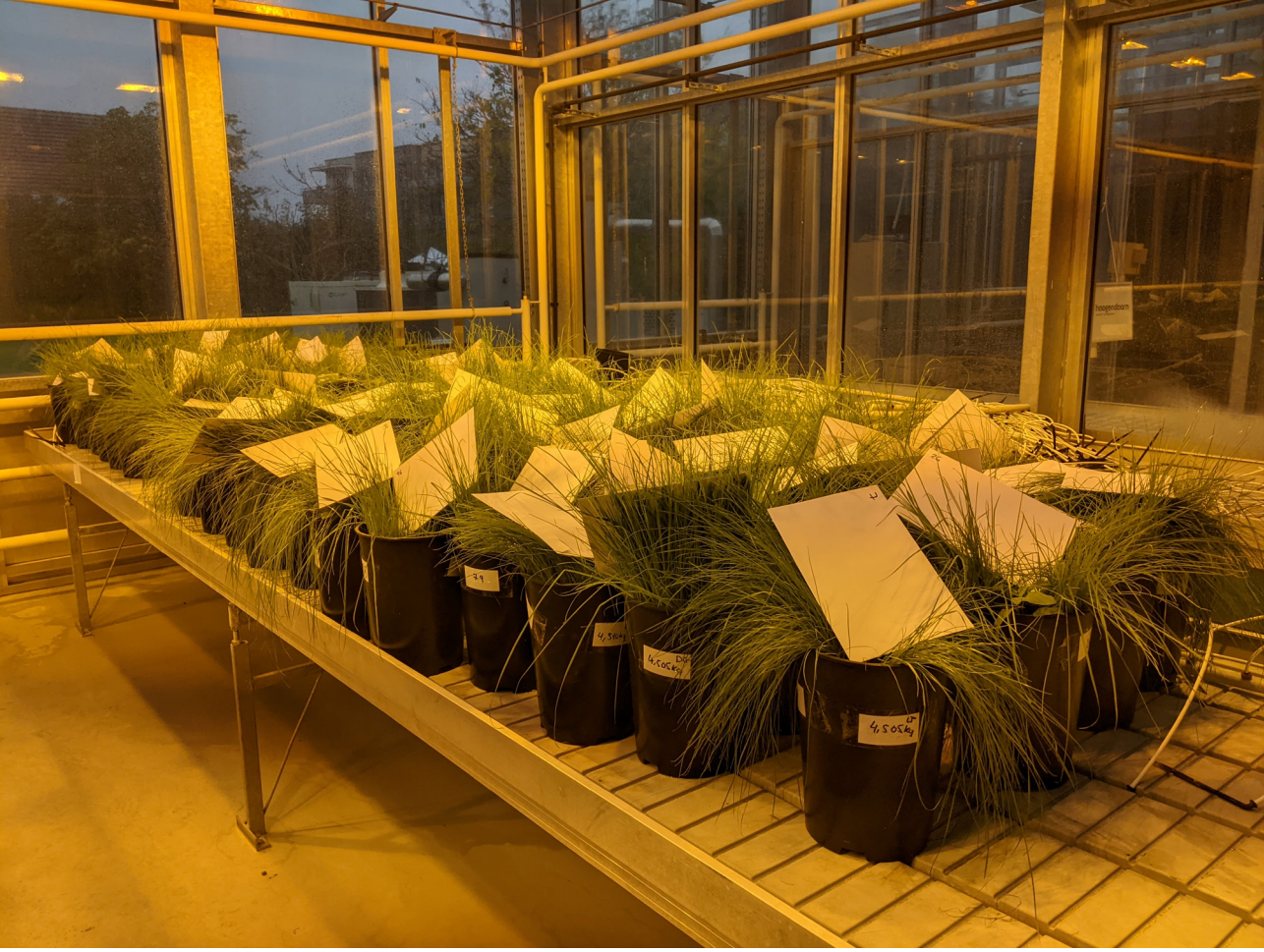


Figure 3.S3. *Boxplot describing a range of soil variables measured from the unamended control treatments at the first measurement dates of the preliminary incubation (I) and the described incubation (II). All variables present significant differences between incubations (Kruskal-Wallis test, $p < 0.05$).*



Grasses before harvest at Unilasalle, Mont-Saint-Aignan, France

Phosphorus fertilization of grasses with dairy processing waste materials and contributions of soil microorganisms towards phosphorus uptake

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Under review

Abstract

Dairy processing waste (DPW) is one of the largest agro-industrial residues globally and has the potential of reducing the dependency on mineral phosphorus (P) fertilizers in grasslands. However, the agronomical value and the effects of DPW and the secondary materials produced from it on soil biota remains largely unexplored. In a 112 day greenhouse experiment, we evaluated the biomass production and P uptake of two grass species with contrasting growth strategies (*Lolium perenne L.* fast-grower *vs Dactylis glomerata L.* slow-grower) under the fertilization of raw DPW sludge, and its secondary materials (ash and hydrochar). We compared the fertilized pots with a positive control with triple-superphosphate (TSP) and a negative control without P application. Our results showed that Olsen P and plant P concentrations were highest in the treatment with TSP after one month of application for both grass species. However, biomass production, P uptake and phosphorus use efficiency (PUE) was the greatest for sludge at day 112 for both species (56.39 ± 3.34 g pot^{-1}, 0.20 g ± 0.02 P and 56.18 ± 12.44%, n = 8). Ash and hydrochar resulted in lower biomass production than TSP and sludge and their PUE was low (< 20%). Fungi and arbuscular mycorrhiza fungi biomass were among the most important variables in explaining P uptake. In summary, we observed that sludge is a promising P fertilizer due to a higher P availability compared to the secondary materials and that DPW material addition did not result in a significant change of the microbial community. The results of our study suggest that application of DPW sludge and management of soil's fungal biomass can reduce the dependency on mineral P fertilizers in grassland.

1. Introduction

Phosphorus (P) mineral fertilizers are among the most used fertilizers in agriculture. Yet, P reserves are poorly distributed across the globe and the production cost of P fertilizers are expected to increase (Penuelas et al., 2023). As a result of this, P, in some areas of the world such as the European Union, has been listed as a critical raw material (Bertrand et al., 2016). Following up on this decision it has been proposed that dependency on mineral P sources in agriculture can be reduced through the recycling of P from organic residues and the transition towards a more circular economy (Chojnacka et al., 2020).

Waste from the dairy industry (Dairy Processing Waste, DPW) has the potential to reduce the dependency on mineral P fertilizers. The dairy sector is among the largest agro-industries globally, generating approximately 2.5 million tons of waste yearly in the European Union alone (Hu et al., 2021). Waste from DPW is rich in P, with concentrations that can exceed 5% of dry weight (Ashekuzzaman et al., 2019). DPW is a promising raw material for the production of secondary materials with a high concentration of P such as precipitated P salts (e.g. struvite), thermal oxidation materials (e.g. ash) and/or pyrolysis and gasification materials (e.g. char) (Delgado Sancho et al., 2019).

The effects of DPW and its secondary materials on crop yields and P uptake remain poorly understood. For instance, the mineral fertilizer equivalent value of raw DPW sludge has been suggested to range between - 80% to + 110% depending on the plant species, sludge's pre-treatment and fertilization rate (Ashekuzzaman et al., 2021; Shi et al., 2022). For DPW secondary materials such as biochar, studies showed varied results on crop yields, ranging from positive yields (Arutselvy et al., 2021), to low effects (Shi et al., 2022) or even decreasing plant available P (Khomenko et al., 2023a). The lower biomass production of recycled P fertilizers compared to mineral ones can be associated with a more complex chemical composition. While P in mineral fertilizers is mostly found in water-soluble calcium-P molecules, such as monocalcium phosphate ($Ca(H_2PO_4)_2$), the forms of P are more diverse in recycled fertilizers, and include poorly bio-available forms such as hydroxyapatite or iron phosphates (Kratz et al., 2019; Velasco-Sánchez et al., 2024).

Soil microorganisms could potentially enhance the fertilizer value of DPW through a range of mechanisms, that contribute to increase the availability of poorly soluble forms of P to plants (Richardson and Simpson, 2011; Velasco-Sánchez et al., 2024). Mechanisms to solubilize poorly available P are diverse and include the production of enzymes, chelates, organic acids and symbiotic associations with plants (Richardson and Simpson, 2011). In grasslands, soil microorganisms play an important role in P nutrition (Mander et al., 2012) and their role is likely to become more important in scenarios of reduced mineral P fertilization (Simpson et al., 2014). However, the effects of DPW or its secondary materials on the composition and activities of the soil microbial community remain largely unknown, and DPW might contain different pollutants that could impact negatively soil microorganisms (López-Mosquera et al., 2000). Some of the few articles that focused directly on the effects of DPW sludge on microbial communities have shown both increases and decreases in enzymatic activities and microbial community functioning (Frąc et al., 2012; Gryta et al., 2014; Oszust et al., 2015). Most of these studies were however short-term studies or were restricted to a few geographical areas. It is crucial to understand the impacts of the application of DPW on soil microorganisms to study their potential in enhancing DPW's fertilizer value.

In this experiment, we therefore aimed to study the agronomic value of DPW and two of its secondary materials, ash and hydrochar, and their impacts on soil microorganisms. In particular, our research objectives were: (i) to study the effects of DPW and its secondary materials on the biomass production and P uptake of two grass species (*Lolium perenne L.* and *Dactylis glomerata L.*), and (ii) to evaluate the impacts of DPW materials on soil microbial communities and their contribution towards P uptake. Our hypotheses were that (i) DPW or its secondary materials would present high contents of available P for grass growth; and (ii) that the importance of soil microorganisms towards P nutrition would be more important for DPW materials than for mineral fertilizers. We also hypothesized that different grass species would result in different P use and different associations with soil microorganisms.

2. Materials and methods

2.1. Soil sampling and preparation

Soil was collected from a Cambisol (WRB) from the top 0.2 m at Haudricourt, Normandy, France (49°42′16.6″ N, 1°41′10.9″ E) in the summer of 2021. This soil is classified a loamy soil, it has a neutral pH-H2O of 7.1, low available P (Olsen P) of 28 mg P_2O_5, potassium oxide (K_2O) content was 177 mg kg^{-1}, magnesium oxide (MgO) 95 mg kg^{-1}, calcium oxide (CaO) 2198 mg kg^{-1}, K2O:MgO 1.86, cation exchange capacity (CEC) 9.15 meq 100 g^{-1} and a soil organic matter content of 3%.

After soil collection, soil was passed through a 5 mm sieve and homogenised. Then soil was subdivided and placed in 80 cylindrical pots of 0.2 m diameter by 0.26 m height (Soparco, Sablons-sur-Huisne, France). The pots were filled up to 30 mm below their top opening. Soil moisture was adjusted to its 70% water holding capacity (WHC). The final soil weight in each pot was 4.3 kg pot^{-1}. Water holding capacity was estimated by placing dry soil in a steel cylinder and then rewetting it by capillarity and immersion. Soil was then drained by placing the cylinder over a dry sand bed for 2 h. The gravimetric water content at this point accounted as 100% water holding capacity. Pots were placed in a completely randomized design in a glasshouse in the facilities of Institute Polytechnique Unilasalle (Mont-Saint-Aignan, France), with an automatic drip irrigation system to maintain soil water content close to 70% WHC by regular water addition, light cycle of 15 h, temperature of 21 °C and air humidity of 60%. Soil was amended with nitrogen (N) and potassium (K) fertilizers at a rate of 100 kg N ha^{-1} and 120 kg K ha^{-1} to ensure no N and K limitation during the experiment and promote seed germination. These fertilizers were added after solubilizing N fertilizer (27%) (YaraBela AXAN) and K fertilizer (muriate of potash, KCl) in water.

2.2. Experimental design

The experiment was divided into two phases: an initial germination phase of 28 days during which the plants were allowed to establish; and a second phase of 112 days during which the pots were fertilized with the different P fertilizers. The experiment consisted of 4 replicates for each grass species (*Lolium perenne L.* (Lp)), a fast-grower grass species and *Dactylis glomerata L.* ((Dg) a slow-grower grass species),

as well as 3 different P fertilizer treatments (DPW's raw sludge; its secondary materials ash and hydrochar for two destructive dates. We also included in our design pots fertilized with mineral P fertilizer (TSP) and a control with no P application resulting in a total of 80 pots. Detailed information about the experimental design can be observed in Supplementary material, Figure 4.S1.

At the beginning of the germination phase, we applied 2 g (Semences de France, La Chapelle-d'Armentières, France) of seeds to ensure proper colonization of the soil surface (Ros et al., 2018). The seeds were buried at 10 – 20 mm depth and the pots were covered with a plastic film to stimulate germination. The plastic film was removed once the grass seeds sprouted.

At the end of the germination phase, grass was well established and was cut 40 mm above the soil surface. Additional N fertilizer was applied (30 kg N ha^{-1}). DPW materials and TSP were then broadcasted over the soil surface at a P fertilizers rate of 45 kg P ha^{-1}, as recommended by the seed commercial provided (Semences de France), based on the total P content of the DPW materials.

2.3. Dairy processing waste materials

The DPW materials used in this study were raw DPW sludge treated with iron (Fe) salts, and its secondary materials ash and hydrochar. Characterization of the DPW materials can be found in Table 4.1. In addition to DPW trials pots was included fertilized with mineral P fertilizer (TSP) and a control with no P application. After the first harvest of leaf biomass and the application of the P fertilisers, we performed 4 harvests, each after a 28-day growth period. Two of these harvests were destructive to collect root biomass and soils for biological and chemical analyses (days 28 and 112, n = 40) and the other 2 were non-destructive and consisted only of harvesting leaf biomass (days 56 and 84) (Supplementary material, Figure 4.S1). After each harvest, a maintenance dose of N fertilizer was added to prevent N limitation (30 kg N ha^{-1}).

Table 4.1. *DPW materials characterization. Values indicate means ± standard deviation. See footnote for method description.*

Element	Hydrochar	Ash	Sludge	Unit	Method
Al	0.37 ± 0.03	0.76 ± 0.08	0.12 ± 0.02	%	A
Ca	6.81 ± 0.97	13.72 ± 1.47	4.68 ± 0.69	%	A
Fe	13.34 ± 1.07	26.26 ± 3.08	9.74 ± 1.47	%	A
K	0.51 ± 0.05	3.19 ± 0.36	1.07 ± 0.08	%	A
Mg	0.83 ± 0.06	1.86 ± 0.19	0.59 ± 0.09	%	A
Na	1.51 ± 0.80	2.00 ± 0.36	1.72 ± 0.40	%	A
Cd	9.70 ± 0.13	9.40 ± 0.40	0	mg kg^{-1}	A
Co	0	0	0	mg kg^{-1}	A
Cr	35.6 ± 6	40.6 ± 3.6	15.7 ± 5.3	mg kg^{-1}	A
Mn	362 ± 28.3	807 ± 115.9	204.5 ± 36	mg kg^{-1}	A
Ni	0	0	0	mg kg^{-1}	A
Pb	895.4 ± 73.6	1748.7 ± 142.7	645.7 ± 135.5	mg kg^{-1}	A
Zn	214 ± 86.7	495.6 ± 104.7	169.2 ± 114.3	mg kg^{-1}	A
pH	5.048 ± 0.008	5.94 ± 0.006	8.62 ± 0.04	--	B
EC	5.48 ± 0.155	4.287 ± 0.057	4.583 ± 0.189	mS/cm	B
C	38.44 ± 2.56	0.67 ± 0.17	32.53 ± 1.46	%	C
N	6.26 ± 0.94	0.16 ± 0.04	5.81 ± 0.12	%	C
H	5.03 ± 0.15	0.07 ± 0.03	5.27 ± 0.36	%	C
P	4.88 ± 0.33	5.69 ± 0.06	3.05 ± 0.02	%	D
Production method	Adjust DM to 15%, pH to 5, then 225 °C for 1 h	250 °C for 2 h then 550 °C for 2-3 h	-		

2.4. Plant analyses

2.4.1. Biomass sampling

Aboveground biomass (leaves) was collected 4 times over the course of the experiment (days 28, 56, 84 and 112) by clipping the leaves 40 mm over the soil surface. Leaves were dried at 40 °C for one week and weighed on a precision scale (Mettler Toledo, Greifensee, Switzerland) to calculate leaf biomass production. At the destructive harvests (days 28 and 112) roots and basal stem (aboveground biomass between 0 to 40 mm) were also collected. This was done by clipping the basal stems and by washing the soil out of the pot over a 50 µm mesh size. Total biomass was calculated as the sum of leaf, basal stem and root dry biomass. Cumulative leaf biomass was calculated as the sum of the leaf biomass production of days 28, 56, 84 and 112. Cumulative total biomass was calculated by adding cumulative leaf biomass and basal stem and root biomass at day 112.

2.4.2. Biomass elemental composition

Carbon (C) and nitrogen (N) content in plant material were measured in an elemental CHNS analyser (Shimadzu, Kyoto, Japan). P content in 0.2 g of plant biomass was determined after microwave assisted digestion with concentrated nitric acid, HNO_3 (16.24 M), and measured colorimetrically using the molybdate blue method (Murphy and Riley, 1962). The digestion was performed in a MARS-6 instrument (CEM, Matthews, USA) using a program with a ramp and hold time of 15 min, a target temperature of 200 °C, pressure of 800 psi and 900 – 1050 W of power. The complete digestion, including cooling lasted approximately 1 hour. Phosphorus use efficiency (PUE) was calculated by calculating the difference in P uptake with the unfertilized control (Equation 1) (Syers et al., 2008):

$$PUE = \frac{P\ uptake_{fertilized} - P\ uptake_{control}}{P\ added} \quad [1]$$

PUE was calculated on leaf biomass, cumulatively on leaves (cumulative leaf PUE) by considering the cumulative uptake of P by leaves in the 4 different harvests. Similarly, cumulative total PUE was calculated considering as well root and basal stem P uptake at day 112.

2.5. Soil analyses

2.5.1. Soil sampling

Soil samples were collected on the destructive harvests (days 28 and 112). This was done by taking 3 soil cores through the whole profile of the pots with a 20 mm diameter auger. The subsamples were then pooled and sieved at 4 mm. The cores were taken following a line, crossing the centre of the pot and sampling the whole depth of the pot. Afterwards, soil was either dried at 40 °C for soil chemical analyses or frozen at – 80 °C for soil biological analyses.

2.5.2. Soil chemical analyses

Content of total C and N in the soil was measured using a CHNS elemental analyser (Shimadzu, Kyoto, Japan). Moreover, N-NO_3 and N-NH_4 contents in the soil were measured colorimetrically using a Gallery instrument (Thermo Fisher Scientific, Waltham, USA), after 1 h extraction with 1 M potassium chloride (KCl). Plant available P was estimated using the Olsen P method, where P was extracted from 5 g of soil with 100 mL of a 0.5 M solution of sodium bicarbonate ($NaHCO_3$) at pH of 8.5 for 30 min. P was then measured colorimetrically following the molybdate blue method in a spectrophotometer at 882 nm wavelength (Varian Cary 50 Scan UV-Visible Spectrophotometer) (Agilent Technologies, Santa Clara, USA) (Murphy and Riley, 1962). Total P in soil was also determined after microwave assisted digestion with concentrated HNO_3 and measured colorimetrically with the molybdate blue method (Murphy and Riley, 1962). Soil pH was determined in a 1:5 v:v extract using a glass electrode (Mettler Toledo, Greifensee, Switzerland) after being shaken for 1 h.

2.5.3. Soil biological variables

2.5.3.1. Phospholipid fatty acid characterization

Microbial biomass and composition were assessed by measuring and characterizing phospholipid fatty acids (PLFA) and neutral fatty acids (NLFA) (Bligh and Dyer, 1959; Frostegård et al., 1991). In short, lipids from 2 g of freeze-dried soil are extracted using 10 mL of Bligh and Dyer solution (Chloroform:MeOH:citrate buffer 1:2:0.8 v:v:v). Remaining lipids in soil are extracted with a second round of

extraction with Bligh and Dyer solution. Phases are divided by adding 4 mL of chloroform and 4 mL of citrate buffer and stored at - 20 °C for a minimum of 8 h. Then, the chloroform fraction is sampled with a Pasteur pipette and evaporated with a vacuum device where lipids are retained (SpeedVac, Thermo Fisher Scientific, Waltham, USA). The lipids are then mixed with 100 µL of chloroform and separated into NLFA and PLFA with the use of a bond-elute SPE, 1.5 mL of chloroform to collect NLFAs, 6 mL of acetone to clean the SPE column and 1.5 mL of methanol to collect PLFAs. The methanol fraction is evaporated and PLFAs are mixed with 1 mL of a 1:1 v:v toluene/methanol solution. Finally, 0.2 µL of 0.1 mg 19:0 FAME mL^{-1} and 1 mL of 0.2 M KOH in methanol are added to the dried polar lipids and incubated for 15 min at 37 °C. After incubation, 2 mL of hexane, 0.3 mL of acetic acid and 2 mL of ultrapure water were added, vortexed and let for 1-2 hours for layers to separate. The hexane upper layer is pipetted out, evaporated and dried lipids are redissolved in 150 µL of hexane. This 150 µL are then transferred to GC vials. PLFAs and NLFAs were measured in a GC-MS (Agilent Technologies, Santa Clara, USA). PLFAs were divided into Actinomycetes, Gram +, Gram -, Anaerobe, Eukaryote and saprophytic Fungi microbial groups and AMF from NLFAs were also quantified using the MIDI Sherlock Software v.6.3B with PLFA package v.2.00 (Shimadzu, Kyoto, Japan) (Buyer & Sasser, 2012). We also calculated the total amount of bacteria and fungi of the PLFA profile (Saprophytic fungi, Actinomycetes, Gram +, Gram -, Anaerobe), the ratio Fungi $Bacteria^{-1}$ in the PLFA (Saprophytic fungi / (Actinomycetes, Gram +, Gram -, Anaerobe), the total fungi peaks (NLFA-AMF, Saprophytic fungi) and the total bacteria peaks (Actinomycetes, Gram +, Gram -, Anaerobe). Specific peaks assigned to the different microbial groups can be found in the Supplementary material, Table 4.S1.

2.5.3.2. Enzymatic activities

Enzymatic activities (β-Glucosidase (E.C. 3.2.1.21) (Bglu), arylamidase (E.C. 3.4.11.2) (ARYLN) and acid and alkaline phosphatase (E.C. 3.1.4.1) (PAC) and (PAK), respectively, were measured colorimetrically using a microplate technique (Cheviron et al., 2021). In short, we used 25 mL of ultrapure water for Bglu, ARYLN and of Trizma buffer (50 mM) adjusted to pH 5.5 for PAC and 11 for PAK to create a soil suspensions with 4 g of soil. After 10 min at 250 rpm, 125 µL of soil suspension were

placed in 96-well microplates and mixed with their respective substrate solutions: 4-nitrophenyl β-d-glucopyranoside (CAS N°: 2492-87-7) for Bglu, l-leucine β-naphthylamide hydrochloride (CAS N°: 893-36-7) for ARYLN and 4-nitro-phenylphosphate disodium salt hexahydrate (CAS N°: 333338-18-4) for PAC and PAK, respectively. The concentrations of the substrate solutions were 0.05, 0.008 and 0.05 mol L^{-1}, respectively. After mixing, microplates were incubated at 37 °C for 1 h for Bglu, 2 h for ARYLN and 30 min for PAC and PAK. The reaction was stopped with 25 µL 0.5 M of calcium chloride ($CaCl_2$) and 100 mM 100 µL Trizma at pH 12 for Bglu, PAC and PAK and microplates were centrifuged for 5 min at 1500 g. In the case of ARYLN, the reaction was stopped with ethanol 96% and coloration was developed with 100 µL of acidified ethanol and 100 µL of DMCA. ARYLN's plates were incubated in the dark for 20 min at room temperature before measurement. The measurements for all enzyme activities were performed in a Varioskan Flash-Thermo microplate reader (Thermo Fisher Scientific, Waltham, USA). Absorbance was measured at 405 nm for Bglu, PAC and PAK and 540 nm for ARYLN. Results of enzymatic activities were expressed in nmol PNP (paranitrophenol) min^{-1} g^{-1} of dry soil for Bglu, PAC and PAK activities and in β-naphthylamine min^{-1} g^{-1} of dry soil for ARYLN activities.

2.6. Statistical analyses

All statistical analyses were performed using RStudio version 1.4.1717 (RStudio Team, 2021). Figures were done using the package "ggplot2" in the RStudio environment (Wickham, 2016) and further edited in PowerPoint (Microsoft, Redmond, USA).

To test of the effect of P fertiliser application over time and for the two grass species on biomass production, nutrient uptake, chemical properties, soil microbial composition and biomass and enzymatic activities, we performed three-way analysis of variance (ANOVA) using the "aov" function. Each observation of chemical properties, soil microbial composition and biomass and enzymatic activities was treated as a sample and the interactive effects of fertiliser, harvest date and grass species included as factors was tested. In the case of leaf biomass or leaf nutrient uptake, samples were obtained at 4 different dates (at 28, 56, 84 and 112 days after fertiliser application), resulting in a violation of the assumption of independence and

in an unbalanced design (80 observations at day 28 and 40 observations at days 56, 84 and 112). For this reason, we performed an ANOVA type III with fertilizers, grass species and harvest time as fixed factors and the pot's ID as a random factor to account for the lack of independence associated with repeated observations performed on the same location (Shaw and Mitchell-Olds, 1993). The mixed effect ANOVA type III model was performed using the "lmer" and "anova" functions of the "lme4" package (Bates et al., 2015) and "lmerTest" package (Kuznetsova et al., 2017), respectively. When the effects of one of the three fixed factors or the interactions of the factors were found significant, we performed Tukey's post-hoc test using the "emmeans" package (Lenth, 2023). In case of significant interactions, Tukey's post hoc were conducted on single main effects after estimating marginal means using the R package "emmeans". This allows to test the effect of factor while accounting for the interaction effect. Moreover, we tested the effects of grass species and fertilizer on cumulative biomass production or cumulative nutrient uptake by performing a two-way ANOVA. For all models, the assumptions of homogeneity, normality and heteroscedasticity were inspected by analysing the residuals.

To test for potential effects of biological and chemical soil properties on P uptake by grass leaves, we conducted a multiple regression analysis following Moinet et al., (2016). The aim was to determine the relative importance of chemical vs biological variables in explaining P uptake, as well as which biological variables, if any, would most strongly explain it. A set of candidate models with all possibles combinations of chemical soil variables that could explain P uptake (root biomass, pH, soil C, soil C: N and Olsen P) were first computed. To this set of models, different soil biological variables (PAC, PAK, Bglu, ARYLN, Gram + biomass, Gram $^-$ biomass, saprophytic fungal biomass (PLFA peak), AMF biomass (NLFA peak), total PLFA biomass, Fungi Bacteria^{-1} ratio or total fungal biomass) were added sequentially, one at a time to form all possible combinations of models with chemical variables. Then, the interaction of soil biological and soil chemical variables with the fertilizer or with the grass species was added to all possible combinations. We performed the analysis separately for day 28 and 112 as we found strong differences in most variables between harvest dates. This approach lead to a set of 6428 different candidate models for each date that included models with biological variables or without them. The models were ranked using the AIC to determine the Kullback–Leibler (KL) best

model which is based on bias-corrected, maximized log-likelihood (LogLik) of the fitted model with a penalty for the number of parameters used (Burnham and Anderson, 2004). The model with the lowest AIC (AICcmin) is the best explanatory model of P uptake. The ΔAICc value is calculated for each model as ΔAICc = AICc – AICcmin. Differences of ΔAICc < 2 indicate no significant improvement by the next ranked model, whereas ΔAICc > 2 indicate a considerably better explanation of P uptake (Anderson, 2007). A measure of the strength of support is described by the model probability (Akaike weights, Awc), which estimates the probability that a given model is the KL best model, given the dataset and candidate set of models. The sum of Awc in a candidate set of models equates to 1.

3. Results

3.1. Grass biomass production

The application of the different P fertilizers had major significant effects ($p < 0.05$) on biomass production. Particularly, fertilizer had a significant effect on leaf and root biomass, cumulative leaf biomass and cumulative total biomass production (including leaf, basal stem and root biomass) (Table 4.2). The effect of the fertilizer on leaf biomass was influenced by the time of harvest as shown by the significant interaction term (Table 4.2). Grass species had a significant effect on root biomass ($p < 0.001$) and this effect changed over time, as indicated by the significant interaction. This was also the case for leaf biomass (Table 4.2). Cumulative leaf biomass and total biomass production were not affected by grass species.

Table 4.2. *p-values of main terms and interactions from three-way ANOVAs on leaf and root biomass production and two-way ANOVAs for cumulative leaf and cumulative total biomass. Values under 0.05 denote a significant effect and are highlighted in bold. Not applicable = n.a.*

Factor	Leaf biomass	Root biomass	Cumulative leaf biomass	Cumulative total biomass
Fertilizer	**< 0.001**	**0.013**	**< 0.001**	**0.008**
Grass species	0.897	**< 0.001**	0.934	0.148
Time	**< 0.001**	**0.018**	n.a.	n.a.
Fertilizer x Grass species	0.642	0.256	0.734	0.313
Fertilizer x Time	**< 0.001**	0.848	n.a.	n.a.
Grass species x Time	**< 0.001**	**< 0.001**	n.a.	n.a.
Fertilizer x Grass species x Time	0.073	0.778	n.a.	n.a.

Sludge produced on average the highest leaf biomass (Figure 4.1, panel A), and was significantly highest at days 28 and 56 after application, while and no significant differences were observed for days 84 and 112. Sludge also resulted in the significantly highest cumulative leaf biomass and highest cumulative total biomass (Figure 4.1, panel B). The mean ± se (n = 8) leaf biomass production in the sludge treated pots was 30.85 ± 0.58 g pot^{-1} and the total biomass production was 56.39 ± 3.34 g pot^{-1}, both values being significantly higher than the control. Root biomass was, on average, higher in the pots fertilized with TSP and sludge, yet these differences were not significant when conducting Tukey's post-hoc tests ($p < 0.1$). Mean ± se values for leaf, root and basal stem biomass, total biomass, cumulative leaf biomass and cumulative total biomass for the different P treatments, grass species and harvest dates, as well as summary output of ANOVA models can be found in the Supplementary material Table 4.S2.

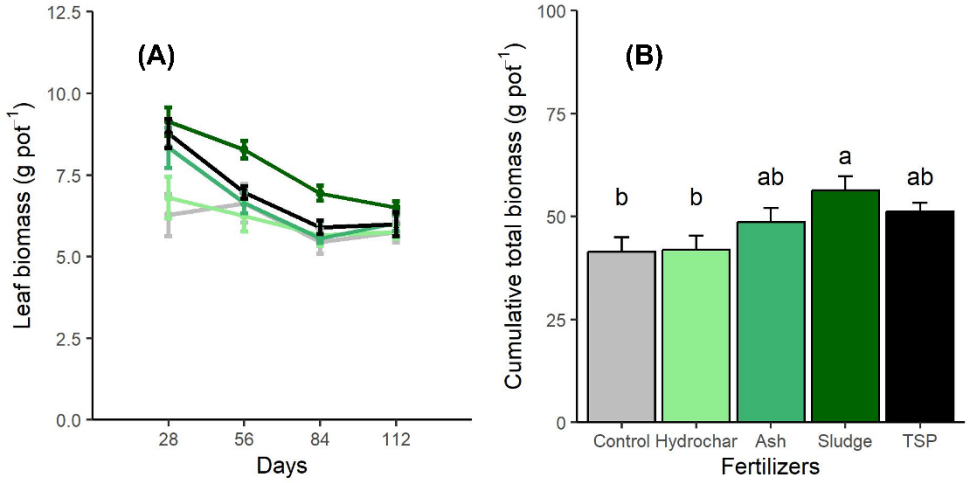

Figure 4.1. *Mean values of both grass species for (A) leaf biomass as affected by the fertilizers and time, and (B) cumulative total biomass (cumulative sum of root, basal stem and leaf biomass) for each fertilizer. Whiskers indicate standard errors, n = 8. Letters denote statistical differences after performing Tukey's post hoc test.*

Regarding the species effect, the biomass production dynamics of both Lp and Dg were affected by the time of harvest. Lp produced significantly more biomass than Dg at day 28, yet this was reverted by day 56, and at day 112 Dg produced significantly more leaf biomass than Lp (Figure 4.2, panel A). At days day 56 and at day 84, leaf biomass was similar for both species. This was due to biomass production of Lp decreasing over time, whereas the biomass production of Dg remained approximately constant throughout our experiment (Figure 4.2, panel A). Root biomass was significantly higher in Lp pots than Dg at days 28 and 112, yet the difference was narrower by day 112. At the end of the experiment, no statistical differences were found between grass species on cumulative leaf biomass or cumulative total biomass (Figure 4.2, panel B). The average cumulative total biomass production was 49.99 ± 2.57 and 45.86 ± 1.97 g pot^{-1} (mean ± se, n = 20) for Lp and Dg, respectively.

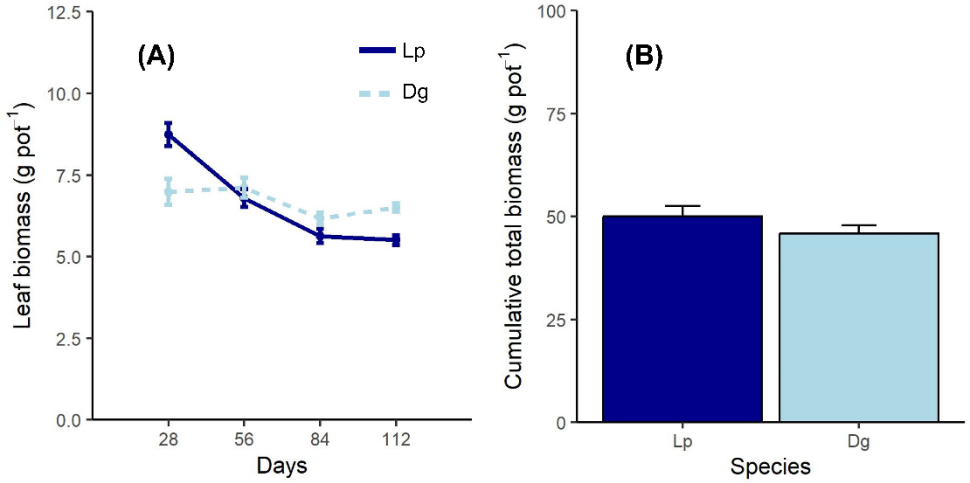

Figure 4.2. *Biomass production of grass species. Panel (A) displays the differences in leaf biomass production between L. perenne (solid) and D. glomerata (dotted) and panel (B) shows total biomass production (root, basal stem and cumulative leaf biomass) for grass species. Whiskers indicate standard errors, n = 20.*

3.2. Nutrient uptake

P concentration, P uptake and PUE were affected significantly by the different fertilizers (Table 4.3). However, the effect of the fertilizer was dependent on harvest time for P uptake and PUE as showed by the significant interactions. Grass species only significantly affected leaf P concentration ($p = 0.013$), showing a higher P concentration in Lp plants compared to that in Dg, with 3.93 ± 0.13 g kg^{-1} and 3.55 ± 0.13 g kg^{-1} (mean ± se, n = 20) for Lp and Dg, respectively. Grass species effect on leaf PUE, cumulative P uptake and cumulative total PUE was also marginally significant ($p < 0.1$), showing greater averages for Lp pots. Mean values and standard errors for each treatment, plant species and harvest time as well as ANOVA summaries can be found in the Supplementary material (Tables 4.S3, 4.S4 and 4.S5).

Table 4.3. *Results of three-way ANOVA tests on leaf P concentration, uptake and PUE and two-way ANOVA for cumulative total P uptake and PUE (p-values). Values under 0.05 denote a significant effect and are highlighted in bold. Cumulative total P uptake and PUE include leaf, basal stem and root biomass. Not applicable = n.a.*

Factor	Leaf P concentration	Leaf P uptake	Cumulative total P uptake	Leaf PUE	Cumulative total PUE
Fertilizer	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Grass species	**0.013**	0.134	0.088	0.098	0.077
Time	**< 0.001**	**0.001**	n.a.	**< 0.001**	n.a.
Fertilizer x Grass species	0.973	0.922	0.855	0.954	0.914
Fertilizer x Time	0.131	**< 0.001**	n.a.	**< 0.001**	n.a.
Grass species x Time	0.121	0.136	n.a.	0.562	n.a.
Fertilizer x Grass species x Time	0.462	0.452	n.a.	0.856	n.a.

TSP was the fertilizer that increased the most, significantly, P concentration in leaves across all treatments (Figure 4.3, panel A). The average P concentration in leaves was 4.87 ± 0.22, 3.65 ± 0.17, 3.56 ± 0.18, 3.39 ± 0.18 and 3.26 ± 0.16 g kg^{-1} for TSP, ash, sludge, hydrochar and control, respectively (mean ± se, n = 8). None of the DPW fertilizers resulted in a significant increase in leaf P concentration compared to the control. P concentration increased over time until day 112 when P concentration started to decrease (Figure 4.3, panel A). Leaf P concentration was higher in Lp than in Dg plants, 3.93 ± 0.29 *vs* 3.55 ± 0.29 g P kg^{-1}, n = 20. Leaf P uptake was affected by harvesting time and the different fertilizer treatments as shown by their significant interaction (Table 4.3). Plants fertilized with TSP increased P uptake only at days 28 and 56, yet no differences were found at days 84 and 112 (Figure 4.3, panel B). The leaf uptake of P was constant for all the fertilizers with the exception of TSP, which decreased over time (Figure 4.3, panel B). Information on root and basal stem P concentration and uptake can be found in the Supplementary material (Table 4.S5).

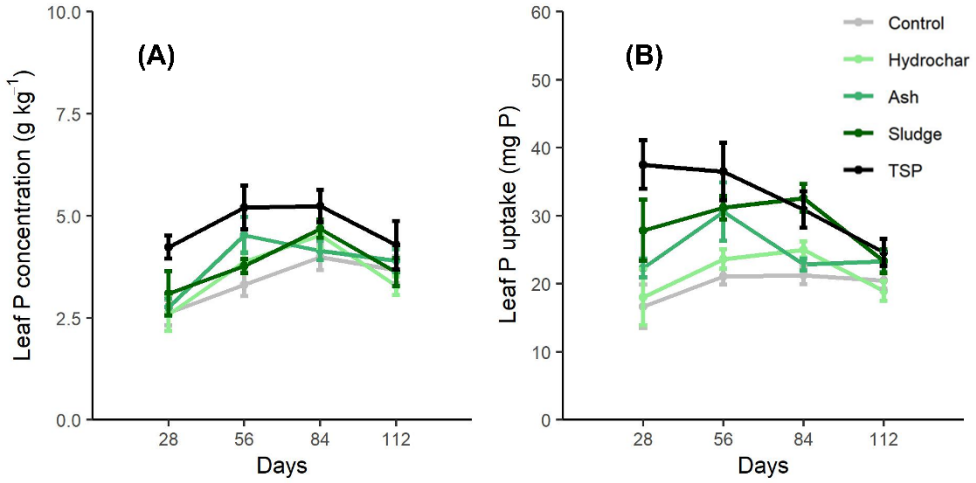

Figure 4.3. *Leaf P concentration (A) and uptake (B) after fertilization. Whiskers indicate standard errors, n = 8.*

Cumulative total P uptake (including leaf, basal stem and root biomass) was the highest in the sludge and TSP treatments, hydrochar and ash treatments did not increase significantly cumulative P uptake in relation to the control (Figure 4.4, panel A). Grass species had no significant different in cumulative total P uptake (Table 4.3). Leaf PUE from the different fertilizer depended on the harvest time, as shown by the significant interaction (Table 4.3). Post hoc analyses on the interaction of harvest time and fertilizer treatment showed no differences at day 28, TSP produced the highest PUE in leaves at day 56 which was equalled by sludge on days 84 and 112. Leaf PUE decreased over time for all the treatments with the exception of hydrochar, which remained constant. Detailed information on leaf PUE can be found in the Supplementary material (Table 4.S3). Cumulative total PUE (including leaf, basal stem and root biomass) was the highest in the pots fertilized with sludge (56.18 ± 12.44%, n = 8) and TSP (48.77 ± 4.66%, n = 8), followed by ash (27.49 ± 7.26%, n = 8) and hydrochar (5.99 ± 6.19%, n = 8) (Figure 4.4, panel B). Grass species had no significant effect on cumulative total PUE (Table 4.3).

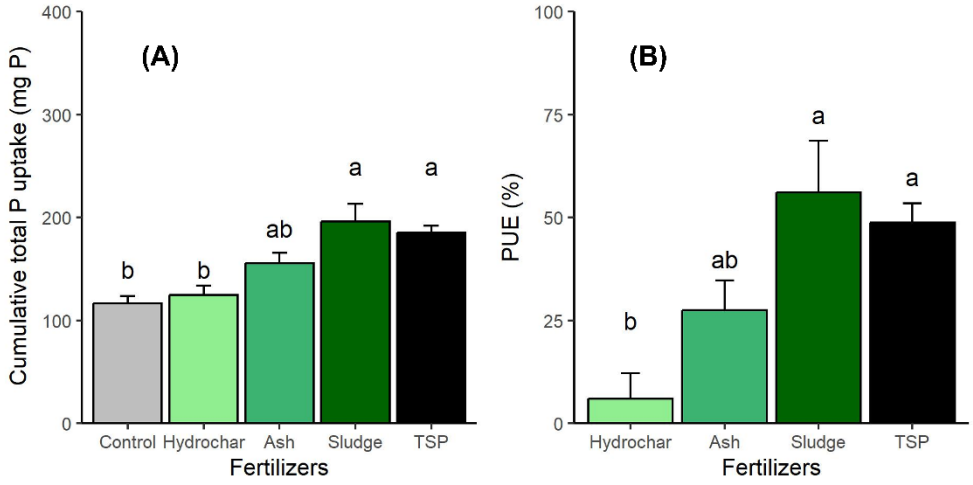

Figure 4.4. *Cumulative total (including leaf, basal stem and root biomass) P concentration (A) and Phosphorus Use Efficiency (PUE) (B) after fertilization. Whiskers indicate standard errors, n = 8. Letters denote statistical differences after performing Tukey's post hoc test.*

The application of the different fertilizers had also impacts on N concentration, uptake and nutrient ratios (Supplementary material, Table 4.S4). For instance, N concentration was significantly highest in the hydrochar fertilized plots and lowest in the TSP, sludge and ash fertilized ones. Cumulative total N uptake (including leaf, basal stem and root biomass) was the highest in the sludge fertilized pots (0.71 ± 0.01 g N pot^{-1}, n = 8). C: N ratio was significantly higher in Dg plants at day 28, and significantly higher in Lp plants at day 112. The C: P ratio was highest in the control and hydrochar fertilized pots and lowest in the fertilized with TSP, the ratio was significantly higher in Dg than Lp plants and decreased over time. Lastly, the N: P ratio in leaf showed significant statistical interactions between fertilizer and harvest time and grass species and harvest time. The N: P ratio was lowest in TSP fertilized pots and highest in the hydrochar fertilized ones at day 28, no differences were found for the remaining days. Detailed information and means ± and ANOVA summaries on N nutrition and nutrient ratios can be found in the Supplementary material, Table 4.S4 and 4.S5.

3.3. Soil chemical variables

Olsen P values were significantly affected by fertilizer addition, grass species and harvest time, however, the effect of the fertilizer depended on the harvest time, as shown by the significant interaction between these two factors (Table 4.4). Olsen P values were significantly higher in the pots amended with TSP at day 28, yet no differences between fertilizers were found at the end of the experiment (Figure 4.5). Moreover, Olsen P decreased significantly over time in the pots fertilized with hydrochar and particularly in the ones fertilized with TSP (Figure 4.5) and was significantly higher in Dg pots compared to Lp. Total P was significantly affected by fertilizer type, showing that hydrochar and ash were the only fertilizers that increased P content compared to the control, with values of 1.06 ± 0.05 and 1.01 ± 0.09 g P kg^{-1} (mean ± se, n = 8), for hydrochar and ash respectively.

Table 4.4. *Results of three-way ANOVA tests on soil chemical properties (p-values). Values under 0.05 denote a significant effect and are highlighted in bold.*

Factor	Olsen P	Total P	$N\text{-}NO_3$	$N\text{-}NH_4$	Total N	Total C	pH
Fertilizer	**< 0.001**	**0.003**	0.264	**0.005**	0.091	0.200	0.634
Grass species	**< 0.001**	0.536	0.157	0.193	0.485	0.248	0.095
Time	**< 0.001**	0.134	0.080	0.868	**0.038**	**< 0.001**	**< 0.001**
Fertilizer x Grass species	0.355	0.975	0.636	0.186	0.280	0.339	**0.049**
Fertilizer x Time	**0.004**	0.205	0.284	0.645	0.211	0.901	0.964
Grass species x Time	0.132	0.131	0.146	0.058	0.984	0.583	0.118
Fertilizer x Grass species x Time	0.312	0.152	0.666	**0.003**	0.520	0.410	0.297

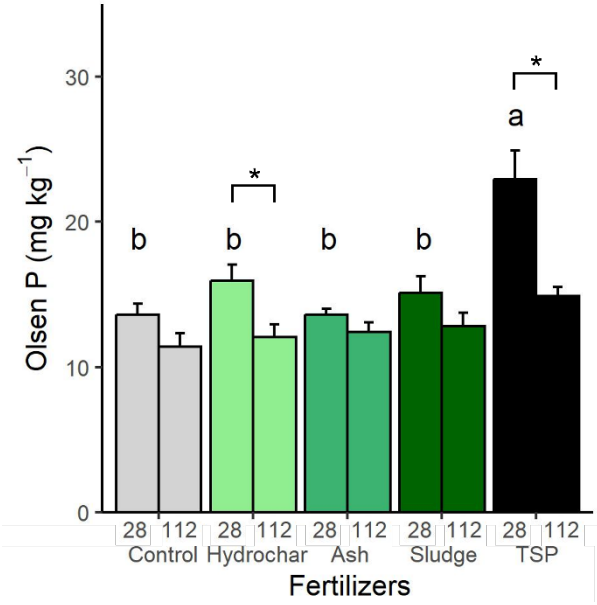

Figure 4.5. *Plant available soil P (Olsen P) 28 days after the application of fertilizers and at 112 days after the application of fertilizers. Whiskers indicate standard errors, n = 8. Letters denote statistical differences after performing Tukey's post hoc test at each harvest time. Asterisks and brackets indicate significant differences over time for a given treatment.*

N-NH_4 content of the soil was affected by the time of the harvest, fertilizer and grass species, as shown by the significant triple interaction. By exploring the triple interaction, we observed that sludge application increased N-NH_4 in the Dg pots at day 28 and in the Lp pots at day 112. N-NH_4 content decreased significantly in the Lp pots fertilized with ash. No other significant statistical differences were found for the other fertilizers or grass species regarding N-NH_4. Soil C was affected by harvest time, showing significantly higher values at day 28 than 112. All mean values ± se and ANOVA summaries for each plant species, plant and treatment can be found in the Supplementary material (Table 4.S6).

The application of P fertilizers had no significant effects on soil microbial communities with the exception of NLFA-AMF and anaerobe biomass. The effect of fertilizer addition on NLFA-AMF biomass was dependent on grass species ($p = 0.022$). Tukey's post-hoc analyses showed that NLFA-AMF biomass was

significantly higher than the control in the Lp pots fertilized with sludge and ash. No differences were found in the Dg pots. Tukey's test also showed that NLFA-AMF was significantly higher in the control Dg pots than the control Lp pots. Total NLFA peaks, PLFA-AMF and NLFA-AMF biomass significantly increased over time. Mean values ± se and ANOVA summaries of PLFA, NLFA microbial groups and ratios for plant species and fertilizer treatments can be found in the Supplementary material (Table 4.S7 and Table 4.S8).

The effects of the different P fertilizers on enzymatic activities were also limited. Only Bglu activities were affected by P fertilization, as shown by the significant interaction of fertilizer and grass species ($p = 0.034$). Post-hoc analysis showed that Bglu activities, in Lp plants, were significantly lowest in soils fertilized with sludge (24.83 ± 1.11 PNP min^{-1} g^{-1}, mean ± se, $n = 4$) and highest in the ones fertilized with TSP and unfertilized control (28.65 ± 0.65 and 28.40 ± 0.82 PNP min^{-1} g^{-1}, mean ± se $n = 4$ for TSP and control, respectively). The actual means ± se of enzymatic activities and ANOVA summaries, for each fertilizer, grass species and sampling time can be found in the Supplementary material (Table 4.S9).

3.4. Drivers of P uptake

P uptake by leaves at days 28 and 112 was modelled by conducting a multiple regression analysis on over 6428 different models (Table 4.5). In table 4.5, the first 8 models ranked based on their AICc at days 28 and 112 are displayed. The variables that was related most to P uptake were root biomass, Olsen P, pH and Soil C or Soil C: N. Moreover, soil microbial variables contributed significantly to improve the models. At day 28, we observed that the microbial variables that contributed most were PLFA-Fungi biomass and total fungal biomass (PLFA-Fungi + NLFA-AMF) and at day 112 the microbial variables that contributed the most were NLFA-AMF and total fungal biomass.

Table 4.5. *Model ranking based on Akaike's information criterion explaining P uptake. Models displayed show models within 0 – 2 ΔAICc range and the following best model. AICc = Aikaike's Information Criterion, ΔAICc= AICc – AICcmin for best model, Aw = Akaike's weight, Awc = Awc cumulative and LogLik = Log likelihood ratio.* [a] *= PLFA-Fungi + NLFA-AMF.*

		Models	AICc	ΔAICc	Awc	Awcc	LogLik
P uptake (day 28)	1	Root biomass + Olsen P + pH + Soil C: N + PLFA-Fungi	262.96	0.00	0.23	0.23	-122.48
	2	Root biomass + Olsen P + pH + Soil C + PLFA-Fungi	263.15	0.19	0.20	0.43	-122.58
	3	Root biomass + Olsen P + pH + Soil C: N + Fungal biomass [a]	264.05	1.09	0.13	0.56	-123.02
	4	Root biomass + Olsen P + pH + Soil C + Fungal biomass [a]	264.42	1.47	0.11	0.67	-123.21
	5	Root biomass + Olsen P + Soil C: N + PLFA-Fungi	266.00	3.05	0.05	0.72	-125.60
	6	Root biomass + Olsen P + Soil C: N + Soil C + pH + PLFA-Fungi	266.26	3.30	0.04	0.76	-122.46
	7	Root biomass + Olsen P + Soil C + PLFA-Fungi	266.35	3.39	0.04	0.80	-125.77
	8	Root biomass + Olsen P + Soil C: N + Fungal biomass [a]	266.98	4.02	0.03	0.83	-126.09
P uptake (day 112)	1	Root biomass + Olsen P + pH + Soil C: N + Fungal biomass [a]	201.36	0.00	0.16	0.16	- 91.35
	2	Root biomass + Olsen P + pH + Soil C: N + NLFA-AMF	201.50	0.14	0.15	0.31	- 91.41
	3	Root biomass + Olsen P + pH + Soil C + Fungal biomass [a]	201.73	0.37	0.13	0.44	- 91.53
	4	Root biomass + Olsen P + pH + Soil C + NLFA-AMF	202.21	0.85	0.10	0.54	- 91.77
	5	Root biomass + Olsen P + Soil C: N + Fungal biomass [a]	203.14	1.77	0.07	0.61	- 93.95
	6	Root biomass + Olsen P + Soil C: N + NLFA-AMF	203.15	1.79	0.07	0.68	- 93.96
	7	Root biomass + Olsen P + Soil C + Fungal biomass [a]	203.22	1.86	0.06	0.74	- 94.00
	8	Root biomass + Olsen P + Soil C + NLFA-AMF	203.59	2.23	0.05	0.79	- 94.18

At day 28, models 1 - 4 explained equally good P uptake. Cumulatively, there is a 67% chance that one of these models is the best in explaining P uptake. All these 4 models included root biomass, Olsen P and soil pH and different combinations of PLFA-

Fungi (saprophytic fungi) and fungal biomass (PLFA-Fungi + NLFA-AMF) or soil C and soil C: N as explanatory factors. At day 112, models 1 – 7 were equally in good in explaining P uptake. In this case, the cumulative likelihood of one of these models being the best at explaining P uptake is 74%. Root biomass and Olsen P were included as explanatory variables in all 7 best ranked models. Soil pH was an important explanatory variable in models 1 – 4. Combinations of either fungal biomass and NLFA-AMF or soil C and soil C: N were important explanatory variables for all the 7 first ranked models.

The best explanatory model at day 28 was "Root biomass + Olsen P + pH + Soil C: N + PLFA-Fungi" ($p < 0.001$; R^2 of 0.77; Table 4.5). The coefficients in the model were 0.67, 2.03, - 3.57, - 0.12 and 0.01 for root biomass, Olsen P, pH, Soil C: N and PLFA-Fungi respectively. At day 112, the best model to predict P uptake is "Root biomass + Olsen P + pH + Soil C: N + Fungal biomass", with a considerable lower R^2 than the best model at day 28 ($R^2 = 0.37$, $p = 0.026$). The estimates for this model were 0.34, 1.00, 2.04, - 0.08 and - 0.01 for root biomass, Olsen P, pH, Soil C: N and fungal biomass respectively.

4. Discussion

4.1. Fertilizer value of DPW

Our first research objectives was to study the fertilizer value of DPW and its secondary materials. We hypothesized that DPW materials would be good candidates to reduce dependency on mineral P fertilizers. Our results indicate that the form of DPW material has a great impact on their agronomic value.

Among all the materials studied, we observed that sludge was the material that increased biomass production, P uptake and PUE the most as compared to the control (Figures 4.1 and 4.4). P uptake and PUE were even higher in sludge fertilized pots than in TSP fertilized ones. We assumed that the higher biomass production of pots fertilized with sludge are related to a higher availability of nutrients compared to TSP and to the presence of labile N and C in sludge. TSP significantly increased P concentration and P uptake, but only at the first harvest (Figure 4.3). After this date, P concentrations and uptake in the sludge treatment equalled with that in the TSP

treatment and even exceeded them for P uptake, as well as PUE. TSP application increased Olsen P in the soil, yet P from TSP could have been rapidly sorbed into the soil mineral phase or leached out from the pot (Jalali and Jalali, 2020; Teles et al., 2020), decreasing plant available P and uptake over time in this treatment. This was corroborated by the decrease over time in Olsen P in the TSP fertilized pots (Figure 4.5). On the other hand, P in the sludge was likely mostly present in organic forms (Yu et al., 2021), which could have performed as slow releasing sources of P for grass and soil microorganisms. It has already been demonstrated that available P from TSP decreases sharply over time whereas organic forms of P are potentially mobilizable by soil microorganisms over a longer period of time and by a re-equilibrium with the mineral phase (Khomenko et al., 2023a; Velasco-Sánchez et al., 2024).

The application of sludge not only increased P uptake but also increased total N uptake and soil N-NH_4 (Table 4.4), regardless of the additions of N fertilizer that, we applied after each cut. This suggests that sludge improved both P and N uptake and that the forms of P and N in sludge are very available for plants over time. Our results also showed a higher limitation of N for the grasses grown in TSP fertilized at day 28, as shown by the significantly lower N: P ratios, and higher P limitation for the plants fertilized with hydrochar (Supplementary material, Table 4.S4). DPW's sludge has also been proposed, in previous research, as a material with a great replacement value of mineral N fertilizers (Shi et al., 2022). In our study we further confirmed that observation, even when enough mineral N fertilizers was applied.

Ash and hydrochar showed lower P uptake and PUE than sludge or TSP (Figure 4.4). For ash, we observed similar dynamics as TSP: a strong increase of P uptake at the beginning of the experiment which decreased at the later stages of the experiment (Figure 4.3). This can potentially be explained by a quick sorption of the plant available fractions of P in ashes to the mineral phase, or by leaching from the system. By the end of the experiment, the PUE of ashes was approximately 25%, nearly half of those from TSP or sludge (Figure 4.4, panel B). Hydrochar was the DPW material that performed worst with values of PUE that were not higher than 0% (Figure 4.4, panel B). We assumed that the P in hydrochar was in very recalcitrant forms (Schneider and Haderlein, 2016). Our results match the observations from other

greenhouse and field experiments in which sludge from DPW resulted in higher or equal biomass production or P uptake as TSP and hydrochar resulted in poor biomass production or even negative effects on crops (Schimmelpfennig et al., 2015; Shi et al., 2022; Khomenko et al., 2023b; Hu et al., 2024)

The results of our experiment present also challenges for nutrient recovery from DPW. Although our results show that raw sludge is the best fertilizer for the studied grass species, the moisture content of DPW sludge can easily reach values of 90% (Ashekuzzaman et al., 2019; Hu et al., 2021). Economical costs related to drying or transportation and farmers perception towards the use of recycled P fertilizers should be taken into consideration, which would potentially reduce the realistic applicability of DPW sludge in agriculture (Behjat et al., 2022; Garmendia-Lemus et al., 2024). Moreover, in our study, we used an iron-chelated sludge. The results could have been different if the studied sludge was treated with aluminium chelants or if limestone was added to the sludge (Shi et al., 2022). The lack of widespread regulation makes even more complicated to predict the potential of P fertilization from DPW sludge (Kellis et al., 2013; Collivignarelli et al., 2019). In our experiment, we tested the secondary materials from technologies that aim to concentrate P by reducing the moisture content of sludge. Thermal oxidation and ashing resulted in PUE that were low (Figure 4.4). These materials have the potential to be used as raw material for the production of precipitated P salts such as struvite (Darwish et al., 2017; Becker et al., 2019), or as soil amendments (Islam et al., 2021; Ma and Rosen, 2021), however their direct use as P fertilizers needs further experiments and confirmation.

4.2. Impact of DPW materials on soil microorganisms and their contribution towards P uptake

The second research question of this study was to evaluate the impacts of DPW materials on soil microbial communities and their contribution towards P uptake. Soil microorganisms have been typically regarded as key players in P nutrition by crops (Richardson and Simpson, 2011). Recently, this vision has also been contested, indicating that most of the experiments associating P nutrition and microbial activities were conducted on artificial conditions (Raymond et al., 2021; O'Callaghan et al., 2022). The results of our experiment showed that the addition of DPW

materials or TSP had no major significant effects on most of the soil microbial variables studied, while, regardless of the plant species or P fertilizer treatment, fungal biomass played an important role in P uptake.

Sludge and ash application only affected AMF and anaerobe communities in the Lp pots (Supplementary material, Table 4.S7) and sludge resulted in a decrease in Bglu activities (Supplementary material, Table 4.S9), probably because of the introduction of labile C sources (de Almeida et al., 2015). It is generally assumed that enzyme production is only triggered if the substrates are present in the soil (Allison et al., 2010). Our results contrast with other studies in which the application of DPW sludge resulted in changes on the microbial composition and functions (Frąc et al., 2012; Gryta et al., 2014). The responses of microbial communities to the addition of ashes and hydrochar from DPW remain poorly studied. However, other studies which tested materials from other sources, such as sewage sludge, indicated significant effects on the soil microbial community. These included increased soil respiration after ash application (Pichtel, 1990) or alteration in the soil microbial composition after application of hydrochar (Zhu et al., 2023). The lack of significant differences found in our study could be associated with the length of our experiment (López-Mosquera et al., 2000) and the chemical composition of DPW materials applied, which tend to be highly heterogeneous (Ashekuzzaman et al., 2019).

Yet, in our study, we observed that regardless of the plant species or P fertilizer treatment, fungal biomass played a key role in P uptake (Table 4.5). Fungal communities were important variables to explain P uptake in at least the first ranked 8 models at dates 28 and 112 (Table 4.5). Our results also suggest that the effects of fungi were superior than those of bacteria. The effects of fungi, however, cannot solely explain P uptake by the different grasses as the association of Olsen P, pH, root biomass and soil C: N were equally important variables in the most explanatory models (Table 4.5). The effects of the fungal community on P uptake were dependent on the sampling date and soil C. Particularly, PLFA-Fungi biomass, which is associated with saprophytic fungi, correlated positively with P uptake at day 28 and was included in the best ranked models based on their AIC (Table 4.5). Saprophytic fungal groups such as *Aspergillum spp., Penicillium spp.* or *Trichoderma spp.* have been shown to be important in P solubilization (Daynes et al., 2008; Bononi et al.,

2020; Nascimento et al., 2021). These saprophytic fungi were possibly important at the earlier stages of the experiment when most C was available (Table 4.4). It has been demonstrated that C availability is a very important requirement for the soil microbial community to solubilize recalcitrant forms of P (Brucker et al., 2020; Velasco-Sánchez et al., 2024). By the end of the experiment, when the C content of the soil had decreased, the relevance of saprophytic fungi was less pronounced. At this date, AMF was the biological variable that stood up in our best models (Table 4.5). The estimate for AMF in the best ranked model was negative, suggesting that AMF colonization was more important in those plants grown under more limiting P conditions (Fornara et al., 2020; Mitra et al., 2023). AMF have been widely associated with improved P nutrition in grasslands (Fornara et al., 2020) and our results tend to confirm this observation.

4.3. Grass species and P utilization

In our experiment, we also tested the effects of DPW on two grass species with contrasting growth strategies: Lp as a fast-grower and Dg as a slow-grower. As such, we expected that the different growth strategies would result in different PUEs and microbial associations. We observed a myriad of significant plant effects and/or significant interactions of grass species with fertilizer application or with harvest time. For instance, Olsen P values were lower in Lp pots and P uptake and concentration was higher in Lp plots (Table 4.4), suggesting a higher utilization of the Olsen P pool by Lp plants. By the end of the experiment, however, no differences were found in cumulative biomass production or PUE (Tables 4.2 and 4.3 and Figure 4.2, panel B), suggesting a more efficient use of P by Dg plants. Dg has already been suggested to have a high use efficiency of P and N (Ryser et al., 1997). The differences in grass biomass production over time (Figure 4.2), as well as the decrease in biomass production in Lp and the constant biomass production in Dg, contrasted with the availability of P (Olsen P), which decreased over time for TSP. This contrast should be considered, since different grass species would benefit from different P application timings (Simpson et al., 2014).

Lp and Dg showed also different associations with soil microorganisms. We observed that the effects of P fertilizers on AMF and anaerobe communities and Bglu were only significant on Lp plants (Supplementary material, Tables 4.S7 and 4.S9).

Similarly, Lp had significantly higher PAC activities than Dg (Supplementary material, Table 4.S9). The associations of soil microorganisms with Lp plants in the rhizosphere through the production of enzymes have already been studied and have shown to be important factors to improve P uptake in P limiting conditions (Scott and Condron, 2003; Sanguin et al., 2016). Similarly, the importance of AMF in increasing P uptake in Lp plants has also been observed (Li et al., 2019).

Finally, we noted that root biomass was one of the most important variables for explaining P uptake, as shown by the AIC model rankings (Table 4.5). For both dates (days 28 and 112), the coefficient of root biomass for the best ranked models was positive, suggesting a positive relationship between root biomass production and P uptake. Our results show the importance of species selection for a more efficient P utilization in grasslands. Selecting grass species, such as Lp, that make use of the more available forms shortly after fertilization would result in a higher PUE, whereas using slow growers like Dg would potentially reduce the required frequencies of P applications. Combinations of grass species with contrasting root traits and growth strategies should be further explored to reduce the dependency on mineral P fertilization (Simpson et al., 2014; de Oliveira et al., 2018; Ros et al., 2018).

4.4. Outlook

Further experiments, on a wider variety of soil, under field conditions and longer term (multiple seasons) should be performed to validate our findings. Moreover, experiments that focus on the combination of grass species with contrasting P acquisition strategies (e.g. different root traits, associations with soil microorganisms, etc.) under fertilization with dairy processing waste materials should be performed. Likewise, more experiments should focus on the toxicology aspect of dairy processing waste application and the long term effects on the environment (greenhouse gas emissions, heavy metals and nutrient leaching).

5. Conclusion

We evaluated the effects of different dairy processing waste products as substitutes of mineral P fertilizer on grass biomass production, P nutrition and, for the first time,

effects on soil microbial communities and activities. Our results revealed that, whereas dairy processing waste sludge has the potential to replace mineral P fertilizers, secondary materials such as ash or hydrochar are not good candidates to reduce mineral P dependency. Ash and hydrochar could be used as soil amendments or as intermediary materials for the production of precipitated P salts, such as struvite. Our results also highlight the pivotal role of fungal communities in P uptake and nutrition in grasses.

6. Acknowledgments

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Supplementary material

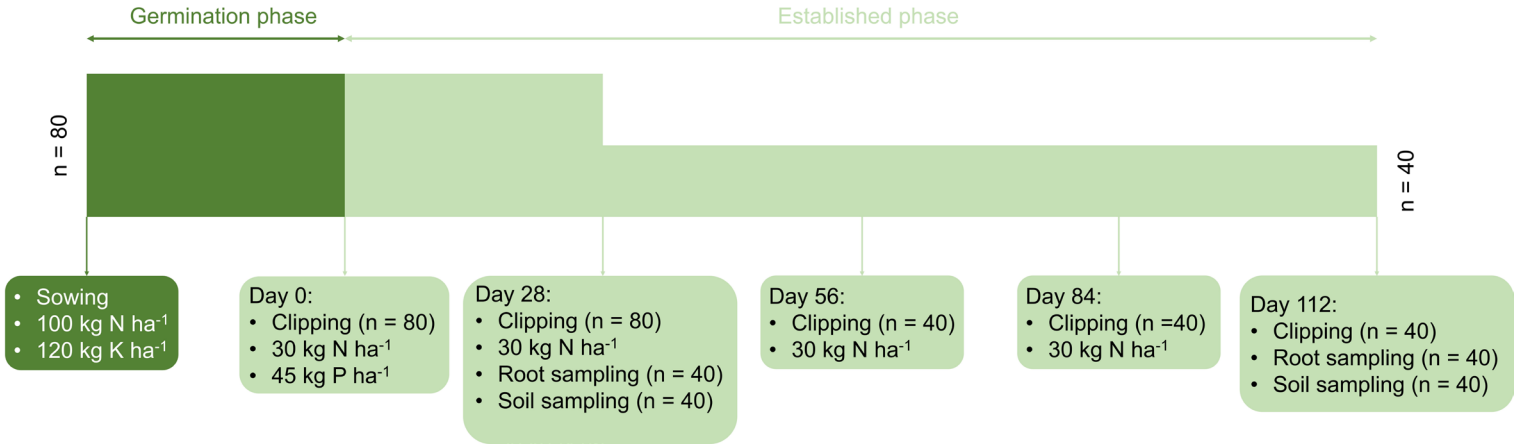


Figure 4.S1. *Experimental design. In dark green germination phase (28 days) is represented and in light green the established phase (112 days) is depicted. P application was performed in the form of triple superphosphate (TSP), dairy processing waste sludge, its ash, its hydrochar and a control with no P application on day 28 after sowing or day 0 of the established phase.*

Table 4.S1. *PLFA and NLFA peaks assigned to different soil microbial groups by MIDI Sherlock Software v.6.3B (Shimadzu, Kyoto, Japan).*

Microbial groups	Peaks
Gram -	10:0 2OH, 10:0 3OH, 12:1 w8c, 12:1 w5c, 13:1 w5c, 13:1 w4c, 13:1 w3c, 12:0 2OH, 14:1 w9c, 14:1 w8c, 14:1 w7c,14:1 w5c, 15:1 w9c, 15:1 w8c, 15:1 w7c, 15:1 w6c, 15:1 w5c, 14:0 2OH, 16:1 w9c, 16:1 w7c, 16:1 w6c, 16:1 w4c, 16:1 w3c, 17:1 w9c, 17:1 w8c, 17:1 w7c, 17:1 w6c, 17:0cyclow7c, 17:1 w5c, 17:1 w4c, 17:1 w3c, 16:0 2OH, 18:0 cyclo w6c, 18:1 w8c, 18:1 w7c, 18:1 w6c, 18:1 w5c, 18:1 w3c, 19:1 w9c, 19:1 w8c, 19:1 w7c, 19:1 w6c, 19:0 cyclo w7c, 19:0 cyclo w6c, 20:1 w9c, 20:1 w8c, 20:1 w6c, 20:1 w4c, 20:0 cyclo w6c, 21:1 w9c, 21:1 w8c, 21:1 w6c, 21:1 w5c, 21:1 w4c, 21:1 w3c, 22:1 w9c, 22:1 w8c, 22:1 w6c, 22:1 w5c, 22:1 w3c, 22:0 cyclo w6c, 24:1 w9c, 24:1 w7c, 11:0 iso 3OH, 14:0 iso 3OH, 17:0 iso 3O
Gram +	11:0 iso, 11:0 anteiso, 12:0 iso, 12:0 anteiso, 13:0 iso, 13:0 anteiso, 14:1 iso w7c, 14:0 iso, 14:0 anteiso, 15:1 iso w9c, 15:1 iso w6c, 15:1 anteiso w9c, 15:0 iso, 15:0 anteiso, 16:0 iso, 16:0 anteiso, 17:1 iso w9c, 17:0 iso, 17:0 anteiso, 18:0 iso, 17:1 anteiso w9c, 17:1 iso w10c, 17:1 anteiso w7c, 18:1 w9c, 19:0 cyclo w9c, 19:0 iso, 19:0 anteiso, 20:0 iso, 22:0 iso
Eukaryote	15:4 w3c, 15:3 w3c, 16:4 w3c, 16:3 w6c, 18:3 w6c, 19:4 w6c, 19:3 w6c, 19:3 w3c, 20:4 w6c, 20:5 w3c, 20:3 w6c, 20:2 w6c, 21:3 w6c, 21:3 w3c, 22:5 w6c, 22:6 w3c, 22:4 w6c, 22:5 w3c, 22:2 w6c, 23:4 w6c, 23:3 w6c, 23:3 w3c, 23:1 w5c, 23:1 w4c, 24:4 w6c, 24:3 w6c, 24:3 w3c, 24:1 w3c, 18:4 w3c
Anaerobe	12:0 DMA, 13:0 DMA, 14:1 w7c DMA, 14:0 DMA, 15:0 iso DMA, 15:0 DMA, 16:2 DMA, 17:0 DMA, 16:1 w9c DMA, 16:1 w7c DMA, 16:1 w5c DMA, 16:0 DMA, 18:2 DMA, 18:1 w9c DMA, 18:1 w7c DMA, 18:1 w5c DMA, 18:0 DMA, 19:0 cyclo 9,10 DMA
Actinomycetes	16:0 10-methyl, 17:1 w7c 10-methyl, 17:0 10-methyl, 22:0 10-methyl, 18:1 w7c 10-methyl, 18:0 10-methyl, 19:1 w7c 10-methyl, 20:0 10-methyl
Saprophytic fungi	18:2 w6c
AMF - NLFA	16:1 w5c

Table 4.S2. *Mean values of biomass production for different P fertilizer treatments, grass species and harvest times. Values indicate mean ± standard error in g of biomass pot^{-1}. For leaf biomass, at day 28 n = 8, for days 56, 84 and 112 n = 4. Letters in bold denote significant differences between groups according to Tukey's post hoc test. Format of letters indicate different significant ($p < 0.05$) comparisons among groups, e.g.* ***"A"*** *indicates significant differences for Treatment effect and* ***"a"*** *indicate differences for the fertilizer x Time interaction.*

Grass species	Day	Treatment	Leaf biomass	Root biomass	Basal stem	(Leaf + Stem) Root^{-1}	Cumulative leaf biom.	Cumulative total biom.
Lp	28	Control	7.18 ± 0.48 **b**	17.68 ± 2.26	0.37 ± 0.07	0.45 ± 0.05		
		Hydrochar	8.32 ± 0.29 **b**	19.73 ± 4.41	0.76 ± 0.30	0.56 ± 0.16		
		Ash	9.31 ± 0.57 **a**	24.10 ± 5.89	0.45 ± 0.13	0.46 ± 0.07		
		Sludge	9.11 ± 0.63 **a**	22.87 ± 5.49	0.96 ± 0.46	0.46 ± 0.09		
		TSP	9.63 ± 0.51 **a**	27.09 ± 4.25	0.56 ± 0.06	0.41 ± 0.04		
	56	Control	6.00 ± 0.44 **ab**					
		Hydrochar	5.78 ± 0.50 **b**					
		Ash	6.69 ± 0.63 **ab**					
		Sludge	8.18 ± 0.44 **a**					
		TSP	7.34 ± 0.13 **ab**					
	84	Control	4.94 ± 0.41					
		Hydrochar	5.30 ± 0.54					
		Ash	5.26 ± 0.12					
		Sludge	7.02 ± 0.20					
		TSP	5.64 ± 0.23					
	112	Control	5.01 ± 0.09	12.76 ± 1.60	2.52 ± 0.41	0.63 ± 0.11	23.03 ± 1.72 **B**	38.29 ± 3.18 **B**
		Hydrochar	5.48 ± 0.34	17.78 ± 4.39	2.34 ± 0.06	0.51 ± 0.10	24.81 ± 1.76 **B**	44.93 ± 6.10 **B**
		Ash	5.46 ± 0.08	20.38 ± 4.79	2.37 ± 0.17	0.45 ± 0.10	26.63 ± 1.17 **B**	49.38 ± 5.18 **AB**
		Sludge	6.42 ± 0.22	28.07 ± 2.79	2.66 ± 0.28	0.33 ± 0.02	31.60 ± 0.78 **A**	62.34 ± 3.70 **A**
		TSP	5.12 ± 0.31	24.65 ± 1.51	3.02 ± 0.42	0.33 ± 0.01	27.34 ± 0.94 **AB**	55.01 ± 2.77 **AB**

Table 4.S2 continues on next page

(Table 4.S2 continued)

Grass species	Day	Treatment	Leaf biomass	Root biomass	Basal stem	(Leaf + Stem) Root^{-1}	Cumulative leaf biom.	Cumulative total biom.
Dg	28	Control	5.48 ± 0.50 **b**	4.44 ± 0.80	0.29 ± 0.14	1.12 ± 0.20		
		Hydrochar	4.70 ± 0.47 **b**	2.78 ± 0.63	0.08 ± 0.02	1.52 ± 0.10		
		Ash	7.53 ± 0.53 **a**	8.14 ± 0.77	0.48 ± 0.10	1.00 ± 0.02		
		Sludge	8.38 ± 0.21 **a**	6.62 ± 1.18	0.36 ± 0.06	1.48 ± 0.27		
		TSP	8.23 ± 0.47 **a**	7.06 ± 1.74	0.49 ± 0.11	1.41 ± 0.31		
	56	Control	7.27 ± 1.10 **ab**					
		Hydrochar	6.70 ± 0.78 **b**					
		Ash	6.60 ± 0.28 **ab**					
		Sludge	8.38 ± 0.38 **a**					
		TSP	6.59 ± 0.24 **ab**					
	84	Control	5.97 ± 0.55					
		Hydrochar	6.01 ± 0.42					
		Ash	5.85 ± 0.10					
		Sludge	6.86 ± 0.45					
		TSP	6.15 ± 0.30					
	112	Control	6.48 ± 0.31	14.56 ± 3.62	4.85 ± 0.74	0.86 ± 0.12	25.20 ± 2.15 **B**	44.61 ± 6.32 **B**
		Hydrochar	6.03 ± 0.17	10.56 ± 2.01	4.24 ± 0.43	1.05 ± 0.16	24.10 ± 1.55 **B**	38.90 ± 3.72 **B**
		Ash	6.58 ± 0.22	16.98 ± 3.71	4.49 ± 0.20	0.77 ± 0.19	26.47 ± 1.41 **B**	47.94 ± 5.08 **AB**
		Sludge	6.59 ± 0.35	15.28 ± 2.64	5.08 ± 0.54	0.81 ± 0.10	30.09 ± 0.76 **A**	50.45 ± 3.86 **A**
		TSP	6.83 ± 0.28	14.90 ± 2.18	4.60 ± 0.57	0.84 ± 0.17	27.91 ± 1.06 **AB**	47.91 ± 1.71 **AB**
Fertilizer			**< 0.001**	**0.013**	0.407	0.231	**< 0.001**	**0.008**
Grass species			0.897	**< 0.001**	**< 0.001**	**< 0.001**	0.934	0.148
Time			**< 0.001**	**0.018**	**< 0.001**	**< 0.001**	n.a.	n.a.
Fertilizer x Grass species			0.642	0.256	0.785	0.254	0.734	0.313
Fertilizer x Time			**< 0.001**	0.848	0.744	0.394	n.a.	n.a.
Grass species x Time			**< 0.001**	**< 0.001**	**< 0.001**	**0.002**	n.a.	n.a.
Fertilizer x Grass species x Time			0.073	0.778	0.648	0.952	n.a.	n.a.

Table 4.S3. *Mean values of P concentration and uptake for different P fertilizer treatments, grass species and harvest times in leaves. Values indicate mean ± standard error. For leaf P uptake and concentration, at day 28 n = 8, for days 56, 84 and 112 n = 4. Leaf P concentration expressed in%, leaf uptake in mg of P and cumulative total in g of P pot^{-1}. Letters in bold denote significant differences between groups according to Tukey's post hoc test. Format of letters indicate different significant ($p < 0.05$) comparisons among groups, e.g.* ***"A"*** *indicates significant differences for Treatment effect and* ***"a"*** *indicate differences for the fertilizer x Time interaction.*

Grass species	Day	Treatment	Leaf P concentration	Leaf P uptake	Cumulative total P upt	Leaf PUE	Cumulative total PUE
Lp	28	Control	0.27 ± 0.03 **B**	19.42 ± 2.95 **b**			
		Hydrochar	0.30 ± 0.04 **B**	25.63 ± 3.67 **b**		0.05 ± 0.05	
		Ash	0.27 ± 0.01 **B**	25.10 ± 2.00 **b**		0.06 ± 0.01	
		Sludge	0.32 ± 0.04 **B**	27.99 ± 3.70 **b**		0.11 ± 0.05	
		TSP	0.44 ± 0.03 **A**	43.27 ± 5.37 **a**		0.14 ± 0.03	
	56	Control	0.34 ± 0.04 **B**	20.02 ± 1.42 **b**			
		Hydrochar	0.40 ± 0.01 **B**	23.24 ± 2.27 **b**		0.06 ± 0.07 **b**	
		Ash	0.49 ± 0.06 **B**	33.69 ± 7.79 **ab**		0.05 ± 0.01 **b**	
		Sludge	0.34 ± 0.02 **B**	27.98 ± 2.47 **ab**		0.11 ± 0.05 **ab**	
		TSP	0.54 ± 0.10 **A**	39.97 ± 7.40 **a**		0.14 ± 0.03 **a**	
	84	Control	0.44 ± 0.04 **B**	21.48 ± 1.77			
		Hydrochar	0.49 ± 0.03 **B**	25.64 ± 1.21		0.09 ± 0.07 **c**	
		Ash	0.47 ± 0.02 **B**	24.57 ± 0.93		0.17 ± 0.06 **bc**	
		Sludge	0.49 ± 0.02 **B**	34.19 ± 1.11		0.25 ± 0.06 **ab**	
		TSP	0.55 ± 0.05 **A**	31.15 ± 2.64		0.35 ± 0.06 **a**	
	112	Control	0.43 ± 0.01 **B**	21.53 ± 0.35	0.12 ± 0.01 **B**		
		Hydrochar	0.32 ± 0.05 **B**	17.73 ± 2.41	0.13 ± 0.02 **B**	0.07 ± 0.08 **c**	0.13 ± 0.12 **B**
		Ash	0.44 ± 0.04 **B**	23.82 ± 2.19	0.16 ± 0.02 **AB**	0.19 ± 0.07 **bc**	0.32 ± 0.14 **AB**
		Sludge	0.39 ± 0.07 **B**	24.62 ± 3.28	0.21 ± 0.02 **A**	0.28 ± 0.08 **ab**	0.69 ± 0.14 **A**
		TSP	0.51 ± 0.11 **A**	25.33 ± 4.02	0.20 ± 0.01 **A**	0.38 ± 0.06 **a**	0.56 ± 0.05 **A**

Table 4.S3 continues on next page

(Table 4.S3 continued)

Grass species	Day	Treatment	Leaf P concentration	Leaf P uptake	Cumulative total P upt	Leaf PUE	Cumulative total PUE
Dg	28	Control	0.27 ± 0.03 **B**	14.94 ± 3.22 **b**			
		Hydrochar	0.22 ± 0.03 **B**	9.99 ± 1.40 **b**		-0.03 ± 0.01	
		Ash	0.30 ± 0.04 **B**	21.62 ± 2.37 **b**		0.02 ± 0.01	
		Sludge	0.26 ± 0.05 **B**	21.21 ± 3.29 **b**		0.04 ± 0.04	
		TSP	0.52 ± 0.05 **A**	42.82 ± 4.71 **a**		0.15 ± 0.04	
	56	Control	0.32 ± 0.05 **B**	22.11 ± 1.89 **b**			
		Hydrochar	0.37 ± 0.05 **B**	24.01 ± 2.18 **b**		-0.01 ± 0.02 **b**	
		Ash	0.42 ± 0.07 **B**	27.48 ± 4.23 **ab**		0.06 ± 0.02 **b**	
		Sludge	0.41 ± 0.01 **B**	34.42 ± 0.85 **ab**		0.14 ± 0.05 **ab**	
		TSP	0.50 ± 0.06 **A**	33.02 ± 4.57 **a**		0.23 ± 0.06 **a**	
	84	Control	0.36 ± 0.05 **B**	21.00 ± 2.23			
		Hydrochar	0.41 ± 0.04 **B**	24.43 ± 2.19		0.01 ± 0.02 **c**	
		Ash	0.36 ± 0.02 **B**	21.13 ± 0.89		0.06 ± 0.03 **bc**	
		Sludge	0.45 ± 0.04 **B**	31.08 ± 4.12		0.21 ± 0.06 **ab**	
		TSP	0.49 ± 0.06 **A**	30.67 ± 5.08		0.30 ± 0.08 **a**	
	112	Control	0.31 ± 0.05 **B**	19.47 ± 2.49	0.12 ± 0.01 **B**		
		Hydrochar	0.33 ± 0.02 **B**	20.16 ± 1.79	0.12 ± 0.01 **B**	0.01 ± 0.01 **c**	-0.01 ± 0.03 **B**
		Ash	0.34 ± 0.03 **B**	22.82 ± 2.68	0.15 ± 0.01 **AB**	0.08 ± 0.03 **bc**	0.23 ± 0.07 **AB**
		Sludge	0.33 ± 0.02 **B**	21.97 ± 1.57	0.18 ± 0.03 **A**	0.22 ± 0.07 **ab**	0.44 ± 0.20 **A**
		TSP	0.35 ± 0.01 **A**	23.86 ± 1.48	0.18 ± 0.01 **A**	0.32 ± 0.09 **a**	0.42 ± 0.07 **A**
Fertilizer			**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Grass species			**0.013**	0.134	0.088	0.098	0.077
Time			**< 0.001**	**0.001**	n.a.	**< 0.001**	n.a.
Fertilizer x Grass species			0.973	0.922	0.855	0.954	0.914
Fertilizer x Time			0.131	**< 0.001**	n.a.	**< 0.001**	n.a.
Grass species x Time			0.121	0.136	n.a.	0.562	n.a.
Fertilizer x Grass species x Time			0.462	0.452	n.a.	0.856	n.a.

Table 4.S4. *Mean values of N concentration, uptake and ratios for different P fertilizer treatments, grass species and harvest times in root and basal stem. Values indicate mean ± standard error. At day 28 n = 8, for days 56, 84 and 112 n = 4. Leaf N concentration expressed in%, Leaf N uptake in g and cumulative total N uptake in g pot^{-1}. Letters in bold denote significant differences between groups according to Tukey's post hoc test. Format of letters indicate different significant ($p < 0.05$) comparisons among groups, e.g. "A" indicates significant differences for Treatment effect, "a" indicate differences for the fertilizer x Time interaction and "a" indicates differences for the triple interaction.*

Grass species	Day	Treatment	Leaf N concentration	Leaf N upt	Cumulative total N upt	Leaf C: N	Leaf C: P	Leaf N: P
Lp	28	Control	1.76 ± 0.14	0.12 ± 0.01		23.33 ± 1.70	160.88 ± 18.67 **B**	7.27 ± 1.06 **b**
		Hydrochar	1.81 ± 0.15	0.15 ± 0.01		22.74 ± 1.81	150.29 ± 26.35 **B**	7.21 ± 1.67 **a**
		Ash	1.63 ± 0.13	0.15 ± 0.01		25.32 ± 1.82	149.38 ± 6.44 **AB**	6.16 ± 0.59 **b**
		Sludge	1.73 ± 0.18	0.15 ± 0.01		23.72 ± 2.22	134.16 ± 15.36 **AB**	5.75 ± 0.53 **b**
		TSP	1.57 ± 0.13	0.15 ± 0.01		26.18 ± 2.12	92.66 ± 7.56 **A**	3.66 ± 0.31 **c**
	56	Control	1.59 ± 0.14	0.09 ± 0.01		25.51 ± 1.94	121.97 ± 14.56 **B**	4.80 ± 0.47
		Hydrochar	1.83 ± 0.21	0.10 ± 0.01		22.63 ± 2.27	100.03 ± 3.42 **B**	4.60 ± 0.63
		Ash	1.52 ± 0.18	0.10 ± 0.01		27.26 ± 3.00	84.93 ± 8.30 **AB**	3.29 ± 0.56
		Sludge	1.54 ± 0.05	0.13 ± 0.01		26.01 ± 0.84	118.72 ± 7.37 **AB**	4.60 ± 0.44
		TSP	1.36 ± 0.04	0.10 ± 0.01		29.21 ± 0.66	84.45 ± 20.80 **A**	2.93 ± 0.79
	84	Control	1.68 ± 0.24	0.08 ± 0.01		25.89 ± 4.77	93.43 ± 7.04 **B**	3.88 ± 0.62
		Hydrochar	1.65 ± 0.22	0.08 ± 0.01		25.99 ± 3.51	83.57 ± 5.48 **B**	3.33 ± 0.30
		Ash	1.59 ± 0.11	0.08 ± 0.01		26.01 ± 1.81	87.68 ± 3.08 **AB**	3.40 ± 0.17
		Sludge	1.39 ± 0.16	0.10 ± 0.01		29.98 ± 2.77	82.94 ± 3.07 **AB**	2.82 ± 0.22
		TSP	1.46 ± 0.12	0.08 ± 0.01		28.53 ± 2.39	75.22 ± 6.33 **A**	2.65 ± 0.17
	112	Control	2.07 ± 0.09	0.10 ± 0.01	0.52 ± 0.02 **B**	19.52 ± 1.39	93.29 ± 2.44 **B**	4.83 ± 0.22
		Hydrochar	1.71 ± 0.08	0.09 ± 0.01	0.57 ± 0.01 **B**	24.86 ± 1.53	137.23 ± 17.30 **B**	5.60 ± 0.87
		Ash	1.77 ± 0.09	0.10 ± 0.01	0.59 ± 0.04 **AB**	23.85 ± 0.83	99.72 ± 13.61 **AB**	4.20 ± 0.59
		Sludge	1.68 ± 0.05	0.11 ± 0.01	0.70 ± 0.02 **A**	24.47 ± 0.83	112.81 ± 14.57 **AB**	4.61 ± 0.59
		TSP	1.67 ± 0.07	0.09 ± 0.01	0.58 ± 0.02 **B**	24.54 ± 1.29	89.78 ± 13.96 **A**	3.67 ± 0.60

Table 4.S4 continues on next page

(Table 4.S4 continued)

Grass species	Day	Treatment	Leaf N concentration	Leaf N upt	Cumulative total N upt	Leaf C: N	Leaf C: P	Leaf N: P
Dg	28	Control	2.66 ± 0.18 **ab**	0.14 ± 0.01		16.08 ± 1.27	180.20 ± 27.42 **B**	11.60 ± 1.78 **b**
		Hydrochar	3.30 ± 0.16 **a**	0.15 ± 0.01		12.40 ± 0.62	209.34 ± 31.91 **B**	17.19 ± 2.61 **a**
		Ash	2.02 ± 0.16 **c**	0.15 ± 0.02		21.20 ± 1.74	152.90 ± 18.35 **AB**	7.65 ± 1.11 **b**
		Sludge	2.22 ± 0.13 **bc**	0.19 ± 0.01		19.04 ± 1.09	190.05 ± 26.13 **AB**	10.11 ± 1.41 **b**
		TSP	2.09 ± 0.21 **c**	0.17 ± 0.02		20.96 ± 2.18	83.53 ± 8.05 **A**	4.37 ± 0.67 **c**
	56	Control	1.83 ± 0.19	0.13 ± 0.01		22.89 ± 2.23	151.49 ± 31.74 **B**	6.56 ± 0.95
		Hydrochar	1.76 ± 0.13	0.12 ± 0.02		23.67 ± 1.74	117.92 ± 18.74 **B**	5.15 ± 1.12
		Ash	1.60 ± 0.11	0.11 ± 0.01		25.90 ± 1.67	103.71 ± 12.71 **AB**	4.13 ± 0.68
		Sludge	1.66 ± 0.18	0.14 ± 0.01		26.08 ± 3.53	100.69 ± 2.26 **AB**	4.02 ± 0.39
		TSP	1.61 ± 0.07	0.11 ± 0.01		25.48 ± 1.22	86.54 ± 13.61 **A**	3.40 ± 0.48
	84	Control	1.64 ± 0.17	0.10 ± 0.01		26.17 ± 2.89	122.80 ± 18.65 **B**	4.89 ± 1.06
		Hydrochar	1.57 ± 0.13	0.10 ± 0.01		27.09 ± 2.33	105.36 ± 13.00 **B**	4.08 ± 0.82
		Ash	1.53 ± 0.08	0.09 ± 0.01		27.55 ± 1.32	116.18 ± 4.45 **AB**	4.25 ± 0.29
		Sludge	1.50 ± 0.12	0.10 ± 0.01		27.97 ± 2.11	94.42 ± 8.64 **AB**	3.46 ± 0.46
		TSP	1.55 ± 0.03	0.10 ± 0.01		26.88 ± 0.43	89.12 ± 12.65 **A**	3.32 ± 0.48
	112	Control	1.60 ± 0.06	0.10 ± 0.01	0.58 ± 0.06 **B**	26.70 ± 1.20	149.14 ± 22.87 **B**	5.57 ± 0.74
		Hydrochar	1.55 ± 0.07	0.09 ± 0.01	0.59 ± 0.06 **B**	26.97 ± 1.12	125.89 ± 7.07 **B**	4.72 ± 0.46
		Ash	1.53 ± 0.05	0.10 ± 0.01	0.62 ± 0.05 **AB**	28.77 ± 1.21	130.36 ± 11.91 **AB**	4.58 ± 0.54
		Sludge	1.81 ± 0.20	0.12 ± 0.01	0.72 ± 0.02 **A**	24.01 ± 2.70	126.81 ± 7.11 **AB**	5.49 ± 0.72
		TSP	1.70 ± 0.11	0.12 ± 0.01	0.61 ± 0.03 **B**	25.01 ± 1.04	121.44 ± 3.77 **A**	4.88 ± 0.27
Fertilizer			0.061	0.074	**0.002**	0.108	**0.004**	**0.005**
Grass species			**0.048**	0.099	0.187	0.218	**0.006**	**0.004**
Time			**< 0.001**	**< 0.001**	n.a.	**< 0.001**	**< 0.001**	**< 0.001**
Fertilizer x Grass species			0.406	0.551	0.969	0.669	0.871	0.556
Fertilizer x Time			**0.021**	0.732	n.a.	0.414	0.132	**0.002**
Grass species x Time			**< 0.001**	0.653	n.a.	**< 0.001**	0.814	**< 0.001**
Fertilizer x Grass species x Time			**0.016**	0.971	n.a.	0.554	0.625	0.116

Table 4.S5. *Mean values of P and N concentration for different P fertilizer treatments, grass species and harvest times in root and basal stem. Values indicate mean ± standard error, n = 4. Values are expressed in% of dry biomass. Letters in bold denote significant differences between groups according to Tukey's post hoc test. Format of letters indicate different significant (p < 0.05) comparisons among groups, e.g.* ***"A"*** *indicates significant differences for Treatment effect, "**A**" indicates post hoc for significant fertilizer x grass species interaction and* ***"a"*** *indicate differences for the fertilizer x Time interaction.*

Grass species	Day	Treatment	Root P concentration	Root N concentration	Basal stem P concentration	Basal stem N concentration
Lp	28	Control	0.21 ± 0.02 **B**	0.78 ± 0.09	0.31 ± 0.03 **C c**	1.09 ± 0.12 **b**
		Hydrochar	0.21 ± 0.03 **B**	0.74 ± 0.15	0.35 ± 0.02 **BC ab**	1.32 ± 0.15 **a**
		Ash	0.23 ± 0.04 **AB**	0.85 ± 0.07	0.32 ± 0.06 **BC c**	0.96 ± 0.19 **b**
		Sludge	0.27 ± 0.05 **A**	0.93 ± 0.10	0.41 ± 0.03 **AB bc**	1.47 ± 0.18 **ab**
		TSP	0.24 ± 0.03 **AB**	0.67 ± 0.10	0.58 ± 0.04 **A a**	1.15 ± 0.07 **b**
	112	Control	0.18 ± 0.02 **B**	0.81 ± 0.04	0.34 ± 0.02 **C**	1.04 ± 0.07
		Hydrochar	0.21 ± 0.03 **B**	0.86 ± 0.13	0.37 ± 0.02 **BC**	0.97 ± 0.09
		Ash	0.21 ± 0.02 **AB**	0.80 ± 0.11	0.45 ± 0.04 **BC**	0.92 ± 0.02
		Sludge	0.31 ± 0.07 **A**	0.69 ± 0.06	0.47 ± 0.02 **AB**	1.16 ± 0.16
		TSP	0.20 ± 0.01 **AB**	0.63 ± 0.06	0.48 ± 0.07 **A**	0.95 ± 0.04
Dg	28	Control	0.23 ± 0.03 **B**	1.19 ± 0.10	0.30 ± 0.02 **C c**	1.48 ± 0.15 **b**
		Hydrochar	0.21 ± 0.01 **B**	1.29 ± 0.09	0.58 ± 0.04 **A ab**	2.18 ± 0.21 **a**
		Ash	0.28 ± 0.03 **AB**	1.15 ± 0.02	0.32 ± 0.04 **BC c**	1.33 ± 0.18 **b**
		Sludge	0.27 ± 0.02 **A**	1.19 ± 0.08	0.33 ± 0.03 **BC bc**	1.45 ± 0.12 **ab**
		TSP	0.27 ± 0.05 **AB**	1.21 ± 0.15	0.49 ± 0.06 **AB a**	1.57 ± 0.04 **b**
	112	Control	0.20 ± 0.02 **B**	0.70 ± 0.08	0.31 ± 0.01 **C**	0.77 ± 0.05
		Hydrochar	0.19 ± 0.02 **B**	0.88 ± 0.07	0.37 ± 0.02 **A**	0.73 ± 0.03
		Ash	0.27 ± 0.03 **AB**	0.75 ± 0.11	0.36 ± 0.03 **BC**	0.76 ± 0.03
		Sludge	0.32 ± 0.12 **A**	1.06 ± 0.11	0.35 ± 0.05 **BC**	0.79 ± 0.07
		TSP	0.22 ± 0.01 **B**	0.83 ± 0.11		0.62 ± 0.04

Table 4.S5 continues on next page

(Table 4.S5 continued)

Grass species	Day	Treatment	Root P concentration	Root N concentration	Basal stem P concentration	Basal stem N concentration
Fertilizer			**0.018**	0.285	**< 0.001**	**0.008**
Grass species			0.211	**< 0.001**	0.055	0.214
Time			0.543	**< 0.001**	0.383	**< 0.001**
Fertilizer x Grass species			0.870	0.351	**0.002**	0.110
Fertilizer x Time			0.571	0.984	**< 0.001**	**0.008**
Grass species x Time			0.953	**< 0.001**	**0.025**	**< 0.001**
Fertilizer x Grass species x Time			0.997	0.133	0.251	0.309

Table 4.S6. *Mean values of soil chemical variables for different P fertilizer treatments, grass species and harvest times in root and basal stem. Values indicate mean ± standard error, n = 4. Olsen P, total P, N-NO_3 and N-NH_4 are expressed in mg kg^{-1} whereas total N and C are expressed in%. Letters in bold denote significant differences between groups according to Tukey's post hoc test. Format of letters indicate different significant (p < 0.05) comparisons among groups, e.g.* ***"A"*** *indicates significant differences for Treatment effect, "**A**" indicates post hoc for significant fertilizer x grass species interaction and* ***"a"*** *indicate differences for the fertilizer x Time interaction.*

Grass species	Day	Treatment	Olsen P	Total P	N-NO_3	N-NH_4	Total N	Total C	pH
Lp	28	Control	13.00 ± 1.39 **b**	753.51 ± 75.31 **B**	20.87 ± 16.09	2.59 ± 0.33	0.22 ± 0.03	2.96 ± 0.35	6.81 ± 0.13
		Hydrochar	14.68 ± 1.80 **b**	1002.30 ± 140.19**A**	5.18 ± 1.04	2.58 ± 0.34	0.19 ± 0.01	2.46 ± 0.04	6.80 ± 0.16
		Ash	13.10 ± 0.52 **b**	998.13 ± 141.51 **A**	4.11 ± 0.80	1.97 ± 0.12	0.24 ± 0.03	3.16 ± 0.33	6.83 ± 0.10
		Sludge	12.54 ± 0.95 **b**	778.85 ± 91.23 **AB**	9.62 ± 6.72	2.53 ± 0.31	0.13 ± 0.03	2.76 ± 0.21	6.99 ± 0.11
		TSP	19.70 ± 1.73 **a**	1090 ± 51.90 **AB**	5.60 ± 2.69	2.59 ± 0.30	0.17 ± 0.01	2.69 ± 0.22	6.70 ± 0.15
	112	Control	10.36 ± 0.86	824.21 ± 38.75 **B**	3.66 ± 0.63	2.97 ± 0.21 **ab**	0.15 ± 0.01	2.22 ± 0.12	6.78 ± 0.23
		Hydrochar	10.43 ± 0.46	1119.53 ± 119.98**A**	18.13 ± 15.47	1.92 ± 0.32 **b**	0.18 ± 0.01	2.39 ± 0.04	6.93 ± 0.07
		Ash	12.84 ± 1.39	956.06 ± 92.04 **A**	7.05 ± 4.01	2.96 ± 0.21 **ab**	0.19 ± 0.02	2.60 ± 0.16	7.12 ± 0.04
		Sludge	11.82 ± 0.98	942.68 ± 128.26**AB**	3.07 ± 0.30	3.41 ± 0.30 **a**	0.17 ± 0.02	2.48 ± 0.09	7.31 ± 0.08
		TSP	14.60 ± 0.60	780.89 ± 125.02**AB**	3.52 ± 1.61	2.60 ± 0.32 **ab**	0.14 ± 0.01	2.32 ± 0.12	7.10 ± 0.14
Dg	28	Control	14.07 ± 0.72 **b**	859.42 ± 92.52 **B**	94.95 ± 67.45	2.66 ± 0.27 **b**	0.19 ± 0.01	2.49 ± 0.14	6.82 ± 0.12
		Hydrochar	17.20 ± 1.25 **b**	1112.61 ± 29.98 **A**	35.21 ± 12.60	2.42 ± 0.26 **b**	0.20 ± 0.04	2.68 ± 0.35	6.86 ± 0.07
		Ash	14.15 ± 0.44 **b**	1304.41 ± 315.64**A**	11.92 ± 4.93	2.84 ± 0.30 **b**	0.17 ± 0.01	2.64 ± 0.03	6.82 ± 0.12
		Sludge	17.65 ± 0.96 **b**	891.30 ± 75.22 **AB**	21.47 ± 6.69	4.53 ± 0.97 **a**	0.17 ± 0.01	3.00 ± 0.29	6.76 ± 0.10
		TSP	26.14 ± 3.01 **a**	1000.09 ± 21.53**AB**	5.78 ± 2.21	2.27 ± 0.09 **b**	0.18 ± 0.01	2.41 ± 0.07	6.92 ± 0.13
	112	Control	12.40 ± 1.61	706.55 ± 64.05 **B**	3.68 ± 0.85	2.53 ± 0.19	0.16 ± 0.02	2.42 ± 0.14	7.26 ± 0.11
		Hydrochar	13.70 ± 1.26	994.50 ± 72.81 **A**	5.01 ± 0.40	2.98 ± 0.48	0.15 ± 0.04	2.39 ± 0.17	7.45 ± 0.18
		Ash	12.10 ± 0.85	846.19 ± 85.92 **A**	3.88 ± 0.52	2.63 ± 0.11	0.17 ± 0.01	2.36 ± 0.19	7.13 ± 0.12
		Sludge	13.85 ± 1.44	859.10 ± 78.07 **AB**	8.71 ± 2.79	2.69 ± 0.14	0.17 ± 0.01	2.44 ± 0.04	7.14 ± 0.11
		TSP	15.16 ± 1.07	999.24 ± 31.32 **AB**	6.76 ± 3.99	2.37 ± 0.11	0.16 ± 0.02	2.11 ± 0.26	7.18 ± 0.06

Table 4.S6 continues on next page

(Table 4.S6 continued)

Grass species	Day	Treatment	Olsen P	Total P	$N\text{-}NO_3$	$N\text{-}NH_4$	Total N	Total C	pH
Fertilizer			**< 0.001**	**0.003**	0.264	**0.005**	0.091	0.200	0.634
Grass species			**< 0.001**	0.536	0.157	0.193	0.485	0.248	0.095
Time			**< 0.001**	0.134	0.080	0.868	**0.038**	**< 0.001**	**< 0.001**
Fertilizer x Grass species			0.355	0.975	0.636	0.186	0.280	0.339	**0.049**
Fertilizer x Time			**0.004**	0.205	0.284	0.645	0.211	0.901	0.964
Grass species x Time			0.132	0.131	0.146	0.058	0.984	0.583	0.118
Fertilizer x Grass species x Time			0.312	0.152	0.666	**0.003**	0.520	0.410	0.297

Table 4.S7. *Mean values of microbial PLFA and NLFA groups for different P fertilizer treatments, grass species and harvest times in root and basal stem. Values indicate mean ± standard error, n = 4. PLFA total and NLFA total are expressed x 10^5 picomol g^{-1} of soil, whereas the rest of microbial groups are expressed in x 10^4 picomol g^{-1} of soil. Letters in bold denote significant differences between groups according to Tukey's post hoc test. Format of letters indicate different significant ($p < 0.05$) comparisons among groups, e.g. "**A**" indicates post hoc for significant fertilizer x grass species interaction and "**a**" indicate differences for the triple interaction.*

Grass species	Day	Treatment	PLFA total[a]	NLFA total[a]	AMF (PLFA)[b]	AMF (NLFA)	Sap Fungi	Gram +	Gram -	Eukaryote	Anaerobe	Actinomycetes
Lp	28	Control	2.71 ± 0.10	0.33 ± 0.03	0.17 ± 0.01	0.14 ± 0.02 **B**	1.79 ± 0.32	1.40 ± 0.04	9.98 ± 0.32	1.26 ± 0.18	0.06 ± 0.01 **b**	0.59 ± 0.01
		Hydrochar	2.86 ± 0.10	0.31 ± 0.03	0.16 ± 0.02	0.11 ± 0.02 **AB**	1.65 ± 0.05	1.36 ± 0.09	10.57 ± 0.50	1.00 ± 0.09	0.04 ± 0.01 **ab**	0.60 ± 0.02
		Ash	2.69 ± 0.15	0.31 ± 0.05	0.15 ± 0.01	0.15 ± 0.02 **A**	1.73 ± 0.23	1.30 ± 0.06	9.97 ± 0.57	0.97 ± 0.16	0.05 ± 0.01 **a**	0.59 ± 0.02
		Sludge	2.83 ± 0.17	0.34 ± 0.07	0.17 ± 0.01	0.17 ± 0.01 **A**	1.50 ± 0.11	1.44 ± 0.04	10.73 ± 0.70	1.00 ± 0.12	0.05 ± 0.01 **ab**	0.63 ± 0.02
		TSP	2.78 ± 0.10	0.40 ± 0.04	0.16 ± 0.01	0.13 ± 0.02 **AB**	2.32 ± 0.59	1.43 ± 0.05	10.19 ± 0.33	1.00 ± 0.13	0.05 ± 0.01 **ab**	0.61 ± 0.02
	112	Control	2.94 ± 0.11	0.34 ± 0.11	0.18 ± 0.01	0.36 ± 0.13 **B**	1.85 ± 0.28	1.37 ± 0.02	10.84 ± 0.35	1.47 ± 0.13	0.04 ± 0.01 **a**	0.58 ± 0.01
		Hydrochar	3.13 ± 0.20	0.57 ± 0.04	0.22 ± 0.01	0.96 ± 0.14 **AB**	1.34 ± 0.14	1.57 ± 0.08	11.39 ± 0.84	1.62 ± 0.09	0.06 ± 0.01 **ab**	0.63 ± 0.03
		Ash	3.06 ± 0.09	0.63 ± 0.13	0.21 ± 0.01	1.03 ± 0.12 **A**	1.88 ± 0.22	1.44 ± 0.06	11.25 ± 0.23	1.58 ± 0.21	0.05 ± 0.01 **ab**	0.58 ± 0.02
		Sludge	2.82 ± 0.08	0.39 ± 0.16	0.21 ± 0.01	1.09 ± 0.15 **A**	1.53 ± 0.20	1.41 ± 0.07	10.37 ± 0.31	1.55 ± 0.15	0.04 ± 0.01 **b**	0.57 ± 0.02
		TSP	2.46 ± 0.64	0.53 ± 0.02	0.20 ± 0.02	0.81 ± 0.13 **AB**	1.25 ± 0.32	1.52 ± 0.05	8.84 ± 2.31	1.13 ± 0.35	0.05 ± 0.01 **ab**	0.61 ± 0.04

Table 4.S7 continues on next page

(Table 4.S7 continued)

Grass species	Day	Treatment	PLFA total[a]	NLFA total[a]	AMF (PLFA)[b]	AMF (NLFA)	Sap Fungi	Gram +	Gram -	Eukaryote	Anaerobe	Actinomycetes
Dg	28	Control	3.02 ± 0.33	0.35 ± 0.09	0.19 ± 0.01	0.23 ± 0.11	1.66 ± 0.13	1.43 ± 0.08	11.01 ± 1.22	1.49 ± 0.42	0.04 ± 0.01	0.61 ± 0.03
		Hydrochar	2.14 ± 0.58	0.33 ± 0.07	0.17 ± 0.01	0.10 ± 0.02	1.68 ± 0.33	1.33 ± 0.05	10.13 ± 0.43	0.94 ± 0.07	0.04 ± 0.01	0.59 ± 0.03
		Ash	2.71 ± 0.14	0.22 ± 0.01	0.18 ± 0.01	0.13 ± 0.01	1.31 ± 0.09	1.42 ± 0.04	9.98 ± 0.46	0.95 ± 0.15	0.04 ± 0.01	0.60 ± 0.01
		Sludge	2.95 ± 0.25	0.28 ± 0.02	0.17 ± 0.01	0.14 ± 0.02	1.56 ± 0.20	1.39 ± 0.08	10.85 ± 0.70	2.22 ± 1.14	0.05 ± 0.01	0.61 ± 0.02
		TSP	2.57 ± 0.19	0.19 ± 0.02	0.16 ± 0.01	0.10 ± 0.01	1.47 ± 0.31	1.33 ± 0.09	9.53 ± 0.57	0.70 ± 0.09	0.04 ± 0.01	0.59 ± 0.03
	112	Control	2.87 ± 0.10	0.56 ± 0.08	0.22 ± 0.01	1.20 ± 0.44	1.68 ± 0.09	1.51 ± 0.06	10.44 ± 0.38	1.33 ± 0.18	0.05 ± 0.01	0.61 ± 0.02
		Hydrochar	2.31 ± 0.62	0.49 ± 0.05	0.22 ± 0.02	0.73 ± 0.06	1.58 ± 0.17	1.47 ± 0.05	11.08 ± 0.77	1.45 ± 0.06	0.04 ± 0.01	0.61 ± 0.02
		Ash	2.90 ± 0.12	0.58 ± 0.03	0.19 ± 0.01	1.02 ± 0.14	2.07 ± 0.19	1.35 ± 0.03	10.36 ± 0.37	1.51 ± 0.10	0.05 ± 0.01	0.57 ± 0.02
		Sludge	2.78 ± 0.01	0.55 ± 0.03	0.21 ± 0.01	0.91 ± 0.14	1.71 ± 0.08	1.47 ± 0.06	10.19 ± 0.10	1.18 ± 0.05	0.04 ± 0.01	0.59 ± 0.03
		TSP	2.60 ± 0.21	0.50 ± 0.04	0.19 ± 0.01	0.77 ± 0.15	1.79 ± 0.23	1.31 ± 0.07	9.36 ± 0.73	1.28 ± 0.18	0.04 ± 0.01	0.55 ± 0.03
Fertilizer			0.450	0.673	0.309	0.360	0.660	0.730	0.183	0.380	0.223	0.752
Grass species			0.293	0.666	0.144	0.303	0.911	0.378	0.804	0.656	0.070	0.422
Time			0.655	**< 0.001**	**< 0.001**	**< 0.001**	0.859	0.078	0.787	0.092	0.660	0.234
Fertilizer x Grass species			0.187	0.083	0.119	**0.022**	0.877	0.107	0.957	0.781	0.664	0.339
Fertilizer x Time			0.786	0.150	0.412	0.488	0.202	0.344	0.487	0.308	0.062	0.405
Grass species x Time			0.705	0.073	0.252	0.500	0.057	0.614	0.699	0.225	0.249	0.951
Fertilizer x Grass species x Time			0.932	0.382	0.182	0.052	0.302	0.363	0.828	0.247	**0.016**	0.796

Table 4.S8. *Mean values of microbial fungal and bacterial biomass and ratios for different P fertilizer treatments, grass species and harvest times in root and basal stem. Values indicate mean ± standard error, n = 4, expressed x 10^5 picomol g^{-1} of soil.*

Grass species	Day	Treatment	Fungi + Bacteria	Fungi Bacteria^{-1}	Fungi + AMF (NLFA)	Bacteria
Lp	28	Control	1.37 ± 0.04	0.14 ± 0.01	0.18 ± 0.01	1.20 ± 0.04
		Hydrochar	1.43 ± 0.08	0.14 ± 0.01	0.18 ± 0.03	1.26 ± 0.06
		Ash	1.37 ± 0.09	0.15 ± 0.02	0.19 ± 0.03	1.20 ± 0.06
		Sludge	1.44 ± 0.08	0.12 ± 0.01	0.17 ± 0.01	1.29 ± 0.07
		TSP	1.46 ± 0.08	0.19 ± 0.04	0.24 ± 0.06	1.23 ± 0.03
	112	Control	1.41 ± 0.04	0.10 ± 0.01	0.18 ± 0.02	1.29 ± 0.03
		Hydrochar	1.55 ± 0.08	0.14 ± 0.02	0.28 ± 0.03	1.36 ± 0.09
		Ash	1.52 ± 0.05	0.14 ± 0.02	0.29 ± 0.03	1.33 ± 0.02
		Sludge	1.39 ± 0.04	0.12 ± 0.02	0.24 ± 0.03	1.24 ± 0.03
		TSP	1.49 ± 0.05	0.12 ± 0.01	0.21 ± 0.04	1.34 ± 0.04
Dg	28	Control	1.47 ± 0.16	0.13 ± 0.02	0.19 ± 0.04	1.31 ± 0.13
		Hydrochar	1.34 ± 0.04	0.11 ± 0.01	0.14 ± 0.01	1.21 ± 0.04
		Ash	1.37 ± 0.06	0.14 ± 0.01	0.18 ± 0.01	1.20 ± 0.05
		Sludge	1.45 ± 0.09	0.12 ± 0.01	0.17 ± 0.02	1.29 ± 0.08
		TSP	1.30 ± 0.10	0.13 ± 0.02	0.16 ± 0.03	1.15 ± 0.07
	112	Control	1.42 ± 0.06	0.12 ± 0.01	0.28 ± 0.05	1.26 ± 0.04
		Hydrochar	1.53 ± 0.09	0.16 ± 0.02	0.28 ± 0.02	1.32 ± 0.08
		Ash	1.40 ± 0.04	0.14 ± 0.01	0.27 ± 0.01	1.23 ± 0.04
		Sludge	1.40 ± 0.01	0.14 ± 0.01	0.26 ± 0.02	1.23 ± 0.01
		TSP	1.31 ± 0.10	0.16 ± 0.02	0.26 ± 0.04	1.13 ± 0.08
Fertilizer			0.727	0.166	0.548	0.479
Grass species			0.316	0.931	0.717	0.287
Time			0.344	0.929	**< 0.001**	0.270
Fertilizer x Grass species			0.330	0.926	0.449	0.370
Fertilizer x Time			0.493	0.311	0.213	0.485
Grass species x Time			0.618	**0.016**	**0.050**	0.223
Fertilizer x Grass species x Time			0.932	0.325	0.478	0.917

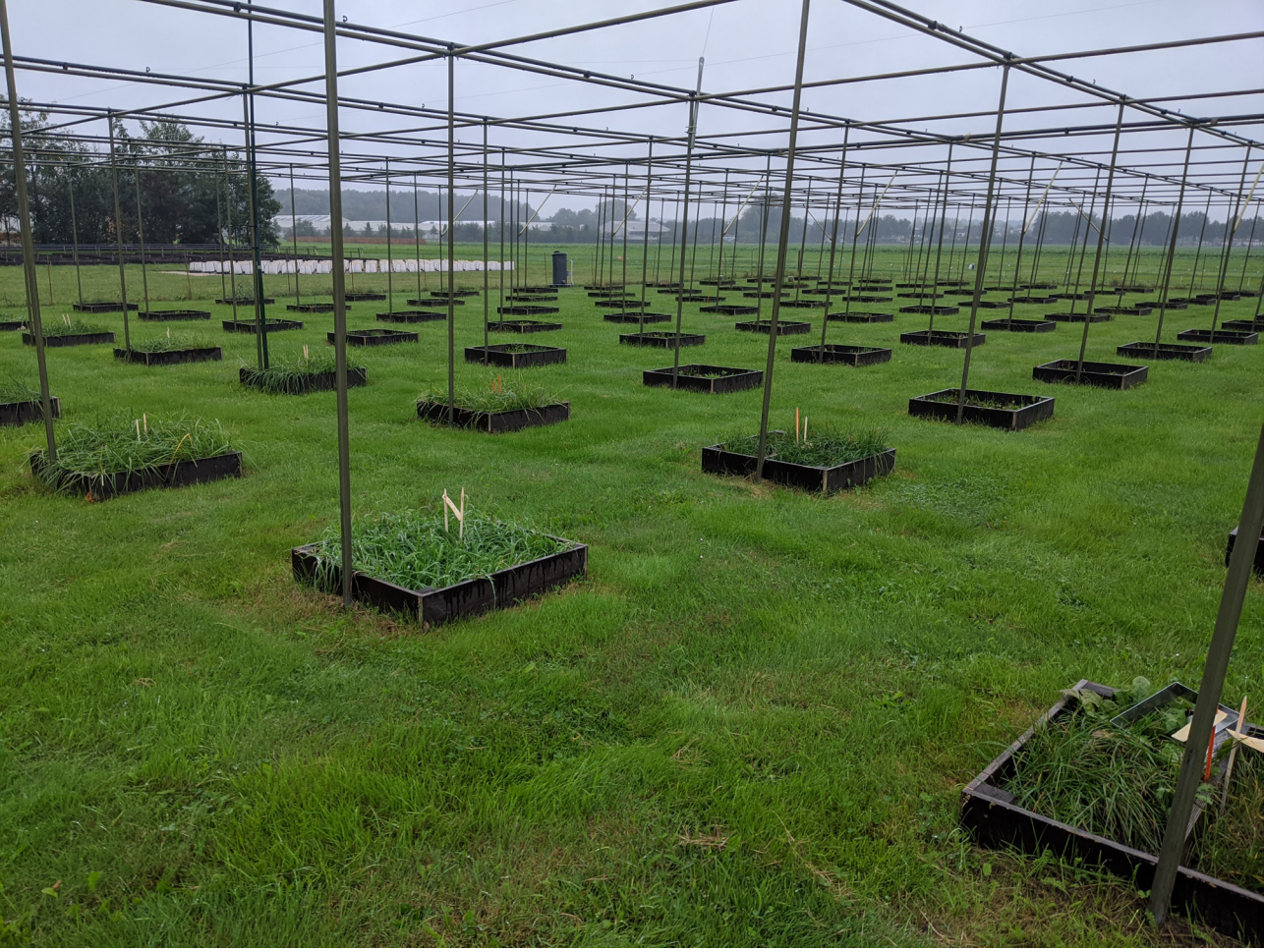
Table 4.S9. *Mean values of soil enzymatic activities for different P fertilizer treatments, grass species and harvest times in root and basal stem. Values indicate mean ± standard error, n = 4. Bglu, PAC and PAK are expressed in PNP (paranitrophenol) min^{-1} g^{-1} of soil, whereas ARYL is expressed β-naphthylamine min^{-1} g^{-1} of soil. Letters in bold denote significant differences between groups according to Tukey's post hoc test. Format of letters indicate different significant ($p < 0.05$) comparisons among groups, e.g. "**A**" indicates post hoc for significant fertilizer x grass species interaction.*

Grass species	Day	Treatment	Bglu	PAC	PAK	ARYL
Lp	28	Control	29.13 ± 1.27 **AB**	99.27 ± 5.86	32.46 ± 0.82	3.31 ± 0.15
		Hydrochar	29.33 ± 0.64 **AB**	108.67 ± 6.23	30.78 ± 1.38	3.00 ± 0.06
		Ash	28.59 ± 1.12 **AB**	99.19 ± 1.90	33.45 ± 1.19	2.98 ± 0.08
		Sludge	26.74 ± 1.50 **B**	105.63 ± 4.36	36.61 ± 2.56	3.31 ± 0.22
		TSP	29.64 ± 0.29 **A**	113.77 ± 8.76	32.37 ± 1.15	3.08 ± 0.22
	112	Control	27.66 ± 1.07 **AB**	99.76 ± 3.44	36.19 ± 0.80	3.53 ± 0.51
		Hydrochar	24.46 ± 0.77 **AB**	100.94 ± 5.63	38.99 ± 1.89	3.49 ± 0.27
		Ash	25.34 ± 0.90 **AB**	93.14 ± 1.32	39.33 ± 3.00	3.22 ± 0.21
		Sludge	22.92 ± 1.05 **B**	96.92 ± 4.60	38.45 ± 1.86	3.33 ± 0.12
		TSP	27.65 ± 1.10 **A**	90.10 ± 6.26	36.62 ± 3.51	3.29 ± 0.20
Dg	28	Control	25.80 ± 1.46	97.65 ± 3.43	32.63 ± 1.10	3.07 ± 0.19
		Hydrochar	28.89 ± 0.48	87.90 ± 5.89	39.53 ± 5.14	3.22 ± 0.17
		Ash	26.93 ± 1.05	99.86 ± 0.92	33.51 ± 0.52	2.96 ± 0.12
		Sludge	28.04 ± 0.72	103.66 ± 4.18	35.87 ± 1.08	4.07 ± 0.43
		TSP	25.34 ± 0.76	100.25 ± 2.87	32.53 ± 2.00	2.92 ± 0.07
	112	Control	25.40 ± 2.79	92.66 ± 5.10	37.46 ± 0.32	3.18 ± 0.09
		Hydrochar	24.66 ± 1.44	90.92 ± 2.40	38.71 ± 4.63	3.36 ± 0.28
		Ash	27.77 ± 2.53	97.16 ± 2.60	36.44 ± 1.35	3.31 ± 0.19
		Sludge	23.27 ± 0.73	92.95 ± 2.28	38.45 ± 1.10	3.20 ± 0.09
		TSP	23.94 ± 0.92	86.56 ± 0.50	34.11 ± 0.36	2.95 ± 0.03

Table 4.S9 continues on next page

(Table 4.S9 continued)

Grass species Day Treatment	Bglu	PAC	PAK	ARYL
Fertilizer	0.252	0.911	0.133	0.075
Grass species	**0.049**	**0.005**	0.725	0.751
Time	**< 0.001**	**< 0.001**	**< 0.001**	0.346
Fertilizer x Grass species	**0.034**	0.078	0.373	0.259
Fertilizer x Time	0.130	0.056	0.947	0.131
Grass species x Time	0.349	0.419	0.204	0.152
Fertilizer x Grass species x Time	0.729	0.621	0.495	0.571



Field experiment at Unifarm, Wageningen, The Netherlands

Combination of *Lolium perenne L.* and *Festuca arundinacea Schreb.* improve yields under low phosphorus availability

Á. Velasco-Sánchez, L.M.E. Ferron, D.T.C. Mani, N. Bennegadi-Laurent, I. Trinsoutrot-Gattin, J.W. van Groenigen & G.Y.K. Moinet

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Abstract

Phosphorus (P) is one of the main nutrients for all plants, including grasses. However, sources of P fertilizer are not renewable, are not evenly distributed and overfertilization 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 *Lolium perenne L* and *Phleum pratense L.* Plants were grown in an unfertilized low P soil and in P fertilized soil for two growing seasons. We measured biomass production, root traits, nutrient uptake, microbial biomass and enzymatic activities. In the unfertilized plots the combination of *Lolium perenne* and *Festuca arundinacea* generated the highest cumulative yields (25951 ± 4059 kg 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 *Festuca arundinacea* and *Lolium perenne* diploid. *Festuca arundinacea* produced higher root biomass than *Lolium perenne* diploid at deeper soil layers (98 *vs* 44 g m^{-2}; $p < 0.05$). On the other hand, *Lolium perenne* diploid had significantly finer roots than *Festuca 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.

1. Introduction

Phosphorus (P) is one of the main nutrients for plants and therefore essential for grassland production (Aydin and Uzun, 2005). However, P is a non-renewable resource that is concentrated in very few countries (Penuelas et al., 2023). 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, overfertilization with P in the past has turned almost 60% of European grassland soils unresponsive to P fertilization (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 fertilization by reducing the overall demand for P fertilizers 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 (Khan et al., 2009; Xue et al., 2016; Giles et al., 2017). 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 (Rogers et al., 2019; Becker et al., 2020). 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., 2014), providing a 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 fertilizer 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., 2014, 2018). 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 a 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 fertilization, 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 (Supplementary material, Figure 5.S1). The mesocosms consisted of wooden boxes (0.75 m wide x 0.75 m tall x 0.40 m deep) that were installed in the field. 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 (WRB) and was collected from the top 0 – 0.25 m from an extensively managed grassland that had not been fertilized with P for more than 25 years. This soil had a low $CaCl_2$ extractable P (0.5 mg P kg^{-1}) fraction, acidic pH (5.59) and a cation exchange capacity of 7 cmol kg^{-1}. More information about this soil is available in Supplementary material (Table 5.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, Nijmegen, The Netherlands) which was 4 and 6 g seeds 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 g m^{-2} respectively. In addition, to improve the palatability of the Lp2Fa grass combination, we studied a 4-species combination (4sp) with timothy (*P. pratense*) (Pp) and a tetraploid variety of *Lolium 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 g 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. The experiment resulted in a randomized block design with 6 plant mixtures replicated 4 times, leading to 24 boxes each split in two plots (with and without P fertilization on the northern and southern sides respectively, not randomized) for a total of 48 plots distributed over 4 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 in the end of the experiment. Specific dates of the harvests can be found in the Supplementary material (Supplementary material, Table 5.S2).

All plots were fertilized with nitrogen (N) and potassium (K). N fertilizer 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. K fertilizer 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 alleviation of any P limitation in the P+ plots. The southern side of each box did not receive any P fertilizer (P-). Rates were based on the Dutch fertilizer guidelines for grasslands (Schoonvelde et al., 2017). Detailed information on the amount of fertilization treatments and sowing can be found in the Supplementary material (Table S2). All fertilizers 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 (Supplementary material, Table S2) by cutting grass shoots 0.05 m above 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).

$$RYT = \sum \text{Yield mixture}_a / \text{Mean yield monoculture}_a \quad [1]$$

Where "a" 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 L^{-1}). A resolution of 600 dpi on a 0.15 x 0.25 m tray was used for scanning. Root length (m m^{-2}) and diameter (mm) were calculated using the software WinRHIZO Pro (Regent Instruments, Quebec, Canada) (Ros et al., 2018). Specific root length (SRL) was calculated by dividing root length by root biomass (m g^{-1}). The remaining roots were dried at 70 °C for 48 h.

N and P content in shoots were measured colorimetrically in a segmented flow analyser (SFA, Skalar, SAN^{++}, Breda, The Netherlands) after digestion with H_2SO_4/Se (Houba et al., 1995). 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 content at the treatment level was calculated as the weighted average of the N and P content 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:

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

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

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 fertilization 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 were 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 colorimetrically 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 colorimetrically using a 96-well microplate technique (ISO 20130:2018) (Cheviron et al., 2021). Enzymes linked to the C, N and P cycle were analysed. The enzymes that were 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 were 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 coloration 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, USA) 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 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, 2023). 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 post-hoc 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 variables and soil 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 post-hoc 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 models residuals were examined for normality, homogeneity and heteroscedasticity assumptions.

Over- or underyielding of the grass species grown in mixture were 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 underyielding 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, 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., 2023a).

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 fertilization (P-). After examination of single main effects we observed that Lp2Fa mixture showed significantly higher cumulative yields 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 1). On average, Lp2Fa produced 25950 ± 8117 kg 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 yields were found between plant treatments in the P+ plots (Figure 5.1). 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 ha^{-1}) and season 2 (15231 ± 2882 kg ha^{-1}) yet the differences were not significant.

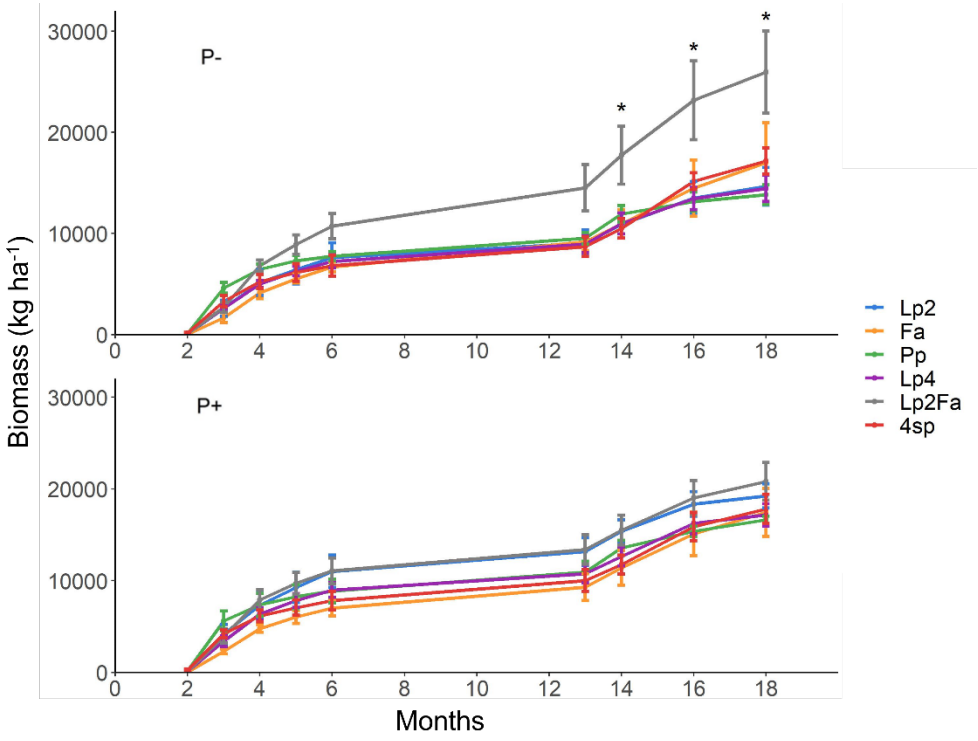

Figure 5.1. *Cumulative biomass production of P unfertilized (P-) and fertilized plots (P+) for the different grass species mixtures. Lp2 = Lolium perenne L. diploid, Fa = Festuca arundinacea Schreb., Pp = Phleum pratense L., Lp4 = Lolium perenne L. tetraploid, Lp2Fa = Lp2 and Fa combination and 4sp = 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.*

The RYT was, generally, higher than 1 in all the harvests of the P- plots for the Lp2Fa treatment but not the 4sp treatment (Figure 5.2). 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.

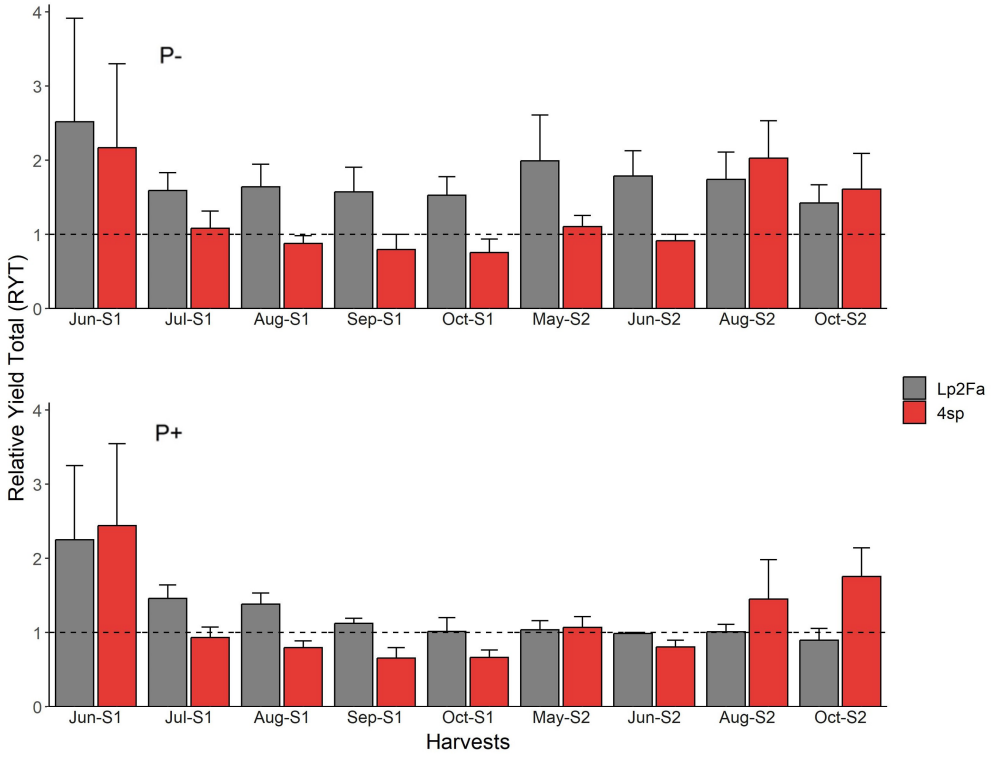

Figure 5.2. *Relative yield total (RYT) of the LpFa and 4sp combinations in the P fertilized (P+) and P unfertilized (P-) plots at each harvest. S1 = First season, S2 = Second season, Lp2Fa = Lolium perenne L. diploid and Festuca arundinacea Schreb. combination and 4sp = Lolium perenne L. diploid, Lolium perenne L. tetraploid, Festuca arundinacea Schreb. and Phleum pratense L. combination. Bars indicate standard error, n = 4. Values of RYT > 1 indicate higher yields than monoculture.*

In the P- plots, Lp2 dominated the harvests of season 1 and Fa dominated the ones of season 2 (Table 5.1). Lp2 produced significantly ($p < 0.05$) higher relative yields (RY > 0.5, overyielding) in the Lp2Fa mixture than in monoculture throughout the grass harvests of the first season (July, August, September and October). Conversely, Fa overyielded significantly throughout the second season (June, August and October). In the 4sp mixture, Lp overyielded in the harvest of May, June and August of season 2. Fa overyielded in the first cut of season 1, yet it significantly underyielded (RY < 0.25) in the harvest of August, September and October in season 1 and in May in season 2. Pp (*P. pratense*) also significantly underyielded in the 4sp mixture in the last cut of season 2.

Table 5.1. *Relative yield (RY) of each grass species grown in mixtures in P fertilized (P+) and P unfertilized plots (P-). Values significantly (p < 0.05) higher or lower than 0.5 in the Lp2Fa mixture indicate overyielding or underyielding 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 overyielding or underyielding. In the 4sp mixture, distinction between Lp2 and Lp4 is not possible (Lp), RY values significantly higher or lower than 0.5 denote overyielding or underyielding. S1 = First season, S2 = Second season, Lp2 = Lolium perenne L. diploid, Fa = Festuca arundinacea Schreb., Pp = Phleum pratense L., Lp4 = Lolium perenne L. tetraploid, Lp2Fa = Lp2 and Fa combination, 4sp = Lp2, Lp4, Fa and Pp combination, + = significant overyielding and - = significant underyielding. 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	Lp2	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 (-)**

Table 5.1 continues on next page

(Table 5.1 continued)

		Jun-S1	Jul-S1	Aug-S1	Sep-S1	Oct-S1	May-S2	Jun-S2	Aug-S2	Oct-S2
						P+				
Lp2Fa	Lp2	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 (-)**

In the P+ plots, the trends were more erratic. Overyielding was found for Lp2 in the Lp2Fa mixture only in the cut of August in season 1 and underyielded in the cut of October in season 2 (Table 5.1). Fa grown in the Lp2Fa mixture overyielded in the harvests of July of season 1 and in the harvests of May and October of season 2. Grass species generally underyielded in the 4sp mixture grown in the P+ plots, Fa underyielded throughout the first season (July, August, September and October) and also in the cut of June in season 2. Similarly, Pp underyielded in the cut of October in season 2. Only Lp (not possible to differentiate Lp2 from Lp4 in the 4sp mixture) overyielded (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 5.3). 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 5.3, panels A 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 mixtures 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 5.3, panel A) and the PNI values indicated near adequate P levels (0.8) in the Lp2Fa and Fa treatments and strong P limitation in the Lp2, Lp4 and 4sp combinations (Figure 5.3, panel B) at the end of S2. In P+ plots, no statistical differences were found among grass species on N: P or PNI and these values did not change across seasons (Figure 5.3, panels C and D). The PNI values in the P+ plots were close to 0.8 in both S1 and S2, suggesting adequate P nutrition (Figure 5.3, panel D).

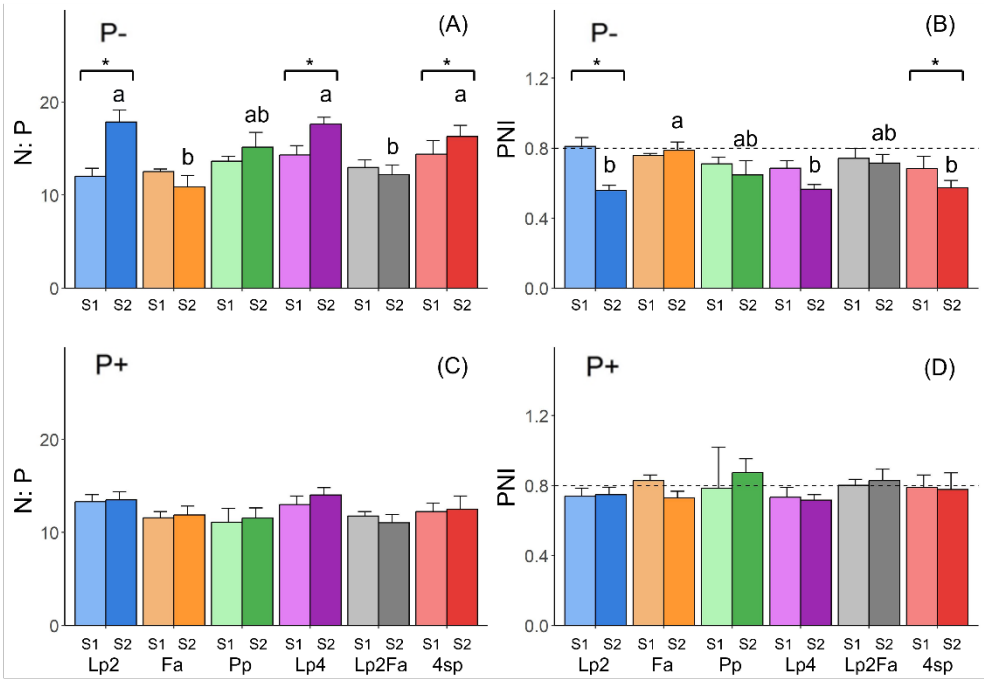

Figure 5.3. *Averaged nutrient uptake in P unfertilized (P-) (panels A and B) and P fertilized (P+) (panels C and D) plots at last harvest of season 1 (S1) and season 2 (S2). PNI = Phosphorus nutrition index. Lp2 = Lolium perenne L. diploid, Fa = Festuca arundinacea Schreb., Pp = Phleum pratense L., Lp4 = Lolium perenne L. tetraploid, Lp2Fa = combination of Lp2 and Fa and 4sp = combination of Lp2, Lp4, Fa and Pp. Letters indicate significant differences among grass species after single main effect evaluation (Tukey test, p < 0.05). Brackets and asterisks indicate significant differences between S1 and S2 (p < 0.05). 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 (Supplementary material, Table 5.S3). In the P+ plots, the PNI values were close to the 0.8 threshold, indicating no strong P limitation (Supplementary material, Table 5.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 (Supplementary material, Table 5.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 (Supplementary

material, Table 5.S4). Detailed information on N and P nutrition as well as NNI and PNI indexes can be found in Supplementary material, Table 5.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 5.4, panels A and D). SRL was found significantly lowest for Fa in both P- and P+ plots (141 ± 8 and 148 ± 15 m 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 5.4, panels C and F).

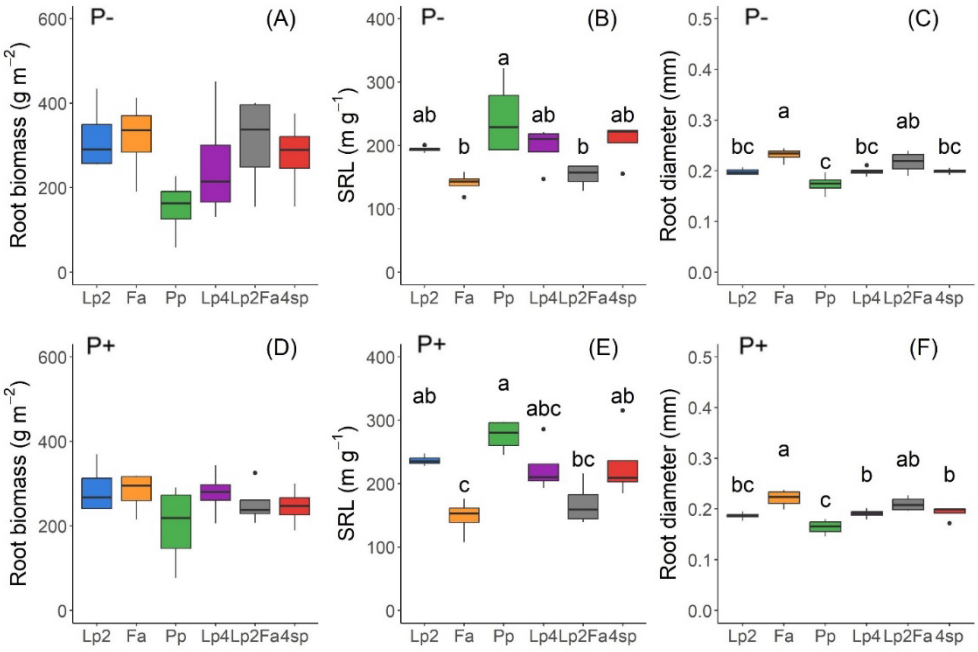

Figure 5.4. *Root traits in P unfertilized (P-) (panels A, B and C) and P fertilized (P+) (panels D, E and F) plots at last harvest in the top soil (0 – 0.15 m). Lp2 = Lolium perenne L. diploid, Fa = Festuca arundinacea Schreb., Pp = Phleum pratense L., Lp4 = Lolium perenne L. tetraploid, Lp2Fa = combination of Lp2 and Fa and 4sp = combination of Lp2, Lp4, Fa and Pp, SRL = Specific root length. Letters are displayed when ANOVA's p < 0.05 and indicate statistically significant differences between plants based on Tukey's HSD test. Boxes describe interquartile range, whiskers represent first and fourth quartiles, the horizontal line shows the median and dots show observations outside of the first and fourth quartiles.*

In the deeper soil layers (0.15 – 0.40 m) we observed significantly ($p < 0.05$) higher root biomass and diameter for Fa (Figure 5.5, panels A, C, D and F). The average root biomass of Fa at the deeper soil layers was 98.14 ± 18.61 g m^{-2} for the P- plots and 93.9 ± 24.69 for the P+ plots g m^{-2}, n = 4 (Figure 5.5, panels A and D). Root diameter was highest in the Fa monoculture and lowest in the Pp monoculture in both P- and P+ plots (Figure 5.5, panels C and F). SRL was also significantly lower in the Fa compared to the rest of 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 5.5, panels B and E).

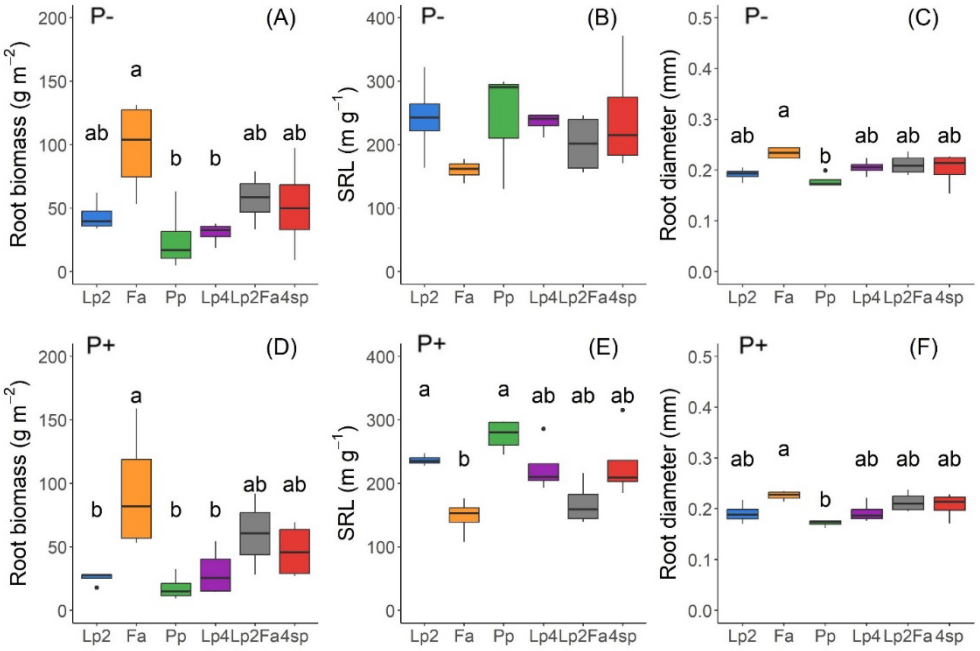

Figure 5.5. *Root traits in P unfertilized (P-) (panels A, B and C) and P fertilized (P+) (panels D, E and F) plots at last harvest in the deeper layers of the soil (0.15 – 0.40 m). Lp2 = Lolium perenne L. diploid, Fa = Festuca arundinacea Schreb., Pp = Phleum pratense L., Lp4 = Lolium perenne L. tetraploid, Lp2Fa = combination of Lp2 and Fa and 4sp = combination of Lp2, Lp4, Fa and Pp. SRL = Specific root length. Letters are displayed when ANOVA's p < 0.05 and indicate statistically significant differences between plants based on Tukey's HSD test. Boxes describe interquartile range, whiskers represent first and fourth quartiles, the horizontal line shows the median and dots show observations outside of the first and fourth quartiles.*

Root traits were also significantly correlated with P nutrition values (Table 5.2). 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. 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, P concentration was negatively correlated with top and deep root diameter and total root biomass.

Table 5.2. *Correlations between P nutrition in grasses and root morphology. Top root: 0 - 0.15 m, deep root: 0.15 - 0.40 m, PNI: Phosphorus nutrition index, SRL: Specific Root Length. Values indicate Pearson's correlation coefficients, highlighted in bold are statistically significant correlations (p < 0.05).*

	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 (kg P ha^{-1})	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 (kg P ha^{-1})	- 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) (Supplementary material, Table 5.S5). Fertilization 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 fertilized 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 5.3). 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.

Table 5.3. *Average values of soil biological variables measured at the end of the experiment in P (P+) fertilized and P (P-) unfertilized plots. BGLU = β-glucosidase, NAG = N-acetyl-glucosaminidase, ARYLN = arylamidase, PAC = Acid phosphatase and PAK = Alkaline phosphatase. Microbial biomass expressed in mg kg^{-1} of dry soil, BGLU, NAG, PAC and PAK are expressed in PNP min^{-1} g^{-1} of dry soil and ARYLN is expressed in β-naphthylamine min^{-1} g^{-1} of dry. In bold, significant effect ($p < 0.05$) of the plant treatment, ANOVA. Different letters indicate significant differences among plants (Tukey's HSD post-hoc test). Values indicate mean ± standard error, n = 4. Lp2 = Lolium perenne L. diploid, Fa = Festuca arundinacea Schreb., Lp2Fa = combination of Lp2 and Fa and 4sp = combination of Lp2, Fa, Phleum pratense L. and Lolium perenne L. 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

4. Discussion

4.1. Root traits and reduced intraspecific competition improve P nutrition

The aim of this experiment was to evaluate the introduction of *F. arundinacea* (Fa) in grass mixtures with *L. perenne* (Lp) alone or with other grass species (tetraploid variety of Lp and *P. pratense* (Pp)) to improve P acquisition and therefore reduce dependency on P fertilisers. We hypothesized that Fa, because of its deeper root system, could potentially explore and make use of more stocks of 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 1) and a RYT consistently greater than 1 in the plots with no P fertilisation (P-), indicating overyielding (Figure 5.2 and Table 5.1).

The increased cumulative yields and RYT of the Lp2Fa combination can be explained by a complementary use of resources over time and space and lower intraspecific competition. During the first season Lp2 grown in the Lp2Fa mixture significantly overyielded compared to the monoculture, whereas, in the second season, Fa was the species that overyielded (Table 4.1). 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 5.3, panels B and D). Lp2 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 m^{-2}). Conversely, at the end of the second season, Fa, a slower-growing grass (Gastal et al., 2010), caught up and started to benefit from its distinct root traits and associated increased access to more nutrients (Cougnon et al., 2014) (Table 5.2). Likewise, Fa could also have benefitted from a lower intraspecific competition, as the seeding rate in the mixture was also reduced compared to the monoculture (3 *vs* 6 g 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 fertilization regime, Lp2 in monoculture had consistently and significantly finer roots than Fa (Figures 5.4, panels C and F and 5, panels C and F) and Fa had significantly more root biomass at deeper soil layers (Figure 5.5, panels A 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., 2014, 2017; 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 in both monoculture and when grown in the Lp2Fa mixture (Figure 5.3 and Table 5.2). 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 5.3, panels A 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; S. G. 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 5.1 and 5.2). In fact, we observed underyielding for some of the species in the mixture at many of the harvests, particularly for Fa (Table 5.1). Other studies have also demonstrated that increasing the number of species in a grass mixture does not necessarily result in increased yields (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).

The adaptation of Fa in different types of soils should be considered. In our mesocosms, we used a 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 (Becker et al., 2020). Moreover, we found contrasting correlations for the P+ and P- plots, suggesting different root pathways of P acquisition depending on soil P status (Table 5.2). Likewise, previous studies have proposed that different root traits, such as root diameter and length, are influenced by soil P availability (Ros et al., 2018; Kumar et al., 2019), aligning with the observed differences in correlations presented in Table 5.2. Lastly, 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, 2021). 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.

4.2. Contribution of soil microorganisms towards P nutrition

Soil microorganisms are able to solubilize poorly soluble forms of P and eventually increase the plant available pool of P (Khan et al., 2009; Richardson and Simpson, 2011). As such, we hypothesized 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 mobilization.

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, the Pp species 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 outcompeted, and had therefore likely provided additional biomass available for microbial degradation. 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; Yue 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 fertilizer in intensively managed grasslands. We hypothesized 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* diploid and *F. arundinacea* resulted in significantly higher yields than the monocultures possibly because of the contrasting root systems of *L. perenne* diploid and *F. arundinacea* and the ability of *F. arundinacea* to withstand P limitation. On the other hand, we did not observe any contribution of the soil microbial community towards higher yields or P mobilization.

More research is needed on the combination of *L. perenne* diploid 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 Fa, potentially including grazing animals in their experimental design. Moreover, experiments conducted on different soils 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.

6. Acknowledgments

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Supplementary material

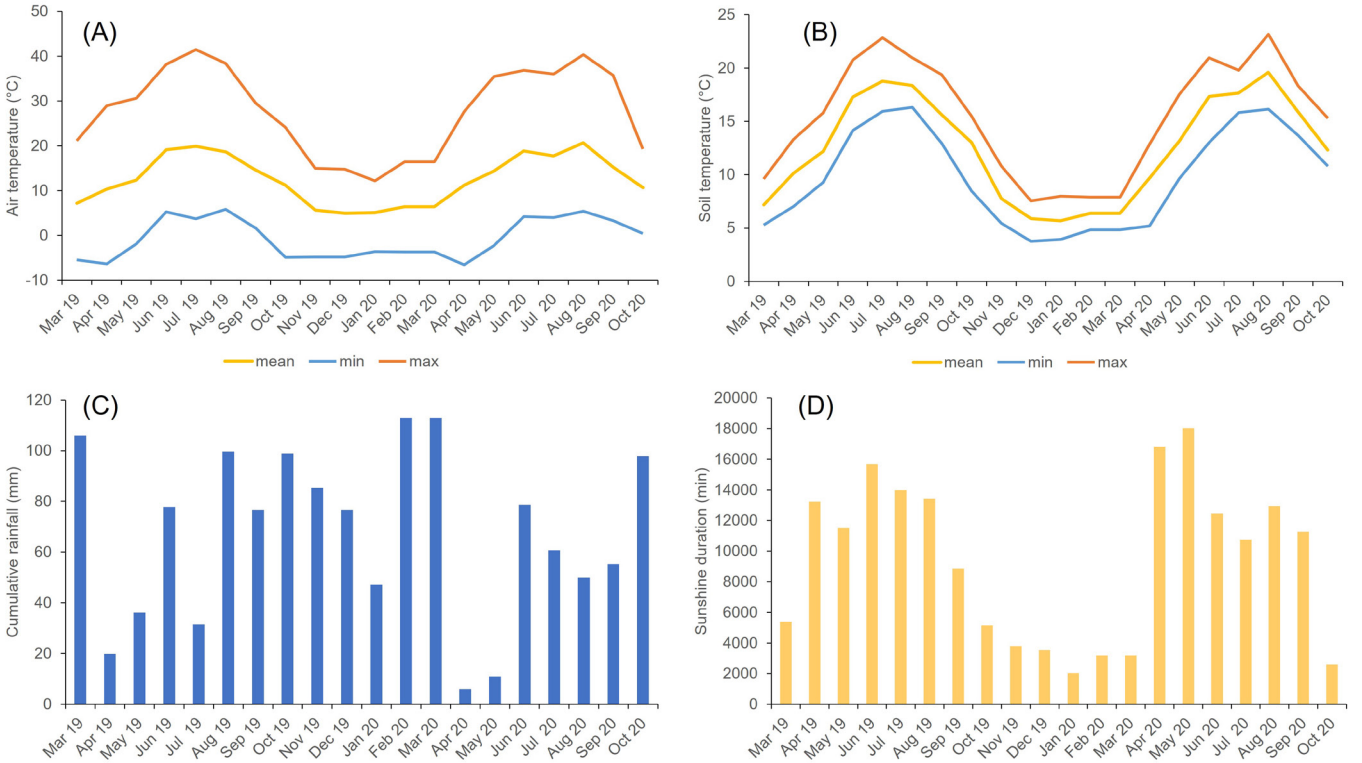

Figure 5.S1. *Detailed climatic information from Veenkampen weather station (Wageningen, The Netherlands) during the 2019 and 2020 growing seasons. Panel A indicates shielded above 0.1 m air temperature, panel B shows soil temperature at 0.1 m depth under grassland, panel C depicts cumulative monthly precipitation and panel D shows cumulative monthly sunshine duration.*

Table 5.S1. *Initial soil analysis. 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.*

Variable	Concentration
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

Table 5.S2. *Timeline of experiment (2019 - 2020). The plots were fertilized with CAN = Calcium ammonium nitrate, TSP = Triple super phosphate and K_2SO_4 = potassium sulphate. P fertilizer was only added in the P+ plots. Fertilizers were applied after each grass cut. Hx represent each harvest. Sowing was carried out in April of 2019 with the recommended sowing rates of the producer (Barenbrug BV). The sowing rates were 4, 6, 1.6 and 6 g m^{-2} for Lolium perenne L. diploid, Lolium perenne L. tetraploid, Phleum pratense L. and Festuca arundinacea Schreb. when grown in monocultures. The sowing rate was reduced by a 50% in the L. perenne and F. arundinacea mixture and by a 75% in the mixture treatment of the 4 grass species.*

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

Table 5.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 fertilized plots (P+) and unfertilized 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 = Lolium perenne L. diploid, Fa = Festuca arundinacea Schreb, Lp2Fa = combination of Lp2 and Fa and 4sp = combination of Lp2, Lolium perenne L. tetraploid, Fa and Phleum pratense L., S1 = end of season 1, S2 = end of season 2. Values show mean ± standard error, n = 4.*

Treatment	Grass species		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 5.S4. *Nutrient (P and N) concentration, uptake and nutritional indicators (PNI, NNI and N: P) at the end of season 1 and season 2 in shoot. PNI = Phosphorus nutrition index, NNI = Nitrogen nutrition index, Lp2 = Lolium perenne L. diploid, Fa = Festuca arundinacea Schreb, Lp2Fa = combination of Lp2 and Fa and 4sp = combination of Lp2, Lolium perenne L. tetraploid, Fa and Phleum pratense L., S1 = end of season 1, S2 = end of season 2. Values show mean ± standard error, n = 4. Significant effects (p < 0.05) 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**

Table 5.S4 continues on next page

(Table 5.S4 continued)

		P (%)	P uptake (kg P ha^{-1})	PNI	N (%)	N uptake (kg N ha^{-1})	NNI	N: P
					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 5.S5. *Average values of soil chemical variables measured at the end of the experiment in P fertilized (P+) and P unfertilized (P-) plots at the end of the experiment. Lp2 = Lolium perenne L. diploid, Fa = Festuca arundinacea Schreb., Lp2Fa = combination of Lp2 and Fa, 4sp = combination of Lp2, Lolium perenne L. tetraploid (Lp4), Fa and Phleum pratense L. (Pp), DOC = dissolved organic carbon and DON = dissolved organic nitrogen. All results with the exception of pH are expressed in mg 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

Livarot cheese, Livarot, Pays d'Auge, France

General Discussion

Á. Velasco-Sánchez

1. Summary of main findings

The main research objective of this PhD thesis was to **study the fertilizing effects of DPW in grasses and the contribution of soil microorganisms to improve their efficiency**. I investigated this overarching research objective by addressing partial research questions in the chapters 2, 3, 4 and 5 of this thesis. The partial research questions (RQ) of this PhD work were:

I. What are the forms of P present in DPW materials? This research question was covered in **chapter 2**.
II. What is the plant availability of P from DPW materials? This research question was addressed in **chapters 2, 3** and **4**.
III. Can soil microorganisms increase the plant availability of P from DPW materials? This research question was investigated in **chapters 3**, **4** and **5**.
IV. Can the selection of grass species contribute to increase the P use efficiency from DPW materials? This research question was studied in **chapters 4** and **5.**

The main findings and conclusions of **chapters 2**, **3**, **4** and **5** are summarized in Figure 6.1.

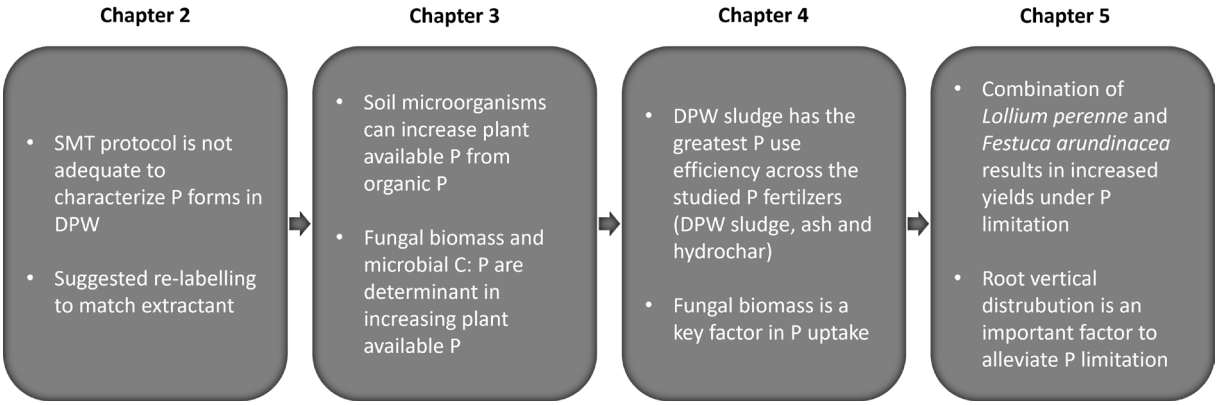

Figure 6.1. *Scheme of main conclusions of chapters 2, 3, 4 and 5. DPW = Dairy processing waste.*

In **chapter 2**, I aimed to modify the SMT fractionation protocol to quantify water-soluble P forms in DPW materials and to assess the solubility in water of their different fractions of P. However, I found aberrant results, e.g. the highest concentration of organic P was found in ashes and there were arbitrary decisions about allocating P into discrete fractions. The results of this chapter align with the suggestions of Barrow et al. (2021) to abandon P fractionation schemes. I have concluded that the SMT procedure provides erroneous results and suggested to rename the supposedly discrete forms of P into more realistic pools related to the extractant used.

In **chapter 3**, I studied the role of soil microorganisms in increasing plant available P from a range of poorly soluble P forms that may be present in DPW or other recycled P fertilizers. I observed contrasting dynamics in P availability between mineral fertilizers and the poorly soluble studied forms. In particular, I observed that plant available P decreased sharply in the triple super phosphate (TSP) amended soils whereas plant available P doubled in the organic P (phytate) amended soils. The increase in P availability was related to the increase in carbon acquisition by the soil microbial community and by the fungal biomass of the soil.

In **chapter 4**, I studied the fertilizer value of DPW sludge, ash and hydrochar for *Lolium perenne* and *Dactylis glomerata* grass species and assessed the role of microorganisms in increasing P uptake. I found that sludge was the material that resulted in the highest biomass production and P use efficiency, even exceeding that of mineral P fertilizer (TSP). I further observed that transforming sludge into ash or hydrochar results in lower P fertilizer use efficiency. I did not observe direct effects of the P treatments on soil microbial communities (activities or composition). However, I did observe that regardless of the P fertilizer treatment, fungal communities were pivotal factors in explaining P uptake, confirming the results obtained in chapter 3.

In **chapter 5**, I studied how the diversification of grass species with different rooting systems (*Lolium perenne vs Festuca arundinacea*) improves access to P. I also studied the role of the soil microbial community in alleviating P limitation. The results of this study showed that combining *Festuca arundinacea* (deep rooting system) with *Lolium perenne* (shallow root system) results in an improved access to

P under P limitation conditions. The improved yields can also be explained by a decreased intraspecific competition. I did not find any positive associations between microbial communities and P uptake.

2. Challenges for fertilization with dairy processing waste

The two first partial research questions of this thesis were to study the forms of P present in DPW materials **(RQ I)** and to investigate plant availability of P from DPW materials **(RQ II)**. After studying these two research questions it becomes clear that the chemical complexity of DPW materials, the difficulties to correctly assess P forms and the high content of P in temperate grassland soils pose challenges for an efficient P fertilization with DPW materials.

Since the green revolution, farmers became accustomed to the utilization of mineral P fertilizers (Ashley et al., 2011). The utilization of mineral fertilizers has many advantages, such as low prices and very homogeneous and readily available chemical forms of P. Because of potential threats in the supply chain and the environmental degradation caused by decades of unrestricted application, society demands the introduction of other forms of P fertilizers in agriculture (Penuelas et al., 2023). The introduction of recycled P from waste as fertilizers might be a solution to reduce the dependency from mineral P while valorising waste materials (Chojnacka et al., 2020). Transitioning towards a circular P fertilization encompasses many technical and scientific challenges, some of these are recurrent to the ones society faced before the widespread utilization of mineral P in the last century (Ashley et al., 2011).

One of the main challenges of recycled P fertilizers is their chemical complexity. While mineral P fertilizers have a very homogeneous and simple chemical P profile, recycled P fertilizers are very heterogeneous and complex "cocktails" of P forms. The availability of P for plants in recycled P fertilizers is often lower than in mineral fertilizers (Römer and Steingrobe, 2018). This is not an exception for DPW or the secondary materials produced from it. Markedly different treatment processes of dairy production wastewater (e.g. lime, aluminium and/or iron application) contribute further to the heterogeneity of P forms in DPW.

In **chapter 2**, I attempted to measure the chemical fractions of P in DPW. The P fractionation of P in recycled fertilizers or waste materials is normally done using the SMT fractionation procedure (Pardo et al., 2003). This protocol has the advantage of being simple and does not require sequential extractions, which are prone to the introduction of analytical mistakes. Unfortunately, the SMT procedure does not make a distinction between plant available P and poorly available P. I modified this protocol by adding a preliminary step in which the percentage of water soluble P within each fraction could be quantified. Characterization of P forms in DPW is highly relevant as different forms of P applied in the soil result in different levels of plant available P and that these forms interact differently with the soil mineral phase and soil microorganisms, as shown in **chapter 3** (Velasco-Sánchez et al., 2024). The results of **chapter 2** highlight, however, the inadequacy of the SMT method to correctly allocate P into discrete forms. Recent studies have also noted the impossibility of allocating P into discrete fractions of P, and provided examples in which experimental results do not match the theory of P being fixed in defined compartments (Barrow, 2021; Barrow et al., 2021). Other techniques, like nuclear magnetic resonance (P-NMR), could help to elucidate the chemistry of P in recycled materials, yet these techniques are expensive and not exempt of limitations and challenges (Cade-Menun, 2017). This represents one of the major challenges for the broad utilization of DPW recycled materials as P fertilizers; how can we correctly and easily assess the fertilizer value of DPW when we cannot easily discern the chemistry of the "cocktail" of P forms?

Similar challenges also exist when attempting to estimate plant available P in soils. Currently, many chemical tests are used to assess P availability in soils (Figure 6.2). For instance, in the Netherlands it is common to use $CaCl_2$ together with ammonium lactate to measure plant available P, while in France three different tests are used (Olsen, Dyer and Joret-Hébert) depending on soil type (Jordan-Meille et al., 2012). The lack of homogeneity in available P testing across Europe makes it even more complicated to give estimates on P fertilization rates. Using different soil P tests

under fertilization with DPW or recycled materials ("cocktails") poses another challenge to the estimation of plant available P, as different reagents have different affinities for specific forms of P. It has been found, for instance, that the Morgan's P

test (commonly used in Ireland) results in an overestimation of plant available P from limed DPW materials (Ashekuzzaman et al., 2021). Similarly, when analysing plant available P from a range of P forms in **chapter 3,** I also detected an overestimation of plant available P from hydroxyapatite when using the Joret-Hébert method (data not shown). Thus, fertilizing with DPW or recycled materials would potentially affect the applicability of the already heterogeneous routinary plant available P tests (Figure 6.2).

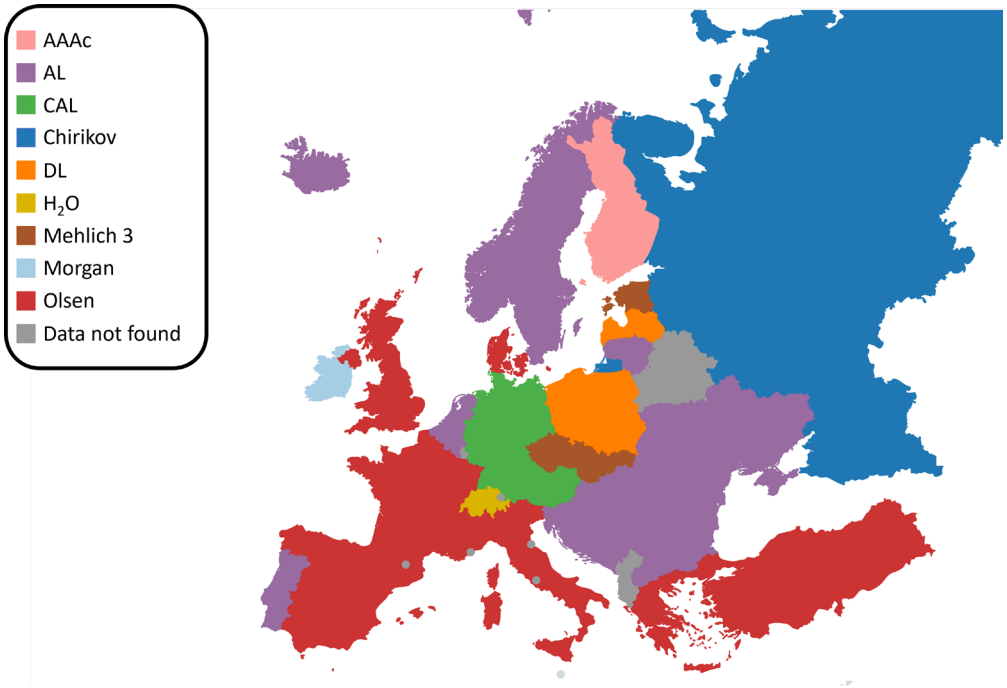

Figure 6.2. *Heterogeneity in commonly used soil P tests in Europe. AAAc = acid ammonium acetate, AL = ammonium lactate, CAL = calcium acetate + calcium lactate + acetic acid, Chirikov = acetic acid, DL = calcium lactate + hydrochloric acid (Egner and Riehm), Mehlich 3 = ammonium fluoride + acetic acid + ammonium nitrate + nitric acid, Morgan = acetate-acetic acid and Olsen = sodium bicarbonate. Note = Some countries use more than one soil P test. See footnote for references.*

(Garbouchev, 1980; Kewai et al., 1997; Fernandes et al., 2000; Lončarić et al., 2006; Ailincăi et al., 2008; Komljenović et al., 2010; Jordan-Meille et al., 2012; Khristenko and Ivanova, 2012; Guðmundsson et al., 2014; Iatrou et al., 2014; Jordanoska et al., 2014; Manea et al., 2016; Salković et al., 2018; Shafeeva et al., 2021; Janković et al., 2023; Khomenko et al., 2023b)

Chapter 4 aimed to study the fertilizing values from DPW and its secondary materials. The results of this study showed that DPW's raw sludge generated the highest phosphorus use efficiency and crops yields, compared to ash, hydrochar and mineral P fertilizer. Particularly, the effects on P uptake and yields from hydrochar were very low, similar to the unfertilized control. These results suggest that DPW fertilizers could be classified into materials that can be used as fertilizers (sludge) and materials that should not be categorized as fertilizers (hydrochar and ash). The conclusions of this study are similar to the ones from recent different agronomic trials that used DPW materials as fertilizer products (Shi et al., 2022; Khomenko et al., 2024). DPW's sludge has the potential to replace mineral fertilizers as it may provide a slower release source of P compared to mineral fertilizers. However, widespread utilization of DPW's sludge is challenging as it contains high water content (up to 90% of its weight can be water) which complicates its transportation. Also, sludge is prone to biological contamination (such as faecal coliforms or salmonella spp.) and the chemical species of P can be very diverse (Ashekuzzaman et al., 2019; Velasco-Sánchez et al., 2023).

The necessity of applying highly soluble P fertilizers in grassland should also be critically reconsidered. In the last 50 years an excessive P fertilization has led to the build-up of P pools in soils in temperate areas. In European agricultural soils, it is estimated average surplus of 0.11 kg P ha^{-1} yr^{-1}, which in some geographical areas can exceed 2 kg P ha^{-1} yr^{-1} (Muntwyler et al., 2024). Recent studies have also suggested that most of European grasslands, particularly those with high P content, are not responsive to P fertilization anymore (Ros et al., 2020; Recena et al., 2022). I have observed this trend in **chapter 5**, in which no large differences in biomass production were found between the P fertilized and unfertilized plots even in a soil with a very low P content. The continued application of P resulting in the increase of soil P, normally in plant unavailable forms, is often referred as the legacy P pool (Kleinman et al., 2011; Gatiboni et al., 2020). Managing soil pH through liming, promotion of P solubilizing microorganisms or the selection of grass species or combinations that can access to less available pools of P have been suggested as potential practices to make use of the legacy P pool (Pavinato et al., 2020). Such management practices could also help to improve P fertilization value of DPW

materials such as ashes or chars, which are poorly available for plants, as shown in **chapter 4**.

3. Soil microorganisms and organic P

Research question **(RQ III)** of this PhD thesis focused on the contribution of soil microorganisms in increasing plant available P from DPW materials. The results from my experiments indicate that soil microorganisms, particularly fungal communities, are pivotal in P nutrition in grasslands and that organic forms of P are the preferred sources of P for soil microorganisms.

Organic forms of P (P-Org) in soils can make up to an average of 35% of the total P content (Blume et al., 2016). In grasslands, particularly, P-Org can account for more than 50% of the total P content (Nash et al., 2014). This pool can be divided into different groups: monoesters, diesters, phytate, polyphosphates and phosphonates (Darch et al., 2014). The stability of these forms is also markedly different, with diesters and monoesters being easily mineralizable forms of P-Org compared to phytate, polyphosphates and phosphonates (Darch et al., 2014). Mineralization of P-Org is also dependent on soil properties, being faster in calcareous soils (Doolette et al., 2010). Phytate, because of its resistance to mineralization (Darch et al., 2014), and monoesters (up to 75% of total P-Org) (McLaren et al., 2015) are considered the predominant forms of P-Org in soils.

The conversions of P-Org into orthophosphates ($H_2PO_4^-$ and HPO_4^{2-}) are intrinsically driven by microbial processes. Microorganisms normally have three pathways to make use of P: immobilization of P in soil solution, solubilization of inorganic P sources or mineralizing P from necromass and soil organic matter (Bünemann, 2015) (Figure 6.3). Improving P-Org cycling by microbial mineralization has been suggested as a strategy to sustain production in arable lands and grasslands (Nash et al., 2014; Amadou et al., 2021). As such, I carried out experiments to assess the role of microorganisms in increasing plant available P. **Chapter 3** showed that microorganisms have a higher affinity towards P-Org compared to other non-organic forms (iron phosphate III and hydroxyapatite), which increased P availability in phytate amended soils. **Chapter 4** also showed that

sludge, a DPW material with high P-Org content, increased plant P uptake and yields in grasses more than other forms of P fertilisers, including mineral P fertiliser. Our results suggest that microbially driven mineralization of P-Org in DPW and soils is able to increase plant available P, thus decreasing the demand for external inputs of P.

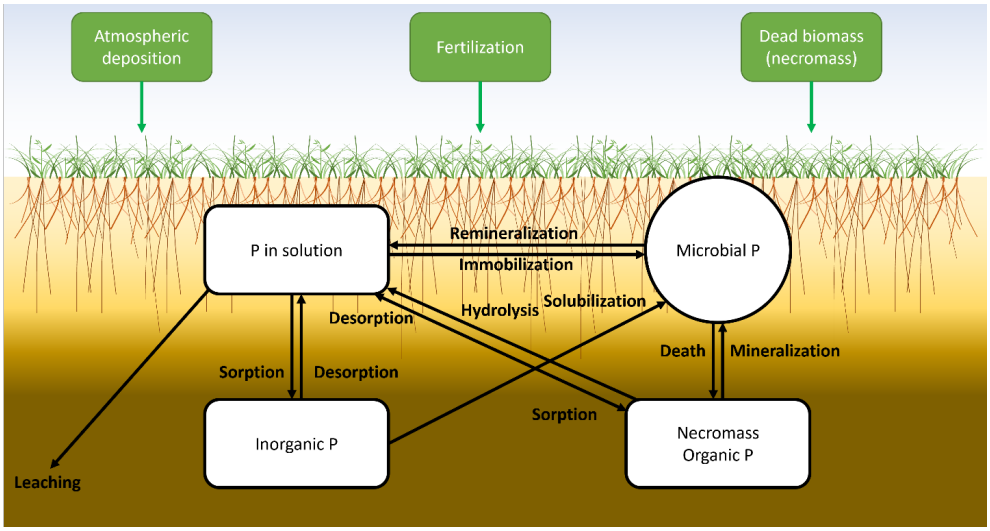

Figure 6.3. *Microbial transformations of organic phosphorus. Boxes represent the pools of phosphorus and the arrows show the processes by which P is exchanged among pools. Adapted from* Bünemann (2015).

Microbial mineralization of P is, however, a species-specific process (Amy et al., 2022), as not all soil microorganisms are able to mineralize P in the same way. Both in **chapters 3** and **4,** I have observed the importance of fungal communities in increasing plant available P. In **chapter 3** this was observed by the number of 18S gene copies and in **chapter 4** the increase in P uptake was related to saprophytic and arbuscular mycorrhiza fungi (AMF). In both cases, the effects of fungi in increasing P availability were superior to those of bacteria under our experimental conditions. The differences detected between bacteria and fungi can be associated with their different growth strategies. It has been suggested that bacteria are faster-paced growing organisms (*r*-strategists) whereas fungi are more conservative in their reproductive strategy (*k*-strategists) (Yuan Sun et al., 2021). The different strategies

of soil microorganisms are ultimately linked to carbon (C) acquisition. C is considered the main limiting factor for microbial growth in soils (Demoling et al., 2007). Fungi have been suggested to have higher C use efficiencies than bacteria (Six et al., 2006). In this sense, the P solubilization mechanisms of bacteria would require a higher amount of C compared to fungi.

In **chapter 3**, I also showed that the microbial C: P ratio was determinant in increasing plant available P and was positively correlated with the changes in plant available P, suggesting that C incorporation into the microbial biomass is a requirement to improve P availability. The microbially driven processes of P cycling have been associated with the microbial needs for C and stoichiometric homeostasis (Spohn and Kuzyakov, 2013; Spohn, 2016). The utilization of different C sources might be dependent on the groups of fungi as shown in **chapter 4**, where saprophytic fungi contributed most to P uptake at high C availability and AMF became more important as the labile forms of C were respired. In addition, the importance of C in P cycling was also showed in **chapter 3** as cellulose was required to achieve P mineralization. The need for C to solubilize P from minerals have also been identified in other experiments (Brucker et al., 2020). The positive results in which microorganisms are screened for their P solubilizing abilities are normally conducted in C rich medias which may differ greatly from the actual C availability in the soil. The lack of microbial contribution towards P uptake in **chapter 5** can also be associated with the poor C content of the soil and its coarse texture (> 75% sand).

Promotion of fungal biomass in soils might be a way to increase plant available P in grasslands. Different methods have been proposed to increase fungal biomass in soils, including incorporation of different organic materials like manures, compost or slurry (Lucas et al., 2014; Shi et al., 2018) or conservation tillage (Chen et al., 2020). Different types of organic amendments have been proven to increase soil fungal biomass, ranging from paper pulp and sawdust (Clocchiatti et al., 2020) to manures and composted waste (Shi et al., 2018; Chen et al., 2020; Liu et al., 2023). My research showed the potential of DPW's sludge to do the same as it contains a high C content (31% of total dry weight), a high water soluble C (3% of total dry weight) and a low C: N ratio (5), suggesting a high lability of C (Velasco-Sánchez et al., 2023). These properties not only make DPW's sludge an interesting source of P,

but also a labile source of C that might allow the proliferation of soil microorganisms and increased mineralization of P from organic P sources over time.

4. New fertilizers require grassland adaptations

The last partial research question of this PhD thesis **(RQ IV)** dealt with the selection of grass species that could result in a improvement in P use after fertilization with DPW materials or alleviation of P limitation under poor P conditions. The results from my experiments confirm the great importance of grass species selection to improve P use from soils.

Grass species management in grassland has been suggested as a mechanism to increase access to soil P (Ros et al., 2018). In **chapter 4**, I observed that two commonly grown grass species in European grasslands have markedly different growth dynamics. *Lolium perenne* was observed to grow faster than *Dactylis glomerata*. These differences influenced P uptake: *Lolium perenne* took up P much quicker than *Dactylis glomerata*, yet by the end of the experiment both grass species had similar biomass production. These different growth patterns have direct implications for the use of P fertilizers, as the addition of P fertilizers should match the demands of the different grass species. In this sense, introducing mixtures of grass species, like *Dactylis glomerata* and *Lolium perenne,* with different requirements for P over time, could result in an improved P use efficiency over time compared to growing the grass species in monoculture. The different use of resources over time is considered part of the niche differentiation or complementarity mechanisms studied in species in intercropping or species mixtures experiments (Phoenix et al., 2020).

In **chapter 5**, I studied the associations of grass species further. In this chapter, I used also commonly grown grasses in European grasslands including *Lolium perenne* (most cultivated grass species in temperate areas) and *Festuca arundinacea* in monocultures, in combination and in a four species combination with species with a high palatability index. We chose *Festuca arundinacea* in our study because of its deep rooting pattern, which has gained attention for its resilience towards drought events (Cougnon et al., 2014). The complementary vertical distribution of roots of

Lollium perenne and *Festuca arundinacea* contributed to improve P nutrition and yields in P limited soils. Among the root traits studied, the vertical distribution of root biomass was shown to be more important than root length or root diameter. The results of our study are in line with previous research on P nutrition and root traits (Ros et al., 2018). Improving access to more pools of P (vertically) can result in a higher use efficiency of P, making use of potentially poorly available sources of P and decreasing the overall demand of P fertilization.

In addition, I studied the relationships between soil microorganisms and different grass species to improve P uptake. The positive relationships of grass species in mixtures and soil microorganisms in increasing P availability are often referred as indirect interspecific facilitation by ecologists (Duchene et al., 2017). The results of **chapters 3 and 4** suggested that soil microorganisms, primarily fungi, would improve P uptake. In **chapter 4,** I observed that the effects of DPW fertilization on the soil microbial community (particularly AMF) are dependent on the grass species, as microbial communities significantly differed between the different DPW treatments only when applied to *Lolium perenne*. In **chapter 5**, however, potentially because of the very low C content of the soil and its sandy texture, I did not observe relationships between soil microorganisms and P uptake. The importance of C acquisition by soil microorganisms has also been discussed in **chapters 3 and 4** and in previous research (Brucker et al., 2020). Also, studying the temporal contribution of soil microorganisms (i.e. studying microbial activities over time) might have yielded different results (Deng et al., 2019). It is also possible that the associations with soil microorganisms that contribute to the solubilization of P are soil dependent (Singh et al., 2007; Moreno-Lora et al., 2023). Potentially, in different soils, different types of mechanisms would be more relevant for P acquisition. For example, in C rich soils the microbial pathway would become more predominant and in soils with finer texture other root traits, such as root length or diameter might contribute more to P uptake (Ros et al., 2018). Therefore, management of grassland to make use of poorly available P pools might require specific selection of species for particular soil conditions and properties.

Fertilization with DPW materials or other recycled P fertilizers would also require species adaptation to meet the P chemistry of the recycled fertilizers (Martínez-

García et al., 2018). The solubility of P in the recycled fertilizers would determine the vertical movement of P. Similarly, the concentration of organic P or inorganic P would also result in different root adaptations that could have impacts in grassland management. For example, when materials are applied with a high concentration of organic P, it would be advisable to select grass species that establish associations with soil microorganisms that can mineralize P, as shown in **chapters 3 and 4** fungal communities are more pivotal in P nutrition (Velasco-Sánchez et al., 2024). Similarly, if the concentration of inorganic P is high in the newly added fertilizers, selection of species that can exudate organic acids and modify soil pH would be recommended (Louw-Gaume et al., 2017).

5. Conclusions

The results and insight of this PhD thesis have provided evidence of the positive effects of DPW on biomass production in grassland and I have shown the positive effects of soil microorganisms in enhancing soil P availability and plant P uptake. The main conclusions for the partial research questions of this thesis are depicted in Table 6.1.

Table 6.1. *Main conclusions to the partial research questions (RQ) of this PhD thesis.*

RQ	Conclusions
I	DPW materials are highly heterogeneous and such heterogeneity affects their P plant availability and interactions with soil microorganisms. Traditional P fractionation methods such as "SMT" fail to identify such groups of P. New methods are required to quantify P forms in waste materials and soils.
II	The availability of P from DPW for plants depends on the chemical forms of P. Organic forms of P can act as slow-release sources of P. Transforming DPW's sludge into ash or hydrochar results in a decrease of plant available P.
III	Soil microbial communities increase plant available P and play a pivotal role in P uptake. The detected effect on soil microorganisms is superior for fungal communities than bacteria. The contribution of the fungal community to increase P availability is related to C acquisition. Microorganisms increase plant available P, primarily, from organic P forms.
IV	Grass species selection is crucial in increasing P uptake and fertilization efficiency. Particularly, different grass species establish different associations with soil microorganisms and this should be further studied. Moreover, grass species with higher root biomass and combinations of grasses with different vertical root distribution have the potential to increase P uptake.

In general, sludge is a promising material with a high P concentration and a low concentration of contaminants. In my thesis, I have shown that raw sludge can be a substitute for mineral fertilizers by providing mineralizable sources of P and the required C to enhance the activities of soil microorganisms. Transforming DPW into ash or (hydro)char with the aim to further increase the P concentration and to ease transportation, however, may result instead in the transformation of P into very poorly available forms of P. Materials such as ash or hydrochar should then be used as amendments to reduce environmental pollution or as intermediary materials in the production of precipitated P salts (e.g., struvite) that may offer better P availability for plants, rather than being used directly as fertilizer.

It is also important to reflect on the necessity to fertilize grasslands with P. My results underline claims in the scientific literature that P fertilization only makes marginal contributions to increase yields, particularly on heavily managed systems, whereas it poses significant environmental threats. Managing the native soil P pools by enhancing the activities of soil microorganisms and by selecting grass combinations that can access different pools of P could be advisable to reduce the dependency of P fertilization in grasslands. Ultimately, to achieve a more efficient use of soil P reserves and to provide better recommendations on P fertilization with recycled P fertilizer, newer methods should be developed that can handle the chemical complexity of recycled P fertilizers and more efficiently consider the plant utilization of P.

6. Recommendations for further research

The results of this thesis have provided new insights on the fertilization with DPW materials, the contribution of soil microorganisms towards plant P uptake and on the importance of grass species selection in the alleviation of P limitation. In parallel, I have been able to identify many knowledge gaps that should be addressed in future research.

Further experiments should aim to improve the characterization of P forms in recycled P fertilizers and in soils. Protocols should aim to be simple, so they can be easily performed and understood by the wider public. Characterization of P forms in

soils and waste materials should include the plant available pool, which is presumably the most relevant in agriculture. Currently, soil tests that assess available P for crops continue to be used, regardless of the lack of homogenization and the demonstrated flaws. I propose that new methods that assess plant availability of nutrients should measure concentration in plant tissues. Therefore, I suggest more research on the identification of the optimal stoichiometric values of C, N and P in plant tissues at species level and the utilization of such ratios to provide fertilizer recommendations for farmers.

The valorisation of DPW in agriculture still faces many challenges. Future experiments should compare more types of DPW materials including different production processes (e.g. chemical composition, temperatures, physical properties, etc.). The production of DPW secondary materials should be optimized to increase the plant available fraction. More socio-economic research should be performed to further assess the viability of P fertilization with DPW materials. Comparison of DPW with other waste materials (e.g. sewage sludge, manure, slurry, compost, etc.) should be performed to better understand the fertilizer value of DPW. The environmental threats of fertilizing with DPW materials should be studied in detail, variables such as the introduction of inorganic pollutants (e.g. heavy metals), organic pollutants (e.g. PAHs) and biological pollutants (e.g. salmonella) should be considered. Moreover, the effects of DPW on greenhouse gases or the eutrophication of groundwaters should be considered and compared to other types of mineral or recycled fertilizers.

More research should be performed on the effects of soil microorganisms on P cycling and on the selection of grass species. The results of this thesis should be tested on a wider variety of soils. The identification of the microbial species that perform the mineralization, solubilization or desorption of different forms of P in soils and fertilizers should also be performed. Development of techniques that can result in the increase of fungal communities are also to be explored. Lastly, future experiments should aim to model P cycling with the integration of different fractions of P in fertilizers, different root traits and different soil properties. These models should ideally be simple enough to be utilized by farmers or extension workers, which are the final users of DPW materials.

Nederrijn river near Wageningen, The Netherlands

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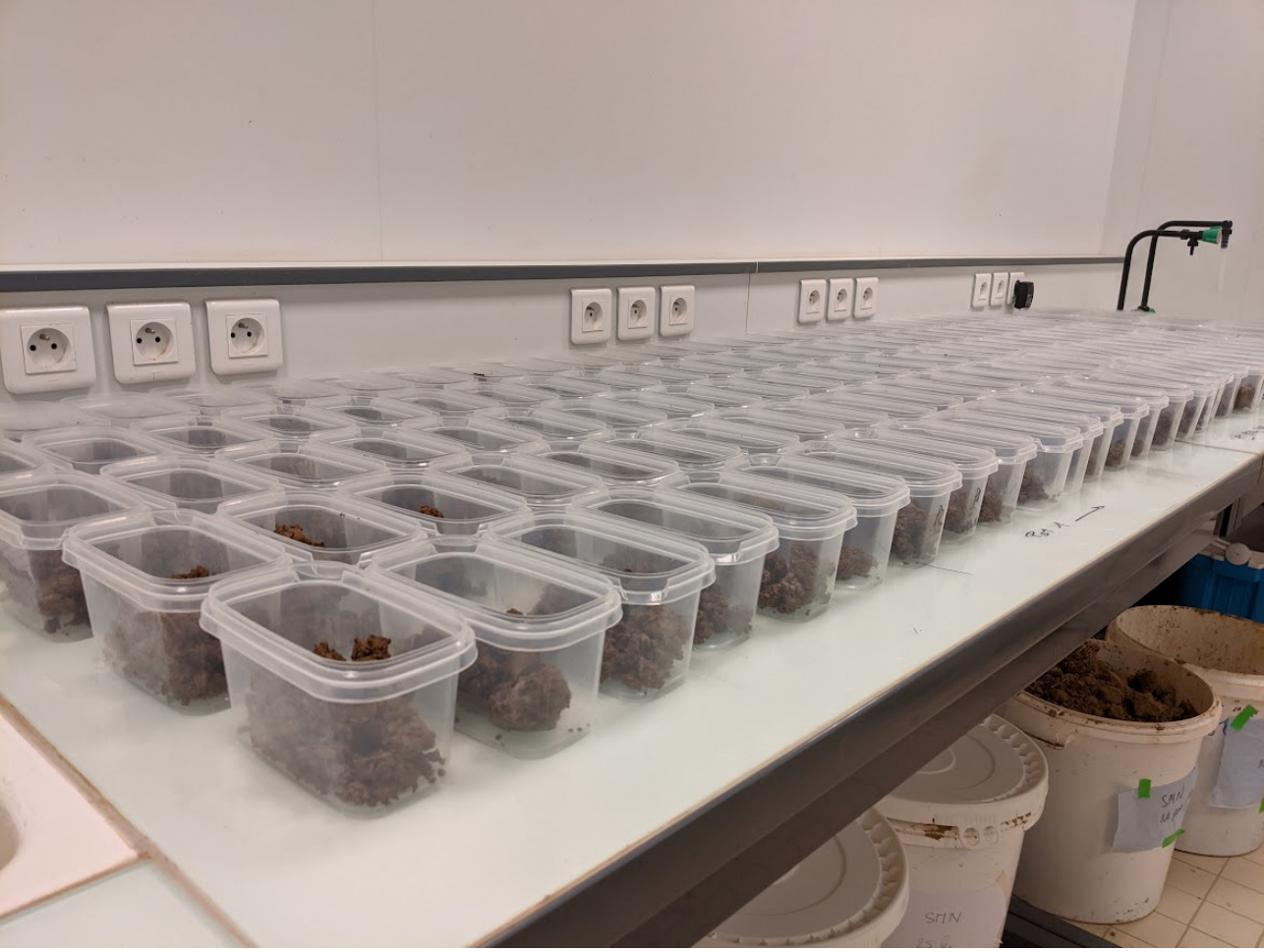

Preparation of incubation experiment at Unilasalle, Mont-Saint-Aignan, France

Summaries

Summaries in English, French and Spanish

Summary

Shortly after the discovery and isolation of phosphorus (P) as a chemical element during the 17th century, the importance of P in agriculture was noticed. Society relied on sources of P such as bird depositions or bones until the green revolution in which the consumption of P from mines (mineral P fertilizers) multiplicated across temperate areas of the world. The utilization of mineral P, often at excessive rates, has led to environmental degradation. Furthermore, the reliance on imports from countries with P deposits has posed a threat on food security. For these reasons, it has become relevant to improve the efficiency of P fertilization and to look for other sources of P that could diminish the overall demand for conventional mineral P fertilizers.

Dairy processing waste (DPW) is one of the most abundant agricultural waste materials in the temperate areas of the world. DPW has been proposed as a material that can replace mineral P fertilizers because of its high P concentration and its low concentration of contaminants. However, their fertilization effects on grasslands as well as the contribution of soil microorganisms in improving P use efficiency remain poorly explored. In this thesis, I have studied the agronomic performance of DPW materials and the contribution of soil microorganisms and grass species selection in increasing P use efficiency. I studied this overarching research question by answering four interconnected partial research questions: (I) What are the forms of P present in DPW materials and can we measure them through P fractionation techniques? (II) What is the plant availability of P from DPW materials? (III) Can soil microorganisms increase the plant availability of P from DPW materials? and (IV) Can the selection of grass species contribute to increase the P use efficiency from DPW materials?

This thesis consists of 6 chapters, from which 4 chapters include the results of experiments carried out in Unilasalle (Mont-Saint-Aignan, France), Wageningen University and Research (Wageningen, The Netherlands) and in Limerick University (Limerick, Ireland). In **chapter 1** I introduced the current issues of P fertilization and outlined the structure of the thesis. In this chapter, I also revisited the P cycle and provided background on the importance of soil microorganisms in P cycling and

the importance of careful grass species selection in improving P acquisition in grassland systems.

In **chapter 2** I studied the chemical forms of P in DPW sludge, ash and hydrochar. I performed the fractionation of the chemical forms of P by using the conventional SMT protocol and attempted to modify this protocol by the addition of a preliminary step in which water soluble P is first quantified in order to compare the solubility of the different assessed fractions. My results indicated that this procedure is inadequate to measure P fractions in DPW materials. I explained why this method fails in correctly identifying P forms, drawing comparisons with similar criticism of P fractionation schemes within the scientific community. I suggest to re-name the assessed fractions of P by the SMT to operationally defined pools of P instead of referring to incorrect chemical P pools.

In **chapter 3** I assessed the contribution of soil microorganisms in enhancing plant available P (Olsen P). I incubated a soil with a low P status with hydroxyapatite (P-Ca), iron phosphate III (P-Fe), phytate (P-Org), a mixture of P-Ca and P-Org (P-Mix) and triple superphosphate (TSP). These chemical forms of P can be found in DPW as well as in other recycled P fertilizers. My results showed that whereas plant available P in TSP amended soils decreased sharply over time, plant available P was roughly doubled in P-Org amended soils. Fungal biomass and microbial C: P were found to be the main factors increasing plant available P.

The agronomic performance of DPW materials (sludge, ash and hydrochar) and the contribution of soil microorganisms in increasing P use efficiency by two different grass species (*Lolium perenne vs Dactylis glomerata*) were evaluated in **chapter 4**. My results indicate that while DPW sludge had an overall high P use efficiency, ash and hydrochar had very low ones. The application of DPW materials had no direct impact on soil microbial communities. Yet, I showed that fungal biomass was a pivotal variable in explaining P uptake. *Lolium perenne* and *Dactylis glomerata* resulted in similar cumulative yields, but showed contrasting growth patterns (*Lolium perenne* fast grower and *Dactylis glomerata* slow grower) that might impact grassland management.

In **chapter 5** I studied combinations of grass species with contrasting root morphologies and their interaction with soil microorganisms to improve P nutrition under P limiting conditions. In this chapter I found that combining *Lolium perenne* with *Festuca arundinacea* resulted in higher yields and improved P nutrition. The improved performance of this grass combination was associated with the vertical niche differentiation of root biomass (*Lolium perenne* shallow and *Festuca arundinacea* deep). I did not find any relationships between soil microbial activities or biomass and P nutrition. , I explained this lack of significant relationships as related to the low carbon (C) content of the soil and its coarse texture.

Finally, in **chapter 6**, I have summarized the main findings of chapters 2, 3, 4 and 5, provided discussion on the major outcomes of these chapters and suggested further experiments. Particularly, I have discussed that even though DPW's sludge is a promising material to reduce dependency on mineral P fertilizer, its transformation into ash and (hydro)char reduces significantly their fertilizer value. Sludge is also a material that is chemically heterogeneous and difficult to transport. I have also critically revisited the necessity to apply P, as P fertilization showed only no or marginal increases in yields. Most grassland soils in Europe have already high P contents. Transformation of this P into plant available P, by increasing the soil organic P fraction, by increasing fungal biomass and by careful selection of grass species are management strategies that should be considered to decrease mineral P dependency.

Résumé

Peu de temps après la découverte et l'isolement du phosphore (P) en tant qu'élément chimique au cours du XVIIe siècle, l'importance du P en agriculture a été remarquée. La société dépendait de sources de P telles que les fientes d'oiseaux ou les os jusqu'à la révolution verte, au cours de laquelle la consommation de P issu des mines (engrais phosphatés minéraux) s'est multipliée dans les zones tempérées du monde. L'utilisation du P minéral, souvent à des taux excessifs, a conduit à une dégradation environnementale. De plus, la dépendance aux importations en provenance de pays dotés de gisements de P constitue une menace pour la sécurité alimentaire. Pour ces raisons, il est devenu pertinent d'améliorer l'efficacité de la fertilisation en phosphore et de rechercher d'autres sources de P pouvant réduire la demande globale en engrais phosphatés minéraux conventionnels.

Les déchets de transformation laitière (DTL) figurent parmi les déchets agricoles les plus abondants dans les régions tempérées du monde. Les DTL ont été proposés comme matériaux pouvant remplacer les engrais phosphatés minéraux en raison de leur concentration élevée en P et de leur faible concentration en contaminants. Cependant, leurs effets de fertilisation sur les prairies ainsi que la contribution des micro-organismes du sol à l'amélioration de l'efficacité d'utilisation du P restent peu explorés. Le travail conduit dans cette thèse ambitionne d'évaluer la performance agronomique des matériaux issus des DTL en considérant la contribution des micro-organismes du sol d'une part et la sélection des espèces de graminées prairiales d'autre part. Cette thématique globale peut se présenter sous la forme de quatre questions de recherche partielles interconnectées : (I) Quelles sont les formes de phosphore présentes dans les matériaux des DTL et peut-on les mesurer à travers des techniques de fractionnement du phosphore ? (II) Quelle est la disponibilité pour les plantes du phosphore des matériaux des DTL ? (III) Les micro-organismes du sol peuvent-ils augmenter la disponibilité pour les plantes du phosphore issus des biosolides issus des DTL ? (IV) La sélection des espèces de graminées prairiales peut-elle contribuer à augmenter l'efficacité d'utilisation du phosphore issus des matériaux des DTL ?

Cette thèse se compose de 6 chapitres, dont 4 comprennent les résultats d'expérimentations menées à l'Unilasalle (Mont-Saint-Aignan, France), à

l'Université de Wageningen (Wageningen, Pays-Bas) et à l'Université de Limerick (Limerick, Irlande). Le **chapitre 1** présente les problèmes actuels liés à la fertilisation en phosphore et expose la structure de la thèse. Dans ce chapitre, le cycle du P est décrit, des informations sur l'importance des micro-organismes dans le cycle du P du sol sont fourni et l'importance de la sélection soigneuse des espèces graminées prairiales dans l'amélioration de l'acquisition du P dans les systèmes de prairies est soulignée.

Le **chapitre 2**, décrit les travaux de caractérisation des formes chimiques du P dans les boues, les cendres et l'hydrochar des DTL. Le fractionnement des formes chimiques du P par le protocole conventionnel SMT et un protocole modifié par l'ajout d'une étape préliminaire d'extraction du P soluble dans l'eau ont été utilisés pour évaluer la disponibilité du P pour les plantes. Mes résultats ont indiqué que cette procédure est inadéquate pour mesurer les fractions de P dans les matériaux issus des DTL. Les lacunes de cette méthode pour identifier correctement les formes de P sont présentées sous la forme de comparaisons avec des schémas de fractionnement du P utilisés par la communauté scientifique. Je suggère de renommer les fractions évaluées de P par le SMT en pools de P définis opérationnellement au lieu de faire référence à des pools de P chimiques incorrects.

Dans le **chapitre 3**, la contribution des micro-organismes du sol à l'amélioration du P disponible pour les plantes (P Olsen) a été évaluée. Un sol à faible teneur en P a été incubé avec différentes formes de P : de l'hydroxyapatite (P-Ca), du phosphate de fer III (P-Fe), du phytate (P-Org), un mélange de P-Ca et P-Org (P-Mix) et du superphosphate triple (TSP). Ces formes chimiques de P sont présentes dans les DTL ainsi que dans d'autres engrais phosphatés recyclés. Les résultats ont montré que, tandis que le P disponible pour les plantes dans les sols amendés avec du TSP diminue fortement avec le temps, le P disponible pour les plantes est environ doublé dans les sols amendés avec du P-Org. La biomasse fongique et le rapport C/P microbien se sont avérés être les principaux facteurs augmentant le P disponible pour les plantes.

La performance agronomique des matériaux issus des DTL (boues, cendres et hydrochar) et la contribution des micro-organismes du sol à l'augmentation de l'efficacité d'utilisation du P par deux espèces de graminées prairiales (*Lolium*

perenne vs Dactylis glomerata) ont été évaluées dans le **chapitre 4**. Les résultats ont montré une efficacité élevée d'utilisation du P issu des boues par les plantes tandis que cette efficacité est très faible pour les cendres et l'hydrochar. L'application des matériaux issus des DTL n'a eu aucun impact direct sur les communautés microbiennes du sol. Cependant, la biomasse fongique du sol s'est révélée être une variable cruciale dans l'explication de l'absorption du P. *Lolium perenne* et *Dactylis glomerata* ont donné des rendements cumulatifs similaires, mais ont montré des patterns de croissance contrastés (Lolium perenne croissance rapide et Dactylis glomerata croissance lente) qui pourraient influencer la gestion des prairies.

Dans le **chapitre 5**, les interactions plantes / microorganismes du sol et leur conséquence sur la nutrition de la plante en P, ont été étudiées en utilisant des combinaisons d'espèces prairiales aux traits racinaires contrastées. La combinaison de *Lolium perenne* avec *Festuca arundinacea* a donné des rendements plus élevés et a amélioré la nutrition en P. La performance améliorée de cette combinaison de graminées est à associer à la différenciation de niche verticale de la biomasse racinaire (*Lolium perenne* superficielle et *Festuca arundinacea* profonde). Aucune relation entre les activités ou la biomasse microbienne du sol et la nutrition en P n'a été mise en évidence. Cela peut être dû à la faible teneur en carbone (C) du sol et à sa texture grossière.

Enfin, dans le **chapitre 6**, les principales conclusions des chapitres 2, 3, 4 et 5 sont rapellés, discutés et des expérimentations ultérieures sont proposées. En particulier, l'intérêt de la transformation des boues des DTL en biosolides pour réduire la dépendance aux engrais phosphatés minéraux est discutée à l'aune des processus de transformation en cendres et (hydro)char qui réduisent significativement leur valeur en tant qu'engrais. Les boues sont également un matériau chimiquement hétérogène et difficile à transporter. J'ai aussi réexaminé de manière critique la nécessité d'appliquer du P, en lumière des observations d'effets marginaux de la fertilisation en P sur les rendements dans les chapitres 4 et 5. La plupart des sols de prairies en Europe ont déjà des teneurs élevées en P, et la transformation de ce P en P disponible pour les plantes pourrait avoir un potentiel significatif pour améliorer ou maintenir les rendements. Une telle stratégie de gestion du P devrait être prise en compte pour réduire la dépendance aux engrais phosphatés minéraux et pourrait être atteinte en

augmentant la fraction de P organique du sol, en augmentant la biomasse fongique et en sélectionnant soigneusement les espèces d'herbes.

Resumen

Poco después de su descubrimiento como elemento químico en el siglo XVII, el fósforo (P) fue considerado indispensable en la agricultura. Aunque al principio solo se obtenía de las deposiciones de las aves o de los huesos, luego, tras la revolución verde, el P empezó a obtenerse de las minas (fertilizantes de P mineral) y su uso se multiplicó en áreas desarrolladas del mundo. Este uso, muchas veces excesivo, ha llevado a la degradación del medio ambiente. Además, la dependencia de importaciones de países ricos en minas de P ha representado una amenaza para la seguridad alimentaria. Por estas razones, es necesario mejorar la eficiencia de la fertilización con P así como buscar otras fuentes que puedan disminuir la demanda global de fertilizantes fosfatados convencionales.

Los residuos de procesamiento lácteo (RPL) son uno de los materiales de desecho agrícola más abundantes en las zonas templadas del mundo. Se ha propuesto que los RPL pueden reemplazar a los fertilizantes minerales de P debido a su alta concentración de P y su baja contenido en contaminantes. Sin embargo, los efectos de su fertilización en praderas, así como la contribución de los microorganismos del suelo para mejorar la eficiencia en la utilización del P, siguen siendo poco entendidos. En esta tesis, he estudiado el rendimiento agronómico de los fertilizantes obtenidos de los RPLs y la contribución de los microorganismos del suelo y la selección de especies de pasto en el aumento de la eficiencia en la utilización del P. He abordado esta pregunta de investigación general respondiendo a cuatro preguntas de investigación parciales interconectadas: (I) ¿Cuáles son las formas de fósforo predominantes en los materiales de RPL y podemos medirlas mediante técnicas de fraccionamiento de fósforo? (II) ¿Cuál es la disponibilidad de fósforo para las plantas a partir de los materiales de RPL? (III) ¿Pueden los microorganismos del suelo aumentar la disponibilidad de fósforo para las plantas de los materiales de RPL? (IV) ¿Puede la selección de especies de pasto contribuir a aumentar la eficiencia en la utilización del fósforo de los materiales de RPL?

Esta tesis consta de 6 capítulos, de los cuales 4 incluyen los resultados de experimentos realizados en Unilasalle (Mont-Saint-Aignan, Francia), Wageningen University and Research (Wageningen, Países Bajos) y en University of Limerick (Limerick, Irlanda). En el **capítulo 1**, presenté los problemas actuales de la

fertilización con P y esbocé la estructura de la tesis. En este capítulo, también recordé el ciclo del P y proporcioné antecedentes sobre la importancia de los microorganismos del suelo y de la cuidadosa selección de especies para mejorar la adquisición de P en pastos.

En el **capítulo 2**, estudié las formas químicas de P en lodos, cenizas e hydrochar provenientes de RPLs. Realicé el fraccionamiento de las formas químicas de P utilizando el conocido protocolo SMT e intenté modificar este protocolo agregando un paso preliminar en el que se cuantifica primero el P soluble en agua para comparar la solubilidad de las diferentes fracciones obtenidas posteriormente. Mis resultados indicaron que este procedimiento no es adecuado para medir las fracciones de P en los materiales de RPL. Expliqué por qué este método falla al identificar correctamente las formas de P, comparando mis resultados con criticas similares a esquemas de fraccionamiento de P por la comunidad científica. Sugiero cambiar el nombre de las fracciones evaluadas de P por el protocolo SMT a "pools" de P definidos operacionalmente en lugar de referirse a "pools" de fracciones químicas predefinidas de P incorrectas.

En el **capítulo 3**, evalué la contribución de los microorganismos del suelo para aumentar el P disponible para las plantas (P Olsen). Incubé un suelo pobre en P con hidroxiapatita (P-Ca), fosfato de hierro III (P-Fe), fitato (P-Org), una mezcla de P-Ca y P-Org (P-Mix) y superfosfato triple (TSP). Estas formas químicas de P se encuentran en los RPL, así como en otros fertilizantes de P reciclados. Mis resultados mostraron que mientras que el P disponible para las plantas en suelos enmendados con TSP disminuyó bruscamente con el tiempo, el P disponible para las plantas se duplicó aproximadamente en suelos enmendados con P-Org. La biomasa fúngica y el ratio C: P en la biomasa microbiana resultaron ser los principales factores que explican el aumento del P disponible para las plantas.

El rendimiento agronómico de los materiales de RPL (lodo, ceniza e hydrochar) y la contribución de los microorganismos del suelo en el aumento de la eficiencia en la utilización del P en dos especies de pasto diferentes *(Lolium perenne vs Dactylis glomerata*) se evaluaron en el **capítulo 4**. Mis resultados indican que mientras que el lodo de RPL tenía una eficiencia en la utilización del P generalmente alta, la ceniza y el hydrochar tenían eficiencias muy bajas. La aplicación de los materiales de RPL

no tuvo un impacto directo en las comunidades microbianas del suelo. Sin embargo, mostré que la biomasa fúngica era una variable clave para explicar la absorción de P. *Lolium perenne* y *Dactylis glomerata* dieron rendimientos similares, pero mostraron patrones de crecimiento diferentes (*Lolium perenne* crecimiento rápido y *Dactylis glomerata* crecimiento lento) que podrían influir en la gestión de las praderas.

En el **capítulo 5**, estudié combinaciones de especies de pasto con morfologías de raíces diferentes y su interacción con los microorganismos del suelo para mejorar la nutrición de P en condiciones limitadas de P. En este capítulo, observé que la combinación de *Lolium perenne* con *Festuca arundinacea* resultó en rendimientos más altos y una mejor nutrición de P. El rendimiento mejorado de esta combinación de pasto se asoció con la diferenciación vertical de la biomasa de raíces *(Lolium perenne* superficial y *Festuca arundinacea* profunda). No encontré ninguna relación entre las actividades o la biomasa microbiana del suelo y la nutrición de P. Expliqué esta falta de relaciones significativas por el bajo contenido de carbono (C) del suelo y su textura arenosa.

Finalmente, en el **capítulo 6**, he resumido los principales hallazgos de los capítulos 2, 3, 4 y 5, proporcionado discusiones sobre los resultados principales de estos capítulos y sugerido experimentos adicionales. En particular, he discutido que aunque el lodo de los RPL es un material prometedor para reducir la dependencia de los fertilizantes de P minerales, su transformación en cenizas e hydrochar reduce significativamente su valor como fertilizante. El lodo también es un material químicamente heterogéneo y difícil de transportar. He revisado críticamente la necesidad de aplicar P, ya que la fertilización con P no ha demostrado aumentar en gran medida los rendimientos y la mayoría de los suelos de praderas en Europa ya tienen contenidos elevados de P. La transformación de P en P disponible para las plantas, aumentando la fracción de P orgánico del suelo, aumentando la biomasa fúngica y seleccionando cuidadosamente las especies de pasto son estrategias de manejo que deben considerarse para disminuir la dependencia de los fertilizantes de P minerales.

Group members of REFLOW during Unilasalle's summer school

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Ángel Velasco Sánchez

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About the author

Ángel Velasco Sánchez was born in Sevilla, Spain on the 15th of December of 1993. His interest in agriculture comes from his grandfather's olive plantation in Fuente Tójar, Córdoba. Ángel did a BSc in Agricultural Engineering at the Universidad de Sevilla with an specialization in crop and animal systems. During his BSc thesis he worked on the effects of soil microorganisms in the nutrition of zinc and phosphorus in wheat plants. Ángel continued his studies in soil sciences at Wageningen University and Research where he obtained a MSc degree in Plant Sciences. During his master thesis he worked on the effects of nitrogen fertilization on soil enzymatic activities. In February 2020, Ángel started his PhD within the European framework REFLOW, where he studied the agronomic effects of dairy processing waste and the contribution of soil microorganisms and grass species selection to improve phosphorus fertilization efficiency. He performed his PhD research between UniLasalle, Rouen (France) and Wageningen University and Research (The Netherlands). Apart from his PhD work, Ángel has worked as a research assistant in a plant breeding company, as extension worker in olive groves and has collaborated in an interdisciplinary project led by Leiden University in which he merged soil and archaeological sciences.

Publications

Scientific articles during REFLOW project

Velasco-Sánchez, Á., Ferron, L.M.E., Mani, D.T.C., Bennegadi-Laurent, N., Trinsoutrot-Gattin, I., Van Groenigen, J.W., Moinet, G.Y.K. Combination of Lolium perenne L. and Festuca arundinacea Schreb. improve yields under low phosphorus availability. Nutrient Cycling in Agroecosystems 128, 199–215 (2024). https://doi.org/10.1007/s10705-024-10346-9

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Hu, Y., Khomenko, O., Shi, W., **Velasco-Sánchez, Á.**, Ashekuzzaman, SM., Bennegadi-Laurent, N., Daly, K., Fenton, O., Healy, M.G., Leahy, J.J., Sørensen, P., Sommer, S.G., Taghizadeh-Toosi, A. and Trinsoutrot-Gattin, I. (2021). Systematic Review of Dairy Processing Sludge and Secondary STRUBIAS Products Used in Agriculture. Frontiers in Sustainable Food Systems. 5:763020. https://doi.org/10.3389/fsufs.2021.763020

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Velasco-Sánchez, Á., Hu, Y., Sommers, S.G., Moinet, G.Y.K., Van Groenigen, J.W., Trinsoutrot-Gattin, I., Bennegadi-Laurent, N. Fertilizer value of dairy processing waste materials and contributions of soil microorganisms towards phosphorus uptake in grasses. *Submitted for publication*

Datasets during REFLOW project

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Velasco-Sánchez, Á., Bennegadi-Laurent, N., Trinsoutrot-Gattin, I., van Groenigen, J. W., & Moinet, G. (2022). Assessing the role of soil microbes in the dynamics of P release from poorly soluble P forms (v0.0) [Data set]. Zenodo. https://doi.org/10.5281/zenodo.6901821

PE&RC Training and education statement

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

Review of literature (6 ECTS)

- Dairy industry wastewater recovered P-fertilizers effects on soil microbial communities

Post-graduate courses (6 ECTS)

- LCA and food security; Beta, UVic (2020)
- Socio/techno economic modelling; Unilasalle (2022)
- Introduction to R and R studio; PE&RC (2002)
- Mixed linear models; PE&RC (2022)
- Soil ecology; PE&RC (2022)

Deficiency, refresh, brush-up courses (2 ECTS)

- Crop growth and soil health; Unilasalle (2022)

Laboratory training and working visits (3 ECTS)

- Phosphorus fractionation; University of Limerick, Ireland (2022)

Invited review of journal manuscripts (1 ECTS)

- Journal of arid environments: soil sciences and geoarchaeology (2023)

Competence, skills and career-oriented activities (9.9 ECTS)

- Fundamentals of research practices; University of Limerick (2020)
- Science to policy; Unilasalle (2022)
- Introduction to latex; PE&RC (2022)
- Scientific writing; Wageningen into Languages (2022)

Scientific integrity/ethics in science activities (0.6 ECTS)

- Scientific integrity; PE&RC (2023)

PE&RC Annual meetings, seminars and PE&RC weekend/retreat (1.5 ECTS)

- PE&RC First year retreat (2020)
- PE&RC Last year retreat (2023)

Discussion groups/local seminars or scientific meetings (6 ECTS)

- Science AGHYLE (2020-2023)
- REFLOW products & the future; European Landowners Organization (2022)

International symposia, workshops and conferences (3.8 ECTS)

- European sustainable phosphorus platform; Vienna, Austria (2022)
- Wageningen soil conference; Wageningen, the Netherlands (2023)

BSc/MSc thesis supervision (1 ECTS)

- Soil microbiological methods

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