Impacts of nitrogen and phosphorus inputs and elevated atmospheric carbon dioxide on phosphorus dynamics and bioavailability in pasture soils

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Driss Touhami

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

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Nutrient inputs and climatic conditions can significantly impact soil phosphorus (P) bioavailability and dynamics by influencing chemical, biological, and biochemical processes. This research aimed at investigating the effects of nitrogen (N) and P inputs and elevated atmospheric carbon dioxide (CO_2) on soil P bioavailability and dynamics under New Zealand pasture soils. Inorganic P (Pi) and organic P (Po) fractions, together with biological and biochemical properties linked to P cycling, were assessed. In P-deficient soil (4 mg kg^{-1} Olsen P) with high organic matter content (7.2 %), P addition alone (45 kg P ha^{-1}) or in combination with N (45 kg P ha^{-1} + 200 kg N ha^{-1}) increased plant growth, total P and N uptake, microbial biomass P, organic anion release, and the depletion of moderately labile Pi and stable Po, whereas N addition alone (200 kg N ha^{-1}) had no impact on plant growth and soil P fractions under legumes (*Lupinus angustifolius* and *Trifolium repens* L.) and grasses (*Lolium perenne* L. and *Triticum aestivum* L.). In N-limited soil with an Olsen P of 10 mg kg^{-1}, N addition alone (250 kg N ha^{-1}) or in combination with P (250 kg N ha^{-1} + 50 kg ha^{-1}) increased shoot biomass and shoot P uptake of Italian ryegrass (*Lolium multiflorum* Lam.) by an average of 1.6-fold compared to the control. In contrast, P addition alone (50 kg P ha^{-1}) had no impact on shoot biomass and shoot N uptake but significantly increased Olsen P and shoot P uptake. Acid and alkaline phosphatase activities increased in summer, especially under N addition treatments, which was attributed to increased plant P demand, alleviation of carbon limitation, and higher temperature. Nitrogen addition alone decreased readily available Pi, labile Pi, labile Po, and moderately labile Pi by 55, 19, 28, and 7 %, respectively, compared to the control treatment. On the other hand, the combined addition of N and P promoted labile Po mineralisation (28 %) and the depletion of labile P fractions but did not mobilise the moderately labile Pi pool compared to P addition alone. In a long-term P fertilisation experiment (0, 188, and 376 kg superphosphate ha^{-1} yr^{-1} for more than 65 years) under grazed pastures, soil P availability increased with increasing P inputs, but microbial biomass P was similar under 188 and 376 kg superphosphate ha^{-1} yr^{-1}. The latter result was attributed to microbial

biomass need to maintain nutrient stoichiometry. Alkaline phosphatase activity increased in response to long-term P inputs, whereas acid phosphatase activity decreased. This finding supported the differentiation in origin and nutrient demand between acid and alkaline phosphatase enzymes, where acid phosphatase enzymes are released from plant roots and inhibited by high P availability, whereas alkaline phosphatase enzymes are derived from soil microbes and driven by C availability rather than P. In P-deficient soil (7 mg kg^{-1} Olsen P), plant biomass, P uptake, and rhizosphere properties did not respond to elevated CO_2 (700 ppm) but were significantly impacted by plant species (*Lupinus angustifolius*, *Lolium perenne* L., and *Triticum aestivum* L.). Hence, soil P availability and P fractions distribution were similar under ambient and elevated CO_2 due to the negative feedback of P deficiency on photosynthesis. Twenty-two years of CO_2 enrichment (500 ppm) under grazed pasture decreased labile and moderately labile Pi by 39 and 15 %, respectively, while promoting the accumulation of moderately labile and stable Po by 26 and 17 %, respectively, compared to ambient CO_2. Decrease in soil Pi was attributed to plant P uptake and immobilisation of Pi in the microbial biomass. Accumulation of Po was linked to enhanced biological activity, increased inputs of Po from root detritus, and adsorption onto reactive mineral surfaces of aluminium, iron, and calcium. Plant species investigated in this study and their associated rhizosphere microbes were able to mobilise sparingly available and recalcitrant soil P fractions. This result challenges the recalcitrance of some soil P fractions, which is defined by resistance to extraction and clearly not to action by plants or microbes. Legumes, especially blue lupin, have shown higher ability in releasing organic anions and phosphatase enzymes, which concurred with higher mineralisation of organic P. Under real soil conditions, organic anions played a minor role in Pi acquisition under both legumes and grasses. Nutrient addition experiments and long-term studies revealed that besides P, C and N availability played a critical role in P dynamics and cycling by impacting microbial P transformations and phosphatase enzyme activity. Applications of both N and P in excess of plant requirements cannot accelerate soil P cycling but, in the contrary, can cause soil P accumulation and eutrophication of waterways. Phosphorus deficiency can limit plant ability to grow and acquire P under elevated CO_2 conditions. Elevated CO_2 can decrease soil P availability under permanent agroecosystems characterized by organic matter accumulation and high availability of free cations (Al, Fe and Ca). Further research is needed to evaluate changes in soil P fractions in response to N and P inputs under N and P co-limited soils. Investigation of the combined effects of elevated CO_2, increased temperature, and water stress on soil P availability and dynamics is warranted.

Keywords: phosphorus availability, long-term phosphorus fertilisation, nitrogen fertilisation, elevated atmospheric CO_2, FACE experiment, rhizosphere, soil phosphorus depletion, microbial phosphorus immobilisation, organic phosphorus accumulation, soil phosphorus fractionation, organic

anions, phosphatase enzyme activity, soil microbial biomass phosphorus, long-term experiments, pasture soil, grazed pasture, *Lupinus angustifolius*, *Trifolium repens* L., *Lolium perenne* L., *Lolium multiflorum* Lam., *Triticum aestivum* L.

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Chapter 1

Introduction

1.1 Background

1.1.1 Phosphorus facts

Phosphorus (P) was first discovered in 1669 by the German alchemist Henning Brand (Butusov and Jernelöv 2013), and since then, it has attracted considerable interest worldwide. Phosphorus has defined the origin of life and the evolution of species because it is a component of fundamental molecules in all living organisms. From DNA to ATP, P is involved in a plethora of biological processes, such as replication of genes, formation of cell membranes, and transfer of energy (Raghothama 2005; Butusov and Jernelöv 2013). Essential biochemical mechanisms are governed by P, including photosynthesis, respiration, enzymatic transfer, microbial turnover, and organic matter degradation (Cole et al. 1977b; Raghothama 1999; Raghothama and Karthikeyan 2005). One of the most important roles of P resides in agricultural systems, where it significantly impacts crop growth and plant metabolism (Fageria et al. 2017). In fact, sufficient P supply is required for root development, seed quality, and crop strength (Finkl and Simonson 1979).

Despite being the 11th most abundant element on the earth (Tiessen 2008), P is the second most limiting nutrient after nitrogen (N), which means that production systems are highly dependent on this element (Vassilev et al. 2006; Fageria et al. 2017). Total P may be high in soils, but only a limited amount is available for plant uptake (Figure 1.1), with estimates ranging from 1 to 11 % of total P (Pierzynski 1991; Menezes-Blackburn et al. 2018). Adsorption and immobilisation of P into active mineral surfaces (aluminium (Al), iron (Fe), calcium (Ca), clays), organic molecules, and microbial biomass are the main mechanisms decreasing soil P availability in terrestrial ecosystems (Richardson 1994; Rengel 2001; Hinsinger 2001; Pierzynski and McDowell 2005; Condron et al. 2005). Therefore, high amounts of P fertilisers have been applied to agroecosystems to maintain and increase crop productivity (Condron 2003; Frossard et al. 2009; Haygarth et al. 2013). This, in turn, has caused an accumulation of soil P and an impairment of surface water quality. (Sharpley et al. 2001; Bünemann and Condron 2007; Mekonnen and Hoekstra 2018; McDowell et al. 2020). In contrast, P deficiency still exists in many regions of the world (Jaramillo-Velastegui 2011; MacDonald et al. 2011), especially in Africa, Latin America, and parts of Asia, suffering from high fertiliser prices and low input agroecosystems (Vitousek et al. 2009; Lynch 2011; Velde et al. 2014).

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Figure 1.1 Map of global soil P availability (Jaramillo-Velastegui 2011).

The U.S. Geological Survey (2011) reported that worldwide phosphate production peaked at 176 million megatonnes, and world reserves reached 65 billion megatonnes in 2010. A few countries possess 95 % of the total phosphate reserves, including Morocco, followed by China, Algeria, Syria, Jordan, South Africa, and the United States (Fageria et al. 2017). There has been some disagreement regarding the worldwide phosphate reserves and production. Cordell et al. (2009) claimed that the peak of phosphate production would be reached around 2030, and world reserves might be depleted after one century in the best-case scenario. On the other hand, the International Fertiliser Development Centre estimates that at the current production level and the actual costs and technologies can supply phosphate for more than 300 years (Fageria et al. 2017).

Phosphorus fertilisers are exclusively driven from rock phosphate, which is a finite resource, and P fertilisers prices can be subject to high volatility (Cordell et al. 2009; Cordell and White 2015). Therefore, enhancing P use efficiency has become a cornerstone to sustain P utilisation and food security worldwide (Tilman et al. 2002; Richardson and Simpson 2011; Fageria et al. 2017; Ros et al. 2020). This objective cannot be achieved without a profound understanding of the critical mechanisms and factors driving soil P bioavailability and dynamics in agroecosystems (Frossard et al. 2000; Condron 2003; Pierzynski and McDowell 2005).

1.1.2 Phosphorus and plant species

Plant species have evolved in harsh environments, such as those with soil P deficiency by developing a myriad of chemical, biological, biochemical, and morphological mechanisms to increase their P uptake, especially at the soil-plant interface (Raghothama and Karthikeyan 2005; Richardson et al. 2009; Hinsinger et al. 2009, 2011; Shen et al. 2011). Soil acidification and exudation of organic anions and phosphatases enzymes are deemed the most important processes taking place in the rhizosphere of P deficient plants (Tabatabai 1994; Hinsinger 2001; Vance et al. 2003; Jones and Oburger 2011; Nannipieri et al. 2011). Similar mechanisms have been used by root-associated microorganisms to enhance P availability (Hinsinger et al. 2003; Nannipieri et al. 2011; Richardson and Simpson 2011; Panhwar et al. 2014; Mimmo et al. 2018). Changes in root architecture and morphology represent one of the key mechanisms used by plants to enhance P uptake (Raghothama 1999; Singh Gahoonia and Nielsen 2004; Lynch and Brown 2008; Péret et al. 2011),while symbiosis with mycorrhizae fungi is an essential plant adaptation strategy under P deficiency to expand the soil volume explored by plant's roots (Marschner 1995; Pandey et al. 2005; Nadeem et al. 2014; Watts-Williams et al. 2019; Nguyen et al. 2019).

Previous studies have shown that physiological and morphological adaptations to P deficiency are plant-specific. For instance, legumes and forest trees have been found to access more sparingly available P fractions compared to cereal crops due to higher release of phosphatase enzymes and organic anions (Kamh et al. 1999; Chen et al. 2003b, 2004; Scott and Condron 2003; Nuruzzaman et al. 2005; Mat Hassan et al. 2012, 2013). On the other hand, cereals adapt their root morphology and architecture to explore more soil volume, produce more shallow roots to target P fertiliser applied, and invest in symbiotic relationships with mycorrhizae to efficiently uptake soil P (Chevalier et al. 2003; Singh Gahoonia and Nielsen 2004; Wang et al. 2010b; Niu et al. 2013b; Pandey et al. 2015b). Nevertheless, some plant species have been found to use both morphological and physiological root traits to enhance their P acquisition (Pang et al. 2010, 2018b; Pandey et al. 2018; Lal et al. 2019).

1.1.3 Phosphorus and nutrient inputs

Nitrogen and P are co-limiting primary productivity in most terrestrial ecosystems (Elser et al. 2007; Vitousek et al. 2010; Harpole et al. 2011; Du et al. 2020). Consequently, N and P fertilisers are commonly applied to increase the productivity of agroecosystems (Tilman et al. 2002; Haygarth et al. 2013). However, excess inputs of N and P fertilisers have raised many concerns about nitrate leaching, build-up of soil P legacy, and eutrophication of water bodies (Carpenter et al. 1998; Sharpley et al. 2001; Bouwman et al. 2017). Furthermore, imbalanced P and N fertilisation can have significant impacts on soil ecosystem services, plant community composition, and microbial diversity (Jouany et al. 2011; Peñuelas et al. 2013; Carnicer et al. 2015). Therefore, a deeper understanding of

the processes governing soil P dynamics and bioavailability under nutrient inputs is required for efficient management of agroecosystems, aiming to increase productivity, minimise fertilisation footprints, and preserve dwindling rock phosphate reserves.

The main arable crop in New Zealand is wheat, which is predominantly grown in the Canterbury Plains (Stats NZ 2017), while ryegrass and white clover make up the grass-legume mixture representing the building block of New Zealand pasture systems (Haynes 1980; Chapman et al. 1996, 2017; Lee et al. 2012). New Zealand pasture systems have been designed in the way that white clover provides N to the pasture mixture (Chapman et al. 1996, 2017). However, P requirements of white clover are high because P is critical for N fixation (Peoples et al. 1998; Sprent 1999; Lam et al. 2012). Therefore, P fertilisers are needed to maintain adequate clover content and supply enough N to ryegrass (Haynes and Ludecke 1981; Jouany et al. 2011). This has been demonstrated in different long-term experiments (Tate et al. 1991), including the Winchmore fertiliser trial in New Zealand (Fraser et al. 2011).

Pastures represent important ecosystems providing more than one-third of global net primary productivity and sustaining the livelihood of approximately one-ninth of the world's population (Hoekstra et al. 2005; Hynd 2019). In New Zealand, pasture systems are the main land use, comprising grazed beef, sheep, deer, and dairy farms. The last 25 years have seen a massive conversion from beef and sheep farms to dairy due to low land prices, adoption of spray irrigation, and more importantly, higher profitability of dairy products (Dynes et al. 2010; McDowell et al. 2017). Indeed, the number of milking cows in New Zealand increased by more than 1.7-fold between 1995 (2.9 Million) and 2015 (5 Million) (McDowell et al. 2017), while the contribution of dairy farming reached 26 % of total New Zealand exports in 2015/2016 (DairyNZ 2016). This land-use change has driven an increase in nutrient inputs to maximise pasture production and enhance the quality of animal feed (McDowell et al. 2017; Stats NZ 2019). Wheeler et al. (2004) analysed the data of Olsen P concentrations spanning different regions and land uses in New Zealand between 1989 and 2001. They found that dairy farms had higher Olsen P concentrations compared to other land uses and that these concentrations were above optimal agronomic pasture production. Recent findings of McDowell et al. (2020) confirmed this increased trend in Olsen P between 2001 and 2015, mainly under dairy pumice and peat soils in the North Island. Besides P, N inputs have been increasingly applied to New Zealand pasture soils (Parfitt et al. 2012; Pinxterhuis and Edwards 2018). In fact, N inputs in New Zealand increased by more than 6-fold between 1990 and 2015, mainly as urea fertiliser (Stats NZ 2019). McDowell et al. (2017) found that N and P losses to water in New Zealand grazed pastures ranged from 22 to 79 kg $ha^{-1}year^{-1}$ and 0.5 to 4.2 kg $ha^{-1}year^{-1}$, respectively. This represented, on average, 29 times higher N losses than P in grazed pasture systems. Scott et al. (2015) highlighted that soil P status plays a crucial role in limiting N losses under pasture systems. In

their lysimeter study, they found that N leaching was higher under low P fertility pasture soils due to lower plant biomass being able to take up N applied. This emphasised the importance of balanced N and P applications to increase pasture productivity and limit nutrient losses. Nevertheless, the implementation of fertiliser management strategies has to take into consideration some other factors, such as animal grazing, seasons, plant community, and topography (Gillingham et al. 2008; Parfitt et al. 2009; Gillingham 2016).

1.1.4 Phosphorus and climate change

By 2050 world population will reach 9 billion, and the demand for food will peak at its highest level, putting food security at the top of human needs (FAO 2001; Godfray et al. 2010). To meet food security, an increase in crop production is needed (Lynch 2007; Fageria et al. 2017), which requires intensive use of N, P, and potassium fertilisers (Tilman et al. 2002). This situation will challenge the productivity and sustainability of agriculture systems.

Climate change can affect plant production through increased concentrations of atmospheric CO_2, temperature, and more extreme rainfall and drought events (Lobell et al. 2011; IPCC 2014). Elevated CO_2 levels are projected to increase plant productivity by 1.8 % per decade, thereby increasing plant nutrient demand, including P (Lobell and Gourdji 2012; Pang et al. 2018b; Bhattacharya 2019). This, in turn, will increase the pressure on phosphate reserves to produce more P fertilisers along with accelerating soil P depletion in several parts of the world already suffering from P-limitation. This context is relevant to New Zealand agriculture because most of the soil P is adsorbed to Al and Fe oxides and present in organic form (Condron and Goh 1989; Maher and Thorrold 1989; Perrott and Mansell 1989), and grazed pastures are highly dependent on P fertilisers to produce high-quality feed for animals. Therefore, an understanding of P acquisition strategies used by different plant species grown in New Zealand is a prerequisite to ensure more adaptable agriculture for future climate change. Additionally, knowledge about how New Zealand grazed pasture systems react to increased elevated CO_2, especially in terms of P availability and dynamics, is critical to find the best solutions to mitigate climate change impacts on pasture productivity and quality.

1.2 Literature review

1.2.1 Phosphorus cycling and dynamics in agroecosystems

Phosphorus is completely derived from apatite (Larsen 1967; Pierzynski and McDowell 2005; Vitousek et al. 2010), the dissolution of which can lead to the formation of P secondary minerals (Walker and Syers 1976). In agricultural ecosystems, P is removed via plants and animal products and replenished using water-soluble fertilisers, reactive phosphate rock, manure, or animal excreta (Condron et al. 2005; Frossard et al. 2009; Vitousek et al. 2009; Haygarth et al. 2013). The losses of P

by runoff, leaching, or erosion are also an essential component of the P cycle, which lead to eutrophication and water pollution (Sharpley 1995; Daniel et al. 1998; Watson and Foy 2001; McDowell et al. 2003) (Figure 1.2). Phosphorus dynamics are governed by a complex interaction between chemical, biological, and physical processes taking place in soils (Condron and Tiessen 2005). Besides soil processes, plant and rhizosphere processes play a central role in P cycling (Richardson 1994; Pierzynski and McDowell 2005; Shen et al. 2011).

Figure 1.2 Phosphorus dynamics in the soil-rhizosphere-plant continuum. C-P, Carbon P; NO, nitric oxide; OA, organic acids (Shen et al. 2011).

An understanding of soil P dynamics as affected by biotic and abiotic factors requires first an assessment of different P forms present in the soil. In this regard, sequential P fractionation methods have been commonly used to determine inorganic, organic, and microbial P pools. Based on chemical bonds existing between P and different soil compounds (soil minerals and organic molecules), soil P is divided into operationally defined inorganic and organic P fractions. The most common P

fractionation procedures were developed by Chang and Jackson (1957) and Hedley et al. (1982). The first method extracts different inorganic P fractions attached to Al, Fe, and Ca, while the second method represents a more detailed (10 fractions) assessment of inorganic, organic, and microbial P fractions. These methods have been refined over time to match the specific objectives of each study (Condron and Newman 2011). Based on the procedure of Condron et al. (1996) and its modification by Chen et al. (2002), soil P is sequentially extracted with ammonium chloride salt (1 M NH_4Cl) to extract loosely bound inorganic P, by sodium bicarbonate (0.5 M $NaHCO_3$, pH : 8.5) and the first sodium hydroxide (0.1 M NaOH) to determine inorganic and organic P attached to Al and Fe oxides, by hydrochloric acid (1 M HCL) to obtain inorganic P bound to calcium, by the second sodium hydroxide (0.1 M NaOH) to extract inorganic and organic P firmly attached to Al and Fe oxides and incorporated into soil microaggregates. The last fraction, called residual P, is digested by strong acids such as perchloric-nitric or sulfuric acids at high temperatures (Olsen and Sommers 1982; Chen et al. 2003c) to extract remaining inorganic and organic P stabilised by oxyhydroxides of Fe and Al and organic carbon (C) (Velásquez et al. 2016). It is important to reiterate that P fractionation determines operationally defined P fractions according to their chemical but not biological availability. For instance, residual P is considered to be unavailable for plant uptake (Condron and Newman 2011). However, it has been shown that this fraction can be depleted in the rhizosphere of different plant species such as *Pinus radiata* (Chen et al. 2002), rape (Gahoonia and Nielsen 1992), and white lupin (Kamh et al. 1999; Mat Hassan et al. 2012), under intensive cropping systems (Jin et al. 2017), and by forest trees subjected to elevated CO_2 conditions and high N inputs (Huang et al. 2014).

Inorganic phosphorus

Different chemical forms of P exist with diverse behaviours and contributions to the total soil P pool (300 to 1000 mg/kg) (Magid et al. 1996; Frossard et al. 2000). Inorganic P contributes between 35 to 70 % of total soil P (Harrison 1987), mainly as orthophosphates (Bünemann and Condron 2007). The main mechanisms governing soil inorganic P cycling and behaviour are sorption-desorption and precipitation-dissolution (Barrow 1983; Pierzynski 1991; Frossard et al. 1995). Inorganic P in the soil can be adsorbed to Al and Fe oxides and become non-available for plants, especially in acidic and highly weathered soils (Pierzynski and McDowell 2005; Gérard 2016). Inorganic P can be further absorbed if firmly attached to the soil and becomes occluded if coated with another layer of Al and Fe oxides or incorporated into soil aggregates (Arai and Sparks 2007; Condron and Newman 2011). In calcareous soils, inorganic P is bound to calcium and could precipitate in the form of apatite in the presence of free calcium carbonates and high soil pH (Frossard et al. 1995; Hinsinger et al. 2008). Inorganic P is present in the soil solution in equilibrium with P adsorbed to organic molecules and soil solid phase. Menezes-Blackburn et al. (2018) found in their meta-data analysis that, on average, only 6 % of total P is available as inorganic P to plants in agroecosystems. Therefore, desorption of

sparingly available inorganic P, mineralisation of organic P, and supply from plant detritus and microbial necromass can occur to supply the available inorganic P pool when the latter is depleted from the soil solution (Cole et al. 1977b; Buresh et al. 1997; Pierzynski and McDowell 2005; Nannipieri et al. 2011; Dinh et al. 2017).

Organic phosphorus

Organic P is described as the P bound to the C chain, mainly as C-O-P (Condron et al. 2005), and represents between 20 to 80 % of the total soil P (Dalal 1977; Magid et al. 1996), depending on location, soil type, and land use (Nash et al. 2014). In terrestrial ecosystems, organic inputs such as plant roots, organic matter, animal excreta, and microbial residues enhance organic P stocks in soils (Condron and Tiessen 2005). Organic P in soils is mainly present as orthophosphate esters, phosphonates, and anhydrides (Condron et al. 2005).

In P-deficient soils, it has been shown that plants and microorganisms release extracellular enzymes to mineralise organic P (Dalal 1977; Fox and Comerford 1992; Richardson 1994; Tabatabai 1994; Richardson and Hadobas 1997; Badalucco and Nannipieri 2007; Hill et al. 2007; Nannipieri et al. 2011). The release of organic anions and especially Low Molecular Weight Organic anions (LMWOA), defined as aromatic and aliphatic carboxylic acids with a maximum molecular weight of 300 Dalton, can also contribute to the mobilisation of organic P via the dissolution of sparingly available organic forms (Wang et al. 2017b; Hou et al. 2018), or the desorption of organic P from soil particles (Lambers et al. 2015). Interestingly, Clarholm et al. (2015) proposed that the mineralisation of organic P is a three-step mechanism involving organic anions and phosphatase enzymes acting in series. In fact, Giles et al. (2018b) and Giles et al. (2017) demonstrated that the exudation of both citrate and phytases from intermingled tobacco plant roots was responsible for the depletion of organic P and simultaneously increased available inorganic P, especially under P limited conditions.

Apart from the mechanisms mentioned above, the mineralisation of organic P is governed by other factors such as organic P chemical nature, soil characteristics, environmental conditions, and land use (Hedley et al. 1982; Magid et al. 1996; Condron and Tiessen 2005; Nash et al. 2014). For example, inositol hexaphosphate accumulated differently among British soils (forests and temperate grasslands) with contrasting organic matter and Al and Fe oxides (Stutter et al. 2015). Scott and Condron (2005) found that soils with lower C content exhibited higher organic P mineralisation under different plant species, including trees and pasture crops. The need for C by soil microbes can increase the priming effect on soil organic matter, thereby increasing organic P mineralisation (Tian et al. 2016). A long-term experiment carried out by Annaheim et al. (2015) showed a non-significant effect of organic fertilisation on soil organic P, suggesting that organic P was influenced by other factors such as mineralogy, environment, and vegetation. The experiment carried out by Chen et al.

(2003a) showed that organic P was depleted in periods characterised by high plant demand and high microbial activity, while it tended to accumulate in periods with high organic inputs, slow plant growth, and low soil microbial activity. Condron et al. (1990) found that most organic P was present as monoesters in cultivated soils in Canada, whereas soils under grasslands had higher proportions of diesters and teichoic acid P. This indicated that cultivation promoted the mobilisation of more complex but readily mineralisable organic structures due to their lower adsorption to the soil solid phase (Magid et al. 1996). Trees are able to access organic P more efficiently than grass and legumes species (Scott and Condron 2003, 2005). Higher release of soluble C and exudation of root phosphatase are the main factors explaining this higher aptitude of forest trees in mineralising organic P (Chen et al. 2003a, 2004).

Organic P, especially monoesters, can suffer from adsorption to Al and Fe oxides, thereby being less available for microorganisms degradation pathways (Magid et al. 1996; Martin et al. 2004; Berg and Joern 2006; Menezes-Blackburn et al. 2018). Soil microorganisms also represent a sink of organic P that can become available to plants as orthophosphates after microbial turnover (Richardson 2001; Jakobsen et al. 2005; Bünemann and Condron 2007). A recent meta-analysis carried out by Menezes-Blackburn et al. (2018) showed that organic P in the form of monoesters represents between 30 to 35 % of total P in agriculture soils, stressing the huge potential of using organic P to sustain agriculture should innovations in fertilisers, plant breeding, and management practices be implemented.

Phosphorus nuclear magnetic resonance spectroscopy (NMR) is one of the most common methods for assessing the chemical structure of organic P species present in the soil, such as orthophosphate monoesters and diesters, phosphonates, pyrophosphates, and polyphosphates (Condron et al. 2005; Turner et al. 2005; Cade-Menun 2017). The use of this technique in chrono-sequences has advanced our knowledge of organic P transformations over time (Turner et al. 2013). Using ^{31}P NMR under different land-uses revealed that arable cropping systems enhanced organic P mineralisation and the breakdown of more complicated structures such as polyphosphates and orthophosphate diesters to more recalcitrant and stable monoesters (Condron et al. 1990). Monoesters have been found to account for the majority of organic P species in grassland soils in New Zealand and United Kingdom due to their higher adsorption to the soil solid phase, whereas diesters have been suggested to be involved in microbial biomass and biological P cycling (Turner et al. 2003; McDowell et al. 2005).

Microbial phosphorus

Microorganisms compete with plants for nutrients (Marschner et al. 2011), and they also play an essential role in P cycling due to an interplay between mineralisation and immobilisation processes (Dalal 1977; Mackey and Paytan 2009; Richardson et al. 2009; Achat et al. 2010; Richardson and

Simpson 2011; Alegria Terrazas et al. 2016; Spohn and Widdig 2017). For instance, microbial P accounted for up to 78 % of total biomass P in a 120,000-year chromosphere, being the most important biotic P source over 100,000 years of ecosystem development (Turner et al. 2013). Microbial biomass P represented between 5 and 24 % of total P in pasture soils in England (Brookes et al. 1984), while it ranged between 10 to 57 kg ha^{-1} under established pastures in New Zealand (Perrott and Sarathchandra 1989). In the bulk soil, microbial biomass P can represent 2 to 10 % of total P but may exceed 50 % in the rich organic soils as well as in the rhizosphere (Oberson and Joner 2005; Achat et al. 2010). Tamburini et al. (2012) showed for the first time using ^{18}O isotopes that soil microbes are the most important component driving soil P cycling in a 150-year soil chronosequence of a glacier forefield. Accumulated as phospholipids, DNA/RNA, ATP, inositol phosphates, and polyphosphates, microbial P can be released as a result of different stresses, including drying and rewetting, nutrient deficiency, and bacterial or fungal predation (Cole et al. 1977a; Turner and Haygarth 2001; Cheng 2009; Blackwell et al. 2010; Butterly et al. 2011; Irshad et al. 2011; Dinh et al. 2017; Xu et al. 2020a). Through annual turnover, microbial biomass P can contribute as much as 100 mg P kg^{-1} soil yr^{-1} in managed ecosystems, emphasising its central role in supplying available P to plants (Oberson and Joner 2005; Richardson and Simpson 2011).

Microbial biomass P is affected by soil nutrient availability. In P-deficient soils, the addition of C, N, and P has been found to increase microbial biomass, which was attributed to a shift in the microbial community composition (Heuck et al. 2015). Spohn and Kuzyakov (2013) carried out an experiment to study the microbial kinetics of ^{14}C and ^{33}P from glucose-6-phosphate in a Leptosol. They found that microbial biomass incorporated 3-fold more C than P when supplied with an organic P source, suggesting that microorganisms were using organic P mineralisation as a means of C-acquisition. Indeed, the growth of soil microbes is primarily regulated by C availability (energy) (Griffiths et al. 2012; Heuck et al. 2015). Another study under elevated CO_2 conditions showed that microbial community immobilised P in the rhizosphere of wheat in response to higher C inputs under elevated CO_2 (Jin et al. 2014). Spohn and Widdig (2017) observed that P turnover in the microbial biomass increased with P scarcity in the soil solution. This result means that the microbial community tends to immobilise P by optimising its internal P when the environment becomes P-deficient. However, other studies have shown that P addition promoted microbial P immobilisation to maintain microbial stoichiometry (Tate et al. 1991; Dodd et al. 2014; Ikoyi et al. 2018; Shi et al. 2020). The response of microbial biomass P to N addition is not clear. In their meta-analysis, Deng et al. (2017) found that N addition had no impact on microbial biomass P, whereas microbial biomass P decreased in response to long-term N addition in larch trees (Yang et al. 2015). An overall decrease in soil microbial biomass has been reported by different studies after long-term N application, which was linked to a decrease in soil pH (Treseder 2008; Hu et al. 2010; Wang et al. 2015a; Tian et al. 2016).

1.2.2 Processes controlling phosphorus availability and dynamics

Root architecture and morphology

Under P deficient conditions, plants translocate more C assimilates to the root system to increase soil P uptake. Therefore, changes in root morphology and architecture are observed in plants suffering from P deficiency (Lynch 2007, 2011). This strategy helps plants to increase root surface area by producing longer and finer roots and promoting symbiotic relationships with mycorrhizal fungi. For instance, *Zea mays* and *Triticum aestivum* exhibited higher root/shoot biomass ratio, while *Brassica napus* had higher specific root length when grown under low P conditions (Lyu et al. 2016). Investing in longer root hairs was used by P efficient cultivars of cowpea and barley to increase their P uptake (Gahoonia et al. 2001; Krasilnikoff et al. 2003). Under other crops such as common bean and soybean, shallow root growth angles have been described as a more efficient P foraging strategy (Wang et al. 2010b; Lynch 2011). Some Proteaceae species, such as white lupin, can develop bottlebrush-like structures along their root system to thrive under P deprived soils, mainly by releasing organic anions (Lambers 2006; Lambers et al. 2015).

Chemical processes

The rhizosphere represents one of the most dynamic environments where plant roots are in continual interaction with a myriad of soil microorganisms (Philippot et al. 2013). This small volume of soil surrounding plant roots has been demonstrated to sustain life on earth due to its impact on nutrient cycling, plant growth, and plant health (Marschner 1995; Dazzo and Ganter 2009; Hinsinger et al. 2009; Neumann and Römheld 2012; Mimmo et al. 2018). Phosphorus suffers from its slow mobility, low solubility in the soil, and its adsorption to the soil solid phase. Hence, soil P dynamics have been described to be more pronounced under the rhizosphere of plants where a plethora of chemical, biological, and biochemical processes take place to enhance P bioavailability and acquisition by plants (Hinsinger et al. 2009; Marschner et al. 2011; Neumann and Römheld 2012).

Phosphorus deficient plants can modify soil chemistry by changing ionic concentration and soil pH as well as releasing organic anions to increase P availability, especially at the soil-plant interface (Hinsinger 2001; Gregory 2006). Numerous experiments have shown that the depletion of inorganic P was coupled with a decrease in soil pH (Marschner 1995; Chen et al. 2002; Villegas and Fortin 2002; Oburger et al. 2011). This soil P depletion was caused by a shift in the adsorption-desorption and dissolution-precipitation equilibria (Hinsinger 1999). Nitrogen fertilisation improves crops P nutrition via the acidification of the soil, especially using ammonium forms (Nye 1981; Marschner 1995; Gregory 2006). Marschner and Römheld (1983) reported that soil P acidification was related to plant species and N inputs. Indeed, Hinsinger (2001) showed that buckwheat, pigeon pea, and lupin released protons to dissolve rock phosphate, thereby enhancing P bioavailability. The release of

organic anions from roots and microorganisms has been shown to influence rhizosphere pH via the modification of the acid-cation balance (Wallander 2000; Hinsinger et al. 2003; Casarin et al. 2004). The rhizosphere also represents a hotspot of CO_2 release and O_2 consumption carried out by roots and soil microorganisms (Bidel et al. 2000). Thus, root and microbial respiration contribute to rhizosphere acidification (Hinsinger et al. 2003), especially in calcareous soils where P is bound to calcium cations (Nye 1981).

Rhizodeposition has been identified as a C transfer from plant roots to rhizosphere soil (Nguyen 2009). Plants release between 20 to 60 % of their photosynthetic C below-ground (Grayston et al. 1996; Kuzyakov and Domanski 2000; Nguyen 2009; Mimmo et al. 2018). The main organic molecules released in the rhizosphere are lysates, secretions (enzymes) and exudates (organic anions, amino acids, and sugars) and are governed by plant species, environmental factors, and nutrient deficiencies (Neumann 2007). Organic anions, especially LMWOA, have been found to contribute significantly to soil ecology as well as plant nutrition and productivity (Grayston et al. 1996; Gregory 2006; Mimmo et al. 2018). Organic anions have various origins but mainly come from plant roots, microorganisms, and organic matter degradation (Adeleke et al. 2017; Oburger and Jones 2018). Acidification, chelation, and exchange reactions are the principal mechanisms by which organic anions dissolve insoluble minerals in soils (Bolan et al. 1994; Omar 1997; Adeleke et al. 2012).

The release of organic anions has been documented across a wide range of plant species. Particularly in P deficiency, citrate, oxalate, malate, malonate, acetate, fumarate, succinate, and lactate have been reported as the common LMWOA released in the rhizosphere (Hoffland et al. 1989; Bolan et al. 1994; Dinkelaker et al. 1995; Jones 1998; Veneklaas et al. 2003; Rengel and Marschner 2005; Shi et al. 2011; Gerke 2015). The release of these organic anions has been shown to increase P availability several folds with higher P mobilisation efficiency for citric acid followed by malic, oxalic, and acetic acids (Bolan et al. 1994; Jones 1998; Gang et al. 2012; Chen et al. 2013; Gerke 2015; Hou et al. 2018). The effectiveness of organic anions is governed by their concentration and configuration in the soil (Khademi et al. 2010). For instance, organic acid concentration in the rhizosphere varies between 1 to 100 µM and can reach 10 mM for proteoid plants such as white lupin (Jones 1998; Strobel 2001). Additionally, citric acid, a tribasic acid, increases P solubilisation more than oxalic acid, a dibasic acid characterised by its rapid degradation (Bolan et al. 1994; Clarholm et al. 2015).

Being a rich source of C, organic anions can be degraded by soil microbes in need of energy (Marschner et al. 2011; Menezes-Blackburn et al. 2016). Organic anions can also sorb onto the solid phase due to their negative charges or complexed by cations such as Al^{3+}. This adsorption and complexation can reduce the ability of organic anions to solubilise P (Jones 1998); meanwhile, it protects them from rapid microbial degradation (van Hees et al. 2003). Such adsorption and

degradation mechanisms complicate the understanding of the role of organic anions and their contribution to P solubilisation (Jones 1998; Marschner 2012). Moreover, the majority of studies on organic anions have been carried out using hydroponic culture systems or substrates suppressing the effects of soil particles and microorganisms (Groleau-Renaud et al. 1998; Jones 1998; Wang et al. 2015c; Valentinuzzi et al. 2015; Oburger and Schmidt 2016; Oburger and Jones 2018) and were run only for short-term (Wang et al. 2017b). Therefore, Wang et al. (2017b) and Pang et al. (2018a) recommended that more studies be carried out under field conditions with different plant species and growth stages to advance our understanding of the role of organic anions in soil P acquisition. Another challenge in the study of organic anions resides in the difficulty to extract and quantify them. Oburger and Jones (2018) recently reviewed the limitations of different sampling and analytical methods used in the study of root exudates, including organic anions, and provided guidance on the choice of adequate experimental approaches.

Biological processes

Microorganisms contribute significantly to the P cycle by influencing the availability of this nutrient in the soil solution (Richardson and Simpson 2011). Soil microorganisms are involved in many mechanisms that mediate P availability, such as the increase of growth and extension of the root system (Pandey et al. 2005) and the induction of physiological mechanisms to solubilise inorganic P and mineralise organic P (Richardson et al. 2011).

According to Kucey (1983), more than 40 % of soil microorganisms are P solubilising microorganisms. Bacteria and fungi are among numerous free-living microorganisms that have been described as being able to solubilise sparingly available P and mineralise organic P (George et al. 2007; Jorquera et al. 2008). The most common P solubilising bacteria are *Pseudomonas* spp. and *Bacillus* spp., while the most representative genera inside the fungi kingdom are *Aspergillus* and *Penicillium* (Richardson and Hadobas 1997; Richardson 2001). Moreover, bacteria and fungi can mobilise soil P via the release of protons, organic anions, siderophores, and phosphatase enzymes (Richardson 2001; Jakobsen et al. 2005; Richardson et al. 2011). Excess of P mobilised by soil microbes can become available for plant uptake (Mehta and Nautiyal 2001).

In addition to bacteria and fungi, symbiotic associations can have a significant role in P uptake and availability. Arbuscular mycorrhizae fungi (AMF) have been shown to be the most abundant endomycorrhizal association that significantly contributes to the P uptake by plants (Pepper and Bezdicek 1990; Peterson and Farquhar 1996; Marschner 2012). Hyphae represent the structures responsible for P absorption and P depletion from the rhizosphere (Pepper and Bezdicek 1990; Li et al. 1991; Marschner 2012). To be more physiologically efficient in absorbing P, hyphae help roots to have access to a larger volume of soil (Richardson et al. 2009; Marschner 2012). Extraradical hyphae

contribute also to the mobilisation of soil organic P via the release of acid phosphatase enzymes (Tarafdar and Marschner 1994; Koide and Kabir 2000; Sato et al. 2015) and potentially to inorganic P solubilisation through the exudation of organic anions (Klugh and Cumming 2007; Klugh-Stewart and Cumming 2009).

Despite the significant contribution to P mobilisation, soil microorganisms may decrease P availability to plants via different mechanisms (Jakobsen et al. 2005) such as immobilisation of P in microbial biomass, degradation of organic anions and phosphatase enzymes, inhibition of root growth and mycorrhizal extension, and consumption of protons leading to pH increase (Marschner et al. 2011). However, recent findings have shown that soil microorganisms can help mitigate the negative effects of excessive P applications when soils are supplied with an adequate amount of C to promote P immobilisation, thereby decreasing P losses (Xu et al. 2020b). All these effects of microorganisms on P availability highlight the need for more investigation to understand the mechanisms behind the plant-microorganism interactions and tease apart the contribution of soil microorganisms to soil P availability compared to root mechanisms (Jakobsen et al. 2005; Dazzo and Ganter 2009; Richardson et al. 2009, 2011).

Biochemical processes

Phosphatase enzymes play a critical role in plant P nutrition via the cleavage of organically P-bound to release free orthophosphates for plant roots (Dick and Tabatabai 1984; Fox and Comerford 1992; Nannipieri et al. 2011; Spohn and Kuzyakov 2013a; Ragot et al. 2015). Acid and alkaline phosphatase enzymes are the main enzymes mineralising phosphomonoesters in soils with optimal activities depending on soil pH, while phosphodiesterases contribute to the mobilisation of orthophosphate diesters. In addition, phytases which are enzymes degrading inositol hexaphosphate (the most common form of organic P in the soil), have been isolated from fungi and bacteria present in soils and plants' rhizospheres (Richardson 1994, 2001; Richardson and Hadobas 1997; Hill et al. 2007; Jorquera et al. 2008). Several studies have suggested that acid phosphatase enzymes are released by plant roots and inhibited by high P availability, whereas alkaline phosphatase enzymes are mainly derived from soil microbes and are C-dependant (Speir and Cowling 1991; Colvan et al. 2001; Sakurai et al. 2008; Spohn and Kuzyakov 2013b, a; Spohn et al. 2015). Though, acid phosphatase enzymes are also released by arbuscular mycorrhizal fungi (Tarafdar and Marschner 1994; Koide and Kabir 2000; Sato et al. 2015). Nitrogen availability has also been considered among the factors driving soil phosphatase activity due to the essential role of N in the biosynthesis of phosphatase enzymes (Olander and Vitousek 2000; Marklein and Houlton 2012).

Phosphatase enzymes are sensitive to environmental conditions (Chen et al. 2003a; Shi et al. 2013; Sofi et al. 2016). Soil moisture and temperature have been described as major factors controlling

phosphatase activities across different biomes (Margalef et al. 2017; Zuccarini et al. 2020). Ge et al. (2017) showed that increased temperature enhanced the activity of phosphatase enzymes in the rhizosphere of rice, while Wu et al. (2015) found that warming accelerated C turnover and increased phosphatase activity in a meadow. Recent data from forest ecosystems have shown that drought decreased acid phosphatase activity (Zhang et al. 2020), whereas high rainfall decreased organic P mineralisation by inhibiting the activity of alkaline phosphatase enzymes (Sun et al. 2020). Previous studies under grassland and silvopastoral systems indicated that phosphatase activities increased in spring when plant P demand is high. In contrast, organic P accumulated in the winter period characterised by lower temperatures and high soil moisture (Tate et al. 1991; Perrott et al. 1992; Chen et al. 2003a; Scott and Condron 2003).

Phosphatase activity and its spatial distribution have recently raised increasing interest and investigations. In this regard, soil zymography has been extensively used because it offers an easy and low-cost method for imaging phosphatase activity in soils and rhizospheres (Spohn et al. 2013; Liu et al. 2017; Wei et al. 2019b; Zhang et al. 2019; Razavi et al. 2019). The experiment carried out by Spohn and Kuzyakov (2013) using soil zymography pictured for the first time the difference in spatial distribution between acid and alkaline phosphatase activities in the rhizosphere of white lupin. Specifically, they found that acid phosphatases were closely related to the roots, while alkaline phosphatases were widely distributed in the bulk soil. The spatial pattern of phosphatase activity in the rhizosphere is also affected by root morphology and development, where root hairs and root radius can play a significant role in the extension of phosphatase activity further than the rhizosphere (Giles et al. 2018a; Ma et al. 2018). Indeed, Razavi et al. (2016) and Ge et al. (2017) showed that phosphatase enzyme activity could extend up to 3.5 mm from the rhizosphere to the bulk soil.

An adequate interpretation of the impact of any parameter on soil phosphatase activity is challenging due to the difficulty to distinguish between the active phosphatase enzymes and the adsorbed ones (Nannipieri et al. 2011). The adsorption of organic P into the soil solid phase can also restrict our understanding of the actual role of phosphatase enzymes in mobilising organic P (Adams and Pate 1992; Magid et al. 1996). Therefore, Jarosch et al. (2019) pointed out that information on the availability of both phosphatase enzymes and organic P substrates is necessary to determine potential mineralisation or accumulation of soil organic P compounds. Razavi et al. (2016) noted that all these obstacles have caused a modest understanding of enzymes activity and recommended more *in situ* and non-destructive techniques to be used to advance our knowledge in this area of research. Studies combining the assessment of phosphatase activities and associated phosphatase genes have been recently undertaken to enhance our knowledge of how soil phosphatase enzymes drive biogeochemical P cycling (Ragot et al. 2015; Fraser et al. 2017; Gaiero et al. 2018; Chen et al. 2019a).

1.2.3 Factors affecting phosphorus availability and dynamics

Previous studies have shown that P dynamics in agroecosystems are controlled by a complex system, including plant species, soil properties, environmental conditions, and management practices (Tiessen et al. 1983; Chen et al. 2002, 2004; Condron 2003; Richardson et al. 2009; Vitousek et al. 2010; Nannipieri et al. 2011; Huang et al. 2017). The following section focuses on the influence of three particular factors, including plant species, N and P inputs, and elevated CO_2 on soil P dynamics and different processes linked to P cycling.

Plant species

Plant species have used different strategies to overcome P deficiency, mainly based on morphological and physiological adaptations to increase spatial P acquisition and enhance P mobilisation (Raghothama 1999; Vance et al. 2003; Rengel and Marschner 2005; Pandey et al. 2005; Pearse et al. 2006; Lynch 2011; Lyu et al. 2016). In this regard, grasses have been described as morphologically driven crops to respond to P deficiency, whereas legumes have been shown to adapt more physiologically to low P conditions (Lyu et al. 2016; Pang et al. 2018b). These different responses to P deficiency can influence soil P dynamics (Nuruzzaman et al. 2006; Wang et al. 2010a; Mat Hassan et al. 2013). For instance, Mat Hassan et al. (2012) and Pang et al. (2015) found that legumes plants can access sparingly available inorganic and organic P fractions using organic anions and phosphatase enzymes. *Lotus pedunculatus* and *Trifolium repens* decreased soil pH at the soil-plant interface to mobilise NaOH-Pi and H_2SO_4-Pi fractions from rock phosphate and monocalcium phosphate, respectively. On the other hand, grasses invest more in root morphology and architecture by producing finer and longer roots and promoting mycorrhizal symbiosis to increase soil P uptake (Chevalier et al. 2003; Singh Gahoonia and Nielsen 2004; Wang et al. 2010a; Pandey et al. 2015b; Sandaña and Pinochet 2016). However, Pang et al. (2010) and Pang et al. (2018) pointed out that several perennial legumes can combine different strategies to increase soil P acquisition when deprived of P. Some recent studies highlighted similar findings under cereals crops where *Triticum durum* and *Triticum aestivum* were found to rely on both physiological and morphological adaptations to acquire P, especially under P deficiency and elevated CO_2 environments (Pandey et al. 2018; Lal et al. 2019).

Trees have been found to have higher plant P demand, thereby accelerating soil P cycling. For instance, Chen et al. (2004) noted that radiata pine had lower microbial biomass P under a range of New Zealand pasture soils compared to ryegrass due to higher release of microbial P to meet greater plant P demand. Similarly, Chen et al. (2003a) showed that plant P uptake, organic P mineralisation, and microbial P turnover was higher under forest soils than adjacent grasslands. Forest trees can allocate significant amounts of rhizodeposits to the below-ground compartment and exhibit high

phosphatase levels in the soil. This increase in C input and soil microbial activity can enhance the depletion of moderately labile organic P fractions (Chen et al. 2003b, 2004; Scott and Condron 2003).

Plant species behave differently in terms of organic acid exudation. Some plants, such as white lupin, benefit from their specific structures (cluster roots), which represent a hotspot for organic anion release (Gardner et al. 1982; Dinkelaker et al. 1995; Shane and Lambers 2005). Interestingly, some other species, such as red clover, release high quantities of organic anions in the rhizosphere despite lacking such structure (Gerke and Meyer 1995; Gerke 2015). Chen et al. (2013) indicated that root architecture and plant P status controlled the release of organic anions from *Lupinus angustifolius* roots. The role of organic anions in enhancing P availability is well documented. However, controversial findings have been reported in several plant species grown in real soil conditions (Pearse et al. 2007; Pandey et al. 2014; Wang et al. 2015c, 2016a).

Intercropping and cover crops present a great alternative to decrease the dependency on P fertilisers while increasing the use of native soil or accumulated P stocks (Kamh et al. 1999; Mat Hassan et al. 2013; Hallama et al. 2019; Liao et al. 2020). Legumes and cereals have specific microbial communities in their rhizospheres characterised by divergent aptitudes in mobilising inorganic and organic P pools (Jorquera et al. 2008; Zhou et al. 2018). With different root morphology and secretory ability, intercropping cereals with legumes has shown an increase in P uptake and plant biomass, especially for the cereal crop. This was attributed to an increase in the mineralisation of organic P by legumes and the alleviation of competition over P by mobilising different P fractions (Li et al. 2003, 2008a). Under P deficient soils, the integration of legumes such as *Faba bean* and white lupin in crop rotation has been found to be efficient in mobilising sparingly available P forms, which can be available for subsequent crops (Kamh et al. 1999; Nuruzzaman et al. 2005; Mat Hassan et al. 2013; Xu et al. 2020a).

Nutrient inputs

Due to adsorption of P into soil active mineral surfaces and organic moieties, excessive amounts of P fertilisers (soluble inorganic fertilisers, rock phosphate, and manure) have been added to agroecosystems, mainly oriented to increase plant productivity and compensate for P export via plant and/or animal products (Condron 2003; Pierzynski and McDowell 2005; Haygarth et al. 2013). When P balance (inputs - outputs) in agroecosystems is positive, soil P accumulates along with potential P losses to waterways (Sharpley 1995; McDowell et al. 2003; McDowell and Condron 2004, 2012), whereas when P balance is negative, soil P reserves are depleted (Nguyen et al. 1995). Accumulation of P termed as soil P legacy can occur in different inorganic and organic P fractions, including moderately labile P fractions but also in more recalcitrant P fractions such as residual P (Condron and Goh 1989; Maher and Thorrold 1989; Vu et al. 2008; Tian et al. 2017). This

accumulation of P is governed by different factors such as the parent material, land use, and type of fertiliser applied (Steele 1976; Hedley et al. 1982; Annaheim et al. 2015; Fink et al. 2016; Liu et al. 2019). On a global scale, Bouwman et al. (2017) pointed out that soil P legacy has built up in most developed countries, India, and China, whereas some countries in Europe have recently shown negative P balances due to the use of soil P stocks accumulated over the years.

The addition of inorganic and organic P fertilisers can affect soil P pools as well as soil microbial activity and diversity. For instance, Mat Hassan et al. (2012) found that *Faba bean* depleted NaOH-Pi in its rhizosphere despite being fertilised with inorganic P, while white lupin accessed NaOH-Po and residual P under the same conditions. Similarly, Li et al. (2008) noted that two rice genotypes supplied with 100 mg P kg^{-1} soil as calcium phosphate depleted resin-Pi, $NaHCO_3$-Pi, $NaHCO_3$-Po, and NaOH-Pi. Malik et al. 2012 showed that the application of inorganic P fertilisers enhanced the accumulation of P in non-labile P forms. In contrast, the addition of organic residues increased microbial respiration, modified soil microbial community composition, and increased the accumulation of labile and recalcitrant organic P fractions in the soil. Therefore, organic amendments can be a source of energy for soil microbes and, at the same time, a source of slow-release P forms for plants. Compost addition increased microbial P and enhanced the accumulation of inorganic P into recalcitrant and residual P fractions under wheat plants grown in two contrasting soil types (Verma et al. 2016). This accumulated P could supply the labile inorganic P pool in the long run. Applications of manure have been shown to increase phosphatase activities (Saha et al. 2008; Liu et al. 2017) while being a source of organic anions that can contribute to desorption of sparingly available soil P (Adeleke et al. 2017).

Nitrogen applications have been used as an effective way of phytoremediation of high P contaminated soils (Newman et al. 2009; van der Salm et al. 2009). Due to increased plant production in response to N inputs, increased offtake of soil P was observed (Messiga et al. 2014; Liu et al. 2019), along with a significant decrease in P losses (Dodd et al. 2014). Besides the increase in plant yield, N addition can affect soil chemical properties, microbial activity, and root traits, thereby influencing soil P transformations (Fujita et al. 2010; Wang et al. 2015a; Yang et al. 2015; Tian et al. 2016; Heuck et al. 2018; Fan et al. 2020). For example, N addition can decrease soil pH, thus enhancing the availability of more recalcitrant inorganic P forms (Sherman et al. 2006; Fan et al. 2019). Nitrogen is a principal component of phosphatase enzymes involved in organic P mineralisation (Olander and Vitousek 2000; Marklein and Houlton 2012). Nitrogen addition can increase rhizodeposition with a concomitant increase in microbial activity and plant P uptake (Xiao et al. 2019; Bicharanloo et al. 2020). On the other hand, N applications can increase mycorrhizal symbiosis as well as root biomass and density, thereby expanding the soil volume explored and

subjected to rhizosphere processes (Treseder 2004; Yuan and Chen 2012; Fan et al. 2019; Schleuss et al. 2020).

Soils are globally co-limited by N and P (Elser et al. 2007; Vitousek et al. 2010; Harpole et al. 2011; Du et al. 2020); therefore, the availability of one element can feedback on the dynamics and cycling of the other. Using P fractionation combined with ^{18}O isotopes, Bauke et al. (2018) found that an adequate application of NPK increased the recycling of Ca-P bound in the subsoil, which was attributed to an increase of root growth and microbial activity under sufficient nutrient conditions in the topsoil. Moreover, continual P applications under N deficiency increased P accumulation due to lower plant production and higher P inputs compared to plant P demand. On the other hand, Scott et al. (2015) showed that the application of N to high P fertility pasture soil reduced N leaching due to increased plant biomass utilising more N compared to the low P fertility soil where higher N leaching was observed. Similarly, in a recent study, Francisquini Junior et al. (2020) described that P applications not only increased forage yield in a grass-legume pasture but also showed higher N use efficiency, irrespective of the solubility of P source.

The response of soil microbial biomass and activity to N and P inputs in managed ecosystems is not consistent. Although it is widely acknowledged that phosphatase activity is inhibited by P availability (Nannipieri et al. 2011; Widdig et al. 2019), no change and even an increase in phosphatase activity were reported as a result of P addition to soils (Ikoyi et al. 2018; Shi et al. 2020). Nitrogen applications have been found to decrease soil pH, thereby impacting acid and alkaline phosphatase activities and their ratio (Dick et al. 2000). Indeed, Lemanowicz (2011) showed that N addition decreased soil pH with a concomitant increase in acid phosphatase activity. In contrast, Wang et al. (2015) noted repressive effects of long-term N addition on both acid and alkaline phosphatase activities in semi-arid grassland soil. In their meta-analysis, Deng et al. (2017) showed that long-term N addition had no impact on microbial biomass P. Whereas P immobilisation occurs in response to long-term P inputs (Dodd et al. 2014; Dai et al. 2020). Discrepancies between these results could be attributed to different cropping systems, initial soil fertility, pH, and quantity and form of fertilisers used in these nutrient addition experiments.

Previous studies assessing the effects of nutrient addition on soil P availability and dynamics focused mainly on N addition alone, especially in forest ecosystems due to rising concerns about increasing N deposition and its effect on soil P depletion (Yang et al. 2015; Fan et al. 2018, 2019; Chen et al. 2018). On the other hand, experiments conducted on cropping systems have been oriented towards investigating soil P fractions redistribution but under continual P applications (Von Sperber et al. 2017) or different N fertiliser sources (Wang et al. 2021b). In contrast, investigations combining N and P additions under grassland soils did not explore soil P fractions (Schleuss et al. 2020; Tian et al.

2016). Therefore, our understanding of the impact of single and combined additions of N and P to co-limited soils is still incomplete.

Long-term experiments are very useful at understanding soil processes as well as changes in nutrient cycling in response to management practices (Johnston 1997; Debreczeni and Körschens 2003; Damar et al. 2021). Indeed, nutrient inputs not only influence soil nutrient status but drive ecosystem services over the years by impacting primary productivity, plant community composition, microbial diversity, and soil ecology (Fraser et al. 1994; Edmeades 2003; Mander et al. 2012; Smith et al. 2012; Chen et al. 2019a). Therefore, great lessons can be learnt from long-term experiments such as the Winchmore fertiliser trial (> 65years), which ultimately will help design tailored solutions and policies to increase nutrient use efficiencies, sustain food production, and protect the environment (McDowell and Condron 2012; Johnston and Poulton 2018; McDowell et al. 2021).

Climate change

Phosphorus demand by plants is highly likely to increase under elevated CO_2 conditions due to stimulation of photosynthesis and plant growth (Ainsworth and Rogers 2007; Jin et al. 2015). Plants use similar strategies to adapt to elevated CO_2 conditions and P deficiency because elevated CO_2 accelerates plant growth and, in turn, plant P demand. Therefore, plant species tend to modify their root morphology as well as physiological and biochemical root traits to acquire P more efficiently. Root morphological adaptations include an increase in root biomass and density, root surface area, and mycorrhizal colonisation (Ceulemans et al. 1999; Treseder 2004; Pandey et al. 2009, 2015b). Elevated CO_2 may not only impact root morphology and architecture but can also affect C delivery to the below-ground compartment. This can affect soil microbial biomass and activity, thereby influencing P dynamics (Ceulemans et al. 1999; Jin et al. 2015).

The organic anion release has been shown to increase under elevated CO_2 with a potential improvement of soil P availability. For instance, Haase et al. (2007) found an increase in the release of malate of 177 % when *Phaseolus vulgaris* L. was grown under elevated CO_2 conditions. Bhattacharyya et al. (2014) described that elevated CO_2 increased the release of organic anions with a concomitant increase in plant P uptake and P solubilisation under tropical flooded rice. Another study carried out by Delucia et al. (1997) showed that elevated CO_2 increased oxalate exudation, which enhanced P uptake by *Ponderosa pine*. However, the efficacy of this higher organic acid release in enhancing soil P availability is still debatable (Jin et al. 2015). Additionally, several studies have reported no positive effect of elevated CO_2 on organic acid release compared to ambient CO_2 conditions (Norby et al. 1987; Campbell and Sage 2002; Edwards et al. 2005). A bottleneck in understanding the response of organic acid release to elevated CO_2 environments resides in the fact that most of the studies have been carried out under sand or solution cultures (Norby et al. 1987;

Watt and Evans 1999; Campbell and Sage 2002; Johansson et al. 2009). Therefore, future investigation looking at the composition of organic anions under ambient and elevated CO_2 in real soil conditions is needed to close this knowledge gap (Pang et al. 2018b).

Increased plant biomass under elevated CO_2 conditions enhances plant P demand promoting organic P mineralisation, especially under P-limited conditions. However, the response of phosphatase activity to elevated CO_2 has shown discrepancies among studies. Due to increased C fluxes into the below-ground compartment, an increase in alkaline phosphatase activity is expected. Drissner et al. (2007) described an increase in microbial biomass after growing white clover for three years under elevated CO_2, which was strongly correlated with alkaline phosphatase activity mainly derived from bacteria. The release of root phosphatase enzymes decreased when Ponderosa pine was grown under elevated CO_2 conditions (Delucia et al. 1997). However, Niu et al. (2013) reported a stimulation effect of elevated CO_2 on acid phosphatase activity under *Arabidopsis thaliana* grown in a P-deficient hydroponic solution. Other studies have shown no significant effect of elevated CO_2 on both acid and alkaline phosphatase activities under forest trees, legumes, and grasses (Jin et al. 2013; Ochoa-Hueso et al. 2017). Therefore, the interpretation of the impact of elevated CO_2 on phosphatase activities must be done cautiously, taking into consideration several factors, including soil P status, plant species, soil organic matter, and CO_2 enrichment design.

Acceleration of C fluxes at the soil-plant interface due to elevated CO_2 environments can stimulate microbial biomass and activity and shape the microbial community composition (Jin et al. 2014, 2015; Tfaily et al. 2018; Kuzyakov et al. 2019). This trade-off between plant and soil microbes is critical to soil P cycling, plant P nutrition, and C sequestration (Jin et al. 2015; Sofi et al. 2016; Mimmo et al. 2018; Kuzyakov et al. 2019). For instance, Drissner et al. (2007) found a 48 % increase in soil microbial biomass under ryegrass and white clover grown under elevated CO_2. Doubling the concentration of ambient atmospheric CO_2 increased microbial C and microbial respiration in the wheat rhizosphere (Jin et al. 2014). Soil microbes are C-limited, while rhizodeposits represent a valuable energy source in the rhizosphere of plants, especially under elevated CO_2 environments. Indeed, Bhattacharyya et al. (2014) noted that microbial P immobilisation was consistent with an increase in organic anions release in the rhizosphere of flooded tropical rice grown under a CO_2 concentration of 550 ppm. Elevated CO_2 is likely to influence the efficiency of arbuscular mycorrhizae to absorb more P for the host plants (Moorhead and Linkins 1997; Jin et al. 2015). Delucia et al. (1997) found that elevated CO_2 increased mycorrhizal infection of Ponderosa pine seedlings, thereby facilitating more P uptake. Nevertheless, some recent findings on *Zea mays* have reported that the P sufficiency controlled the impact of elevated CO_2 on arbuscular mycorrhizal colonisation (Watts-Williams et al. 2019).

Different designs have been used to study the effect of elevated CO_2 on soil and plant parameters, including glasshouse experiments, closed field chambers, open field chambers, and Free Air Carbon Dioxide Enrichment (FACE) (Newton et al. 2006). Among all those, FACE represents the best experimental design to study the effect of elevated CO_2 on ecosystem services (Ainsworth and Long 2005). Such designs, under real field conditions, minimise bias due to artificial conditions (temperature, moisture, and radiation) and allow for the assessment of cumulative effects of elevated CO_2 on soil, plant, and microorganisms interactions (Leakey et al. 2009). The only FACE experiment in the world under grazed pastures was established in New Zealand in 1997 and represents a valuable research site to study the impact of long-term CO_2 enrichment on different parameters such as soil fertility, nutrient cycling, plant community composition, and soil biodiversity (Gentile et al. 2012; Ross et al. 2013; Newton et al. 2014; Xia et al. 2017). This research site can also provide unique data used for modelling the potential effects of elevated CO_2 on the soil-plant-microbe continuum.

Most studies investigating changes in soil P fractions have been carried out under elevated CO_2 combined with high or adequate P conditions (Bhattacharyya et al. 2014; Jin et al. 2014, 2017), while little is known about P transformations under the interactive effects of P deficiency and elevated CO_2. Additionally, studies investigating changes in soil P availability and dynamics under elevated CO_2 and pasture systems have been mostly carried out for short-term periods and under plant biomass removed (Edwards et al. 2005, 2006; Lam et al. 2012; Gentile et al. 2012), which does not account for the effects of animal grazing on nutrient returns, accumulation of plant detritus, and changes in plant community composition. These two knowledge gaps warrant further investigation.

Overall, the literature review showed that plant species, nutrient inputs, and elevated CO_2 can influence soil P dynamics and availability by impacting chemical, biological, and biochemical processes taking place in soils and rhizospheres. Additionally, several knowledge gaps have been identified from the literature related to the global understanding of soil P dynamics and bioavailability under low P conditions on the one hand and related to the context of New Zealand pasture systems on the other hand. These knowledge gaps are summarised below:

- Plant species have developed contrasting strategies to adapt to P deficiency, including the release of organic anions. Nevertheless, the role of organic anions in P acquisition under real soil conditions is still debatable.

- Soils are globally N- and P-limited; hence N and P are commonly applied to agroecosystems. However, little is known about the combined addition of N and P on short-term changes in soil processes and P dynamics in New Zealand soils.

- Studies looking at the effect of elevated CO_2 on soil P dynamics have been mostly carried out under high or adequate P soils; therefore, our understanding of the interactive effects of elevated CO_2 and P deficient conditions on soil P fractions changes is limited.

- A paucity of information is available on how long-term CO_2 enrichment influences soil P availability and dynamics, especially under grazed pastures in New Zealand.

1.3 Hypotheses, objectives, and thesis structure

The general hypothesis of this research is that plant species, N and P inputs, and elevated CO_2 would impact soil inorganic and organic P fractions by influencing chemical, biological, and biochemical processes taking place in soil and the rhizosphere. Specifically, the hypotheses were that:

- Legume species would exhibit higher physiological ability than grasses in acquiring P from low P soils.

- Plant growth would differ between grasses and legumes in response to N and P inputs, which could influence soil P dynamics.

- Phosphorus addition would increase plant growth while promoting microbial P immobilisation and decreasing the release of organic anions and phosphatase enzymes.

- Nitrogen addition would accelerate P dynamics by increasing plant growth, phosphatase activity, and organic anions release, thereby enhancing the mobilisation of more recalcitrant P fractions.

- Combining N and P inputs would have a more significant impact on plant production, P uptake, and soil P dynamics compared to single nutrient addition.

- Seasonal conditions such as soil moisture and temperature would influence soil properties linked to P cycling regardless of nutrient inputs or P status.

- Elevated CO_2 would increase soil P availability by enhancing organic anion release and mobilising sparingly available P fractions, but this increase in P availability could differ according to plant species.

The overall objective of this research was to determine the impacts of N and P inputs and elevated CO_2 on changes in inorganic and organic P fractions as well as soil processes linked to P cycling under different plant species and pasture soils in New Zealand. This overall objective was divided into two specific objectives.

Specific objective 1: To investigate the short- and long-term impacts of N and P inputs on soil P availability and dynamics and associated processes as well as determine seasonal changes in these processes as affected by nutrient inputs (Chapters 2, 3, and 4).

- In Chapter 2, changes in plant growth, rhizosphere processes, and soil P fractions under legume and grass species were studied as affected by the single and combined additions of N and P to P-deficient soil.

- In Chapter 3, plant growth, soil P fractions, and seasonal changes in soil biological and biochemical processes related to P cycling were assessed in response to N and P inputs to N and P co-limited soil sown with Italian ryegrass.

- In Chapter 4, the longest-running replicated field trial in grazed pastures in the world (Winchmore fertiliser trial) was used to quantify the cumulative effects of long-term inputs of different rates of P fertiliser on key soil parameters linked to biological P cycling, along with assessing the effect of contrasting soil P status on short-term seasonal changes in these parameters.

Specific objective 2: To assess the short- and long-term impacts of elevated CO_2 on soil P availability and dynamics and associated processes (Chapters 4 and 5).

- In Chapter 5, the interactive effects of elevated CO_2 and P deficiency on plant growth, rhizosphere properties, and changes in soil P fractions were investigated under different plant species.

- In Chapter 6, the New Zealand FACE experiment (the longest field experiment on CO_2 enrichment in grazed pastures in the world) was used to give some insights on the impact of long-term elevated CO_2 on soil P fractions and chemical, biological, and biochemical processes linked to soil P dynamics and bioavailability.

Chapter 7 was dedicated to summarising and discussing the results of this research as well as highlighting future research priorities.

Figure 1.3 outlines the overall structure of this thesis.

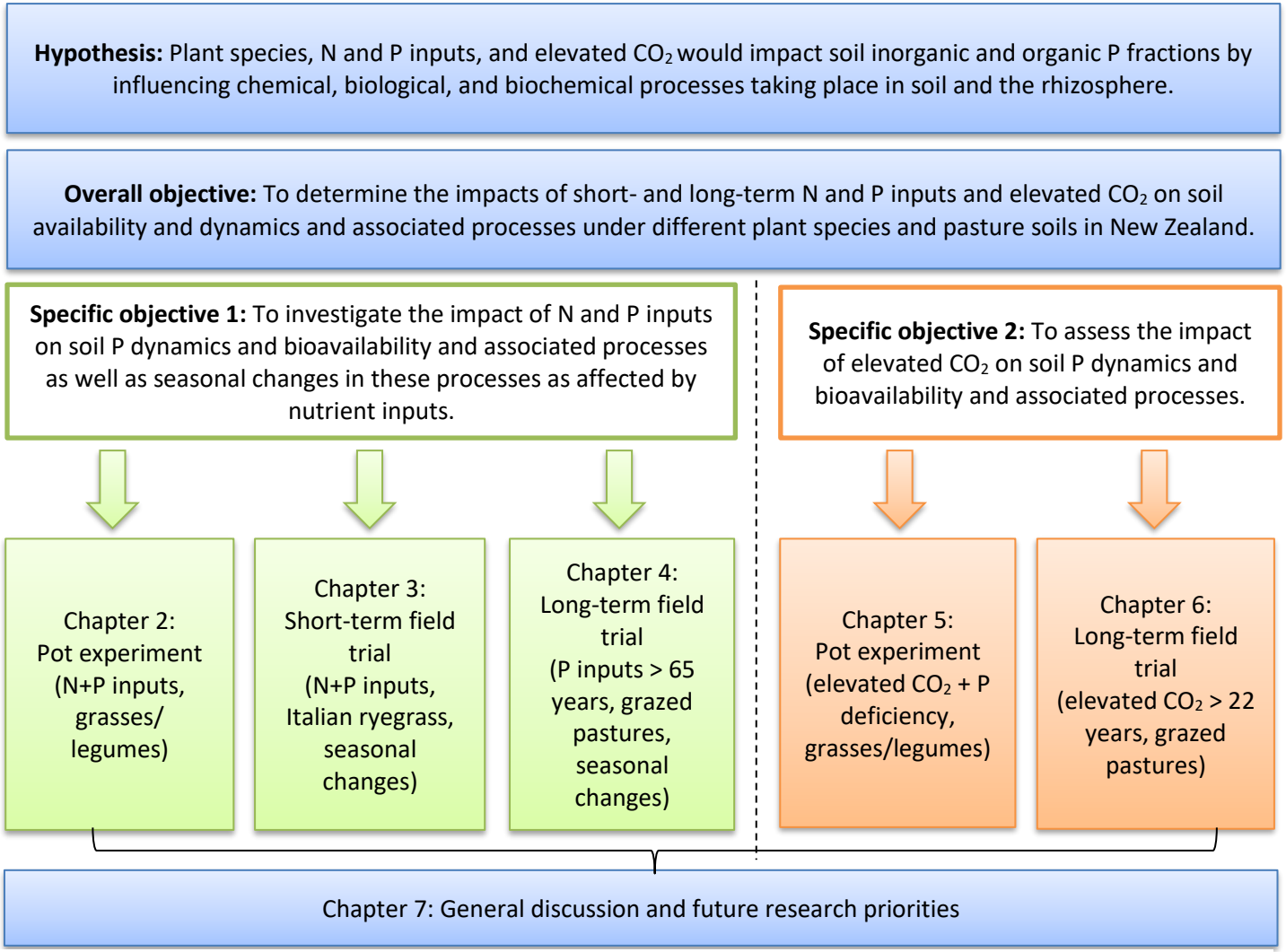


Figure 1.3 Flow diagram of the thesis structure.

Chapter 2

Impacts of Nitrogen and Phosphorus Additions on Plant Growth, Rhizosphere processes, and Soil Phosphorus Fractions under Legumes and Grasses Grown in Phosphorus deficient Pasture Soil

2.1 Introduction

From gene duplication to root system development and photosynthesis, phosphorus (P) is involved in a plethora of biological and biochemical mechanisms critical to crop production (Raghothama 2005; Butusov and Jernelöv 2013). The total amount of P in soils may exceed plants requirements; however, only a small amount is available for plant uptake (Holford 1997). Soil P is present as inorganic and organic forms (Hedley et al. 1982). Inorganic P is subject to adsorption and precipitation with iron (Fe), aluminium (Al), and calcium (Ca) cations as well as with positively charged soil particles (Hinsinger 2001; Pierzynski and McDowell 2005). Organic P represents up to 65 % of total P and requires a mineralisation step to become available for plant uptake (Dalal 1977; Condron et al. 2005; Richardson and Simpson 2011; Richardson et al. 2011; Nash et al. 2014). The availability of P for plants is governed by its degree of attachment to cations (Fe, Al, and Ca) and carbon moieties present in the soil, resulting in different levels of P availability for plant uptake (Condron et al. 2005; Yang and Post 2011). In low P soils, plants have developed a myriad of chemical, biological, and biochemical mechanisms to increase P acquisition (Richardson et al. 2009; Hinsinger et al. 2009; Shen et al. 2011). Root acidification and the exudation of organic anions and phosphatase enzymes are deemed the most important processes taking place in the rhizosphere of P deficient plants (Vance et al. 2003; Jones and Oburger 2011; Nannipieri et al. 2011). The emission of protons from plant roots is used by P-deficient plants to acidify the soil and solubilise inorganic and organic P (Marschner 1995; Chen et al. 2002; Villegas and Fortin 2002; Oburger et al. 2011). Organic anions act via acidification, chelation, and exchange reactions to desorb sparingly available inorganic and organic P forms (Jones 1998; Hocking 2001; Oburger et al. 2011; Wang et al. 2016b). Phosphatase enzymes released by plants and microorganisms contribute to the cleavage of organic P bonds to supply available P to the soil solution (Richardson 1994; Tabatabai 1994; Nannipieri et al. 2011; Gianfreda 2015). Many of these mechanisms of P mobilisation may also be duplicated by microbes (Schefe et al. 2008; Richardson et al. 2011; Gianfreda 2015; Zhou et al. 2018).

Phosphorus dynamics are affected by plant species (Chen et al. 2002; Li et al. 2015; Cabeza et al. 2017; Ye et al. 2018; Fan et al. 2019). For instance, legume crops have been found to mobilise more stable P pools than cereals reflecting divergent P acquisition strategies (Li et al. 2003). Ye et al. (2018) found that efficient wild barely cultivars relied on acid phosphatase activity to mobilise different inorganic and organic P fractions. Other studies highlighted the contribution of organic anions in the acquisition of sparingly available inorganic P forms in the rhizosphere of legumes such as chickpea, white lupin, and red clover (Veneklaas et al. 2003; Nuruzzaman et al. 2006; Lambers et al. 2013; Gerke 2015). However, the role of organic anions in enhancing P acquisition across plant species is unclear and has been found sometimes to be minor (Wouterlood et al. 2005; Pearse et al. 2007; Wang et al. 2016a, 2017b). For instance, Pearse et al. (2007) pointed out that organic anions exudation by different plants species was not consistently related to their ability to mobilise sparingly inorganic P forms. In contrast, Pandey et al. (2014) and Ryan et al. (2014) failed to find a strong correlation between plant yield, plant P uptake, and the release of organic anions. Most of the studies investigating organic anions and their impact on P availability have been carried out on sterilised sand or nutrients solutions, thus hampering our understanding of their role in real soil conditions (Jones 1998; Oburger and Schmidt 2016; Oburger and Jones 2018). Likewise, phosphatase activity was not always correlated to P deficiency (Adams 1992; Clarholm 1993; Starnes et al. 2008), while alkaline phosphatase enzymes are deemed to be principally derived from soil microbes (Nannipieri et al. 2011; Spohn and Kuzyakov 2013a; Wasaki et al. 2018; Wei et al. 2019a). Therefore, a more accurate understanding of the mechanisms of P acquisition by legumes and grasses is a prerequisite to use them in intercropping systems or as potential green manures to acquire and recycle P in soil-plant systems (Richardson et al. 2009; Cordell et al. 2009; Maltais-Landry 2015; Pang et al. 2018b; Zhou et al. 2018).

The concept of nutrient limitation of the growth of biota originated from the 19th-century work of Justus von Liebig. In plants and microbes, this has reduced to a ratio between nitrogen (N) and P, whereby a ratio of greater and less than 7 indicates growth limited by P and N, respectively (Holford 1997; Vitousek et al. 2010). In a N-limited environment, the application of N fertilisers was found to increase microbial activity together with an enhancement of phosphatase activity and rhizodeposition (Marklein and Houlton 2012; Zang et al. 2017; Chen et al. 2018). Adding N shifts growth to P-limitation, thereby activating mechanisms to make P available. It is widely reported that the addition of inorganic P to P-deficient soils inhibits phosphatase activity (Olander and Vitousek 2000; Vitousek et al. 2010; Nannipieri et al. 2011). Nevertheless, N and P additions have revealed inconsistent results on phosphatase activity and microbial biomass. A meta-analysis on the effect of N addition on P-limitation revealed an enhancement of phosphatase activity by 24 %, while no effect on available soil P and microbial P was observed (Deng et al. 2017). Liu et al. (2010) and Yang et al.

(2015) showed that P addition increased alkaline phosphatase activity while N addition had an opposite effect.

Previous experiments combining N and P additions and investigating P dynamics have been focused on forest ecosystems due to increased concerns about atmospheric N depositions and their impact on soil P availability (Yang et al. 2015; Fan et al. 2018, 2019; Chen et al. 2018). Studies on legume and grass species have been mostly carried out in inert sand or soils but with either N or P addition (Nuruzzaman et al. 2006; Mat Hassan et al. 2012; Sugihara et al. 2016; Ye et al. 2018). The application of N and P fertilisers is a common practice among farmers to increase pasture production and is more representative of what happens in the field (McDowell et al. 2017; Pinxterhuis and Edwards 2018). However, few studies have investigated low P pasture soils, and none have examined the complicating factor of combined N and P additions on soil P dynamics. Here we contrasted four plant species focusing on two legumes, one grass, and wheat as a reference crop sown in small PVC tubes to accelerate P cycling and encourage easy detection of plant responses to P deficiency. Moreover, in this study, we aimed to identify the most important mechanism driving P acquisition under different plant species and its potential changes due to nutrient addition in a low P pasture soil. We hypothesised that (1) plant species will show different responses to P and N additions in terms of plant growth and P uptake; that (2) chemical, biological, and biochemical processes involved in P acquisition will be impacted by plant species and nutrient addition; and that (3) plant species will exhibit divergent strategies to mobilise soil P.

2.2 Materials and Methods

2.2.1 Soil preparation and characteristics

The soil used in the experiment was a silt loam Waikiwi soil (USDA soil taxonomy: Inceptisol) from Woodlands Research Station, 19 km east of Invercargill, New Zealand. Soil samples were collected from the 0-20 cm horizon of a permanent pasture that had not received P fertilisers for the last 25 years, air-dried, and passed through a 2 mm sieve to remove any roots and plant materials. The soil had an Olsen P concentration of 4 mg kg^{-1} and an initial pH in water of 5.4 (1:2.5). Before starting the experiment, the soil was limed with $CaCO_3$ at the rate of 4 tonnes per hectare to reach an optimal pH for plant growth (pH 6.4) and incubated at 25 °C for two weeks at 75 % of field capacity. The soil received a basal fertilisation consisting of 265 mg kg^{-1} of K (K_2SO_4), 30 mg kg^{-1} of Mg (MgO), 3 mg kg^{-1} Mn ($MnCl_2,4H_2O$), 2 mg kg^{-1} Zn ($ZnCl_2$), 2 mg kg^{-1} Cu ($CuCl_2,2H_2O$), 3 mg kg^{-1} B (H_3BO_3), and 0.2 mg kg^{-1} Mo ($Na_2MoO_4,2H_2O$). The other basic properties of the soil used in this study are summarised in Table 2.1.

Table 2.1 Basic physiochemical properties of the Waikiwi soil used in the study.

Soil pH	Total C g C kg^{-1} soil	Total N g N kg^{-1} soil	Total P mg P kg^{-1} soil	Olsen P mg P kg^{-1} soil	Exchangeable Potassium mg kg^{-1} soil	Exchangeable Al mg kg^{-1} soil	Soil texture (%) Sand	Silt	Clay
5.4	44	3.9	1236	4	62.4	7.2	16.5	70.9	12.6

2.2.2 Experimental design

For the purpose of this study, PVC tubes (48 mm internal diameter) were cut at 70 mm height, sealed from the bottom with a 20 µm nylon mesh, and filled with approximately 100 g of oven-dry soil (equivalent 130 g of air-dry soil) to simulate a bulk density of 0.89 g cm^{-3}. Before potting, the soil has received two levels of P: 0 and 33 mg kg^{-1} (equivalent 45 kg P ha^{-1}) as NaH_2PO_4 and two levels of N : 0 and 200 mg kg^{-1} (equivalent 250 kg N ha^{-1}) as urea, in a full factorial design resulting in the following four treatments: Control (0P, 0N), P (33 P, 0N), N (0P, 200 N), NP (33 P, 200 N). The fertilisers were mixed thoroughly with the soil. The rates of P inputs were deemed to increase Olsen P to reach an optimum Olsen P (20-30 mg kg^{-1}) for sedimentary soils in New Zealand (Edmeades et al. 2006). Nitrogen rates reflected some common practices in fertilisation programs in dairy farms in the Canterbury region (Pinxterhuis and Edwards 2018). Four plant species, blue lupin (*Lupinus angustifolius*), white clover (*Trifolium repens* L.*,* cv. Demand), perennial ryegrass (*Lolium perenne* L.*,* cv. Samson), and wheat (*Triticum aestivum* L.*,* cv. Graham) were used in this study. Twenty seeds of white clover and ryegrass and 6 of wheat and blue lupin were sterilised for 20 min in 2 % sodium hypochlorite, washed thoroughly with deionised water, pre-germinated in the dark in wet filer papers, and then sown in the tubes. After emergence, seedlings were thinned to 15 plants for white clover and ryegrass, 5 for wheat, and 4 for blue lupin. The purpose of using a high seed density and a small volume of soil was to maximise soil exploitation by roots (all the soil in the tube was assimilated to a rhizosphere soil), accelerate P cycling, and encourage easy detection of plant species responses to P deficiency. A pre-experiment was carried out to determine the optimal number of seeds for each plant species. White clover and blue lupin (legumes) were not inoculated in this study because of the presence of their respective rhizobia strains in New Zealand pasture soils. The tubes were placed in a randomised block design in a glasshouse between August and October 2018 within a temperature range of 10 to 26 °C and a 16:8 dark:light ratio. There were four replicates of each treatment. The tubes were irrigated by capillarity via a sand bed covered with a nylon mesh and connected to a water reservoir via a syphon system. The difference between the water level in the

reservoir and the bottom of the tubes was kept at 5 cm height to supply enough water to the tubes during the experiment (Figure 2.1).

Figure 2.1 Picture of the glasshouse pot experiment.

2.2.3 Plant and soil analyses

After 60 days of growth, plants were removed from the tubes, and the rhizosphere soil was sampled. Due to the high root density, all the soil in the tube was assimilated to a rhizosphere soil. The soil samples were then sieved through a 2 mm mesh sieve and divided into two portions. The first portion was air-dried for chemical analysis. The second was stored fresh at 4 °C for microbial biomass P and phosphatase enzymes measurements. Roots and any remaining soil were transferred to a plastic beaker where they were gently soaked for two minutes in a known volume of 0.2 mM $CaCl_2$ solution (according to root biomass), liberating a rhizosphere soil extract that was used for organic anion determination by HPLC (Pearse et al. 2007; Pang et al. 2015). Shoot and root materials were separated, rinsed with deionised water, oven-dried at 65 °C for 48 hours, and then weighted. Plant materials were ground to pass through a 1 mm sieve. Shoot and root P concentrations were determined by inductively coupled plasma atomic emission spectroscopy (ICP-OES) after digestion with a mixture of H_2O_2 and HNO_3 (2:1) by closed-vessel microwave-assisted digestion (Anderson

1996). Shoot and root N concentrations were determined by the Dumas combustion method in a VarioMAX CN Macro Elementar Analyser. Phosphorus and N uptake in shoots and roots were calculated by multiplying P or N concentrations by the dry matter of the corresponding plant part. Total P and N uptake represented the sum of shoot + root uptake for each element.

Rhizosphere pH was measured after shaking 4 grams of air-dried soil with deionised water for 1 hour in a 1: 2.5 soil to solution ratio. Microbial biomass P was determined following the fumigation-extraction method described by Brookes et al. (1982) with the recommendations of Morel et al. (1996). In short, 1 gram of field moist soil was weighted in triplicates (set A, B, and C). Set A was fumigated using $CHCl_3$ for 24 hours and extracted by 20 ml of 0.5 M $NaHCO_3$ for 30 min. Set B was kept for 24 hours in the same conditions and extracted with 20 ml of 0.5 M $NaHCO_3$ for 30 min. Set C was non-fumigated and extracted with 20 ml of 0.5 M $NaHCO_3$ spiked with 25 mg kg^{-1} P for 30 min. All the extracts were then centrifugated at 3300 rpm for 10 min and filtered through Whatman number 2 for colourimetry measurements of inorganic P following the molybdate-ascorbic acid method (Murphy and Riley 1962). Microbial biomass P was calculated according to the following equation:

Microbial Biomass P (mg kg^{-1}) = 25 * (PA - PB)/(0.40 * (PC - PB))

Acid and alkaline phosphomonoesterase activities were determined according to the procedure described by Tabatabai (1994). The method consists of adding 4 ml of modified universal buffer MUB (pH 6,5 for acid phosphatase and pH 11 for alkaline phosphatase) to 1 g of moist soil followed by adding 1 ml of substrate p-nitro phenyl phosphate and then incubating the mixture for 1 hour at 37 °C (the final concentration of p-nitro phenyl phosphate was 10 mM). The reaction was halted by the addition of 4 ml 0.5 M NaOH and 1 ml of 0.5 M $CaCl_2$. The extract was then centrifugated at 3300 rpm for 15 min, filtered, and diluted as needed for spectrophotometer reading at 410 nm. The activities of acid and alkaline phosphatase enzymes were considered as potential enzyme activities (Margenot et al. 2018). For organic anion analyses, a subsample of the rhizosphere soil extract was filtered through a 0.45 µm Phenex regenerated cellulose syringe (Phenomenex, USA), and one drop of diluted (1:100) Micropur (10 mg/L, Katadyn products, Switzerland) was added to inhibit microbial decomposition (Cheng et al. 2014; Wang et al. 2015c, 2016a). The extracts were stored at -20 °C until analysis by HPLC after adding a drop of concentrated orthophosphoric acid. The remaining rhizosphere extract was then filtered through a Whatman number 1 filter paper and oven-dried at 70 °C for three days to determine the rhizosphere soil dry weight (Wang et al. 2016a; Kidd et al. 2018). Organic anions were determined in Shimadzu HPLC system (Shimadzu Corporation, Kyoto. Japan) using a Prevail™ organic acid column (300x4.6mm, 5µm particle size, Grace Davison Discovery Sciences) with 25 mM KH_2PO_4 (pH: 2.35) as a mobile phase, 0.6 ml min^{-1} as flow rate at 50 °C, an

injection volume of 30 µl, and a detection wavelength of 210 nm. Two standard stock solutions were prepared to calibrate the system. The first one contained a mixture of 10 organic acids and was prepared by dissolving all solid organic acids of tartaric acid (Sigma – Aldrich, 99.5 %), formic acid (ANALAR, 98 %), pyruvic acid (Sigma – Aldrich, 98 %), malic acid (Merck, >99 %), manolic acid (Sigma-Aldrich, 99 %), lactic acid (Acros Organics, 85 %), acetic acid (BDH, 100 %), citric acid (Sigma-Aldrich, 99.5 %), shikimic acid (Sigma – Aldrich, 99 %), and succinic acid (Sigma – Aldrich, 99 %) in 0.2 mM CaCl2 matrix solution to have a concentration of 1000 ppm for each organic acid. The second one contained only fumaric acid (Sigma- Aldrich, 99 %) due the presence of traces of fumaric acid in the organic acid mixture and was prepared by dissolving solid fumaric acid in 0.2mM $CaCl_2$ matrix solution to have a concentration of 5 ppm. From these two stock solutions, 6 different working standard curve solutions (from 0.05 to 80 ppm (depending on organic acids)) were prepared and used for calibration. Organic anions were identified by the comparison of retention time and absorbance of known organic acid standards. Lab solution software (Version 5.87 SP1) was used to process the Data. Different organic anions were detected in the rhizosphere of ryegrass, but due to the inconsistency of the results, only pyruvate was reported for this species; all other anions were omitted from the calculation of the total organic anions.

Soil P fractionation was conducted on the soil at day 0 and the rhizosphere soil after plant growth. For this purpose, the procedure developed by Hedley et al. (1982) was used with the modifications from Condron et al. (1996) and Condron and Newman (2011). Briefly, 0.5 g of air-dried soil was sequentially extracted by 10 ml of 1 M NH_4Cl, 0.5 M $NaHCO_3$ (pH 8,5), 0.1 M NaOH, 1 M HCl, and a second 0.1 M NaOH (to extract P in the micro-aggregates and the P protected by calcium) (Condron and Newman 2011), and then shaken for 16 hours in an end-over shaker to extract the following fractions: NH_4Cl-P, $NaHCO_3$-P, NaOH1-P, HCl-P and NaOH2-P. The last residue was dried at 50 °C and digested with concentrated H_2SO_4 and H_2O_2 for the determination of residual P (Olsen and Sommers 1982). Inorganic P (Pi) in the alkali extracts ($NaHCO_3$-Pi, NaOH1-Pi, NaOH2-Pi) was determined according to Dick and Tabatabai (1977) and He and Honeycutt (2005) to avoid overestimation of inorganic P due to mineralisation of labile organic P. The method of Murphy and Riley (1962) was used to determine Pi in the acid extracts (NH_4Cl-Pi, HCl-Pi, and residual-Pi). Total P (Pt) in the alkali extracts was measured using ICP-EOS according to do Nascimento et al. (2015), while organic P (Po) was calculated as the difference between Pt and Pi in each fraction. The total soil P was calculated as the sum of all nine fractions. The availability of a fraction for plant uptake was presented based on its depletion or accumulation from the rhizosphere (Cabeza et al. 2017), and this via its calculation as the difference between P in the soil at day 0 and the rhizosphere soil after plant growth for each treatment (Figure 2.6 and 2.7). The distribution of different P fractions in the four treatments at day 0 are reported in Table 2.3.

2.2.4 Data analysis

Data were subjected to a two-way analysis of variance (ANOVA) to determine the effects of N and P additions (nutrient effect) and plant species (plant effect) on plant and soil parameters. To test the effect of plant species on the depletion of different rhizosphere P fractions, we used one-way ANOVA or Kruskall-Wallis test when homoscedasticity was not met. The Tukey test was used to distinguish significant differences between treatment means at 5 % probability. In the case of variance heterogeneity, the Games-Howell *post-hoc* test was used to separate homogenous groups at 5 % probability. All statistical analyses were performed with SPSS 25 (IBM Corp, Armonk, NY, USA).

2.3 Results

2.3.1 Plant biomass and plant nutrient uptake

Phosphorus addition had a significant impact on plant growth. Plant species reacted to P addition as follows: white clover > ryegrass > wheat > blue lupin. In the P treatment, shoot biomass was increased by 413, 449, 118, and 146 % for white clover, ryegrass, wheat, and blue lupin, respectively, whereas root biomass increased by 388, 164, 27, and 10 %, respectively, compared to the control (Table 2.2). In the NP treatment, shoot biomass increased by 635, 504, 146, and 20 % for white clover, ryegrass, wheat, and blue lupin, respectively. Root biomass followed the same trend and was increased by 510, 393, 78, and 19 % for white clover, ryegrass, wheat, and blue lupin, respectively. On the other hand, N treatment showed no significant difference in plant biomass compared to the control, irrespective of plant species. Across treatments, the shoot to root ratio was higher in blue lupin with values greater than 1, while this ratio was 0.5, 0.7, and 0.6 for white clover, ryegrass, and wheat, respectively. Plant P concentration was significantly affected by plant species and nutrient addition. Blue lupin had the highest shoot P concentration (1.2 mg g^{-1}), while white clover showed the greatest root P concentration regardless of treatments (1.5 mg g^{-1}) (data not shown). Across plant species, the response of shoot and root P concentrations had a similar trend and significantly increased under P and NP treatments, though lower P concentrations were observed under the NP treatment, while no changes were noted in N treatment compared to the control (data not shown). Total P uptake enhanced by 5.3-, 3.7-, 1.5- and 0.4-fold after P addition in white clover, ryegrass, wheat, and blue lupin, respectively, whereas, in the NP treatment, it increased by 6.3-,4.3-, 1.4, and 0.3-fold for the same plant species (Table 2.2). Total P uptake was statistically similar under P and NP treatments on the one hand and the control and the N treatment on the other hand. Nitrogen addition significantly increased total N uptake across plant species compared to the control, while the effect of P addition on this parameter was even higher. The combined addition of N and P in this study had a synergistic effect on total N uptake being higher than the single nutrient input. Plant N

uptake increased significantly under blue lupin in response to nutrient inputs, but differences between nutrient treatments were not significant.

2.3.2 Rhizosphere pH

Soil pH measured in the rhizosphere of all plant species significantly decreased compared to the pH of the original soil (pH 6.4). A more pronounced decrease was observed in the rhizosphere of blue lupin and white clover, where the pH dropped to an average of 5.6 and 5.7, respectively, followed by ryegrass and wheat, with pH reaching an average of 5.9 (Figure 2.2).

2.3.3 Microbial biomass P

Plant species had a significant effect on microbial biomass P, with ryegrass exhibiting the lowest concentrations across treatments. P addition increased microbial biomass P across plant species with a significant increase by 45 % and 70 % under ryegrass and white clover, respectively (Figure 2.3a). N and NP treatments increased microbial biomass P across plant species, but there were no significant differences between those treatments and the control.

Table 2.2 Root and shoot biomass (g tube^{-1}), total P uptake (mg tube^{-1}), and total N uptake (mg tube^{-1}) for blue lupin, wheat, ryegrass, and white clover under the different nutrient treatments.

	Root biomass	Shoot biomass	Plant P uptake	Plant N uptake
		Blue lupin		
Control	1.2 ± 0.1 b	2.2 ± 0.1 b	3.54 ± 0.25 c	7.27 ± 0.36 b
N	1.4 ± 0.3 b	2.1 ± 0.1 b	3.26 ± 0.49 c	8.11 ± 0.93 a
P	1.7 ± 0.2 a	2.4 ± 0.1 a	4.84 ± 0.69 a	9.62 ± 0.66 a
NP	1.5 ± 0.2 a	2.6 ± 0.2 a	4.67 ± 0.70 b	9.28 ± 0.97 a
		Wheat		
Control	0.9 ± 0.0 c	1.1 ± 0.0 c	1.25 ± 0.08 c	0.56 ± 0.03 d
N	0.9 ± 0.1 c	1.1 ± 0.1 c	1.28 ± 0.13 c	1.20 ± 0.54 c
P	1.9 ± 0.1 b	1.4 ± 0.0 b	3.21 ± 0.09 a	1.91 ± 0.12 b
NP	2.2 ± 0.2 a	2.0 ± 0.2 a	3.08 ± 0.24 b	3.45 ± 0.32 a
		Ryegrass		
Control	0.4 ± 0.0 c	0.2 ± 0.0 c	0.58 ± 0.04 c	0.95 ± 0.07 d
N	0.4 ± 0.2 c	0.3 ± 0.1 c	0.73 ± 0.27 c	2.01 ± 0.15 c
P	1.0 ± 0.1 b	0.8 ± 0.1 b	1.65 ± 0.13 b	2.94 ± 0.17 b
NP	1.9 ± 0.2 a	1.1 ± 0.0 a	2.52 ± 0.25 a	6.23 ± 0.10 a
		White clover		
Control	0.2 ± 0.0[1] c	0.2 ± 0.0 b	0.38 ± 0.03 b	0.97 ± 0.06 d
N	0.3 ± 0.0 c	0.2 ± 0.0 b	0.46 ± 0.08 b	1.77 ± 0.33 c
P	1.0 ± 0.1 b	0.7 ± 0.1 a	2.03 ± 0.17 a	4.23 ± 0.54 b
NP	1.5 ± 0.1 a	0.9 ± 0.0 a	2.38 ± 0.39 a	5.63 ± 0.96 a

[1]Values represent the mean of four replicates ± standard errors. Different letters in columns denote a significant difference ($P < 0.05$) among nutrient treatments for a given plant.

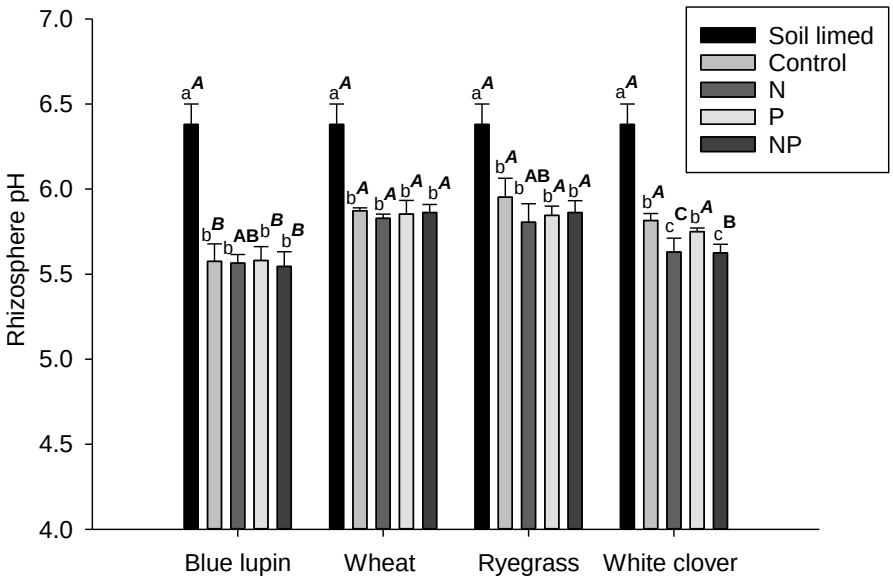


Figure 2.2 Rhizosphere pH in blue lupin, wheat, ryegrass, and white clover for the control (0N, 0P), N (200N, 0P), P (0N, 33P), and NP (200N, 33P) treatments compared to the pH of the original soil. Different lowercase letters represent a significant difference (*P* <0.05) among nutrient treatments for the same plant. Different uppercase letters represent a significant difference (*P* <0.05) among plant species for the same nutrient treatment.

2.3.4 Phosphatase activity

Acid phosphatase activity was not affected by plant species and nutrient treatments showing an average value of 6 µmol g^{-1} h^{-1} (Figure 2.3b). However, alkaline phosphatase activity was significantly impacted by plant species but was similar across nutrient treatments (Figure 2.3c). In fact, alkaline phosphatase activity under blue lupin (average of 2 µmol g^{-1} h^{-1}) was 60, 55, and 120 % higher compared to wheat, ryegrass, and white clover, respectively. Acid phosphatase activity was on average 4.4-fold higher than alkaline phosphatase activity in this study.

2.3.5 Organic anions

Organic anions released in the rhizosphere varied significantly among plant species and nutrient treatments (Figure 2.4). A total of nine organic anions were detected in this study, including citrate, malate, malonate, acetate, pyruvate, lactate, succinate, fumarate, and shikimate (Figure 2.4b). Legumes released significantly more organic anions than grasses. White clover had the highest total organic anions concentration with an average of 16 µmol g^{-1} dry soil, followed by blue lupin (11 µmol g^{-1} dry soil), wheat (1.2 µmol g^{-1} dry soil), and ryegrass (0.05 µmol g^{-1} dry soil). Interestingly, after P addition (P and NP treatments), the total concentration of organic anions increased by 11-, 10-, 5-, and 2-fold in the rhizosphere of blue lupin, white clover, wheat, and ryegrass, respectively, compared

with the control and N treatments (Figure 2.4a). The same trend was observed when total organic anion concentrations were expressed by unit of root dry matter (Figure 2.5).

In the rhizosphere of white clover, citrate was the prominent organic anion released with 42 % of total organic anions followed by malonate (21 %), acetate (20 %), and malate (13 %), whereas the blue lupin rhizosphere was enriched with citrate (41 %) followed by malate (36 %) and malonate (7 %). The wheat rhizosphere showed malate accounting for 81 % of total organic anions, while pyruvate was the only organic anion reported in the rhizosphere of ryegrass due to the inconsistency found in the other organic anions detected (Figure 2.4b).

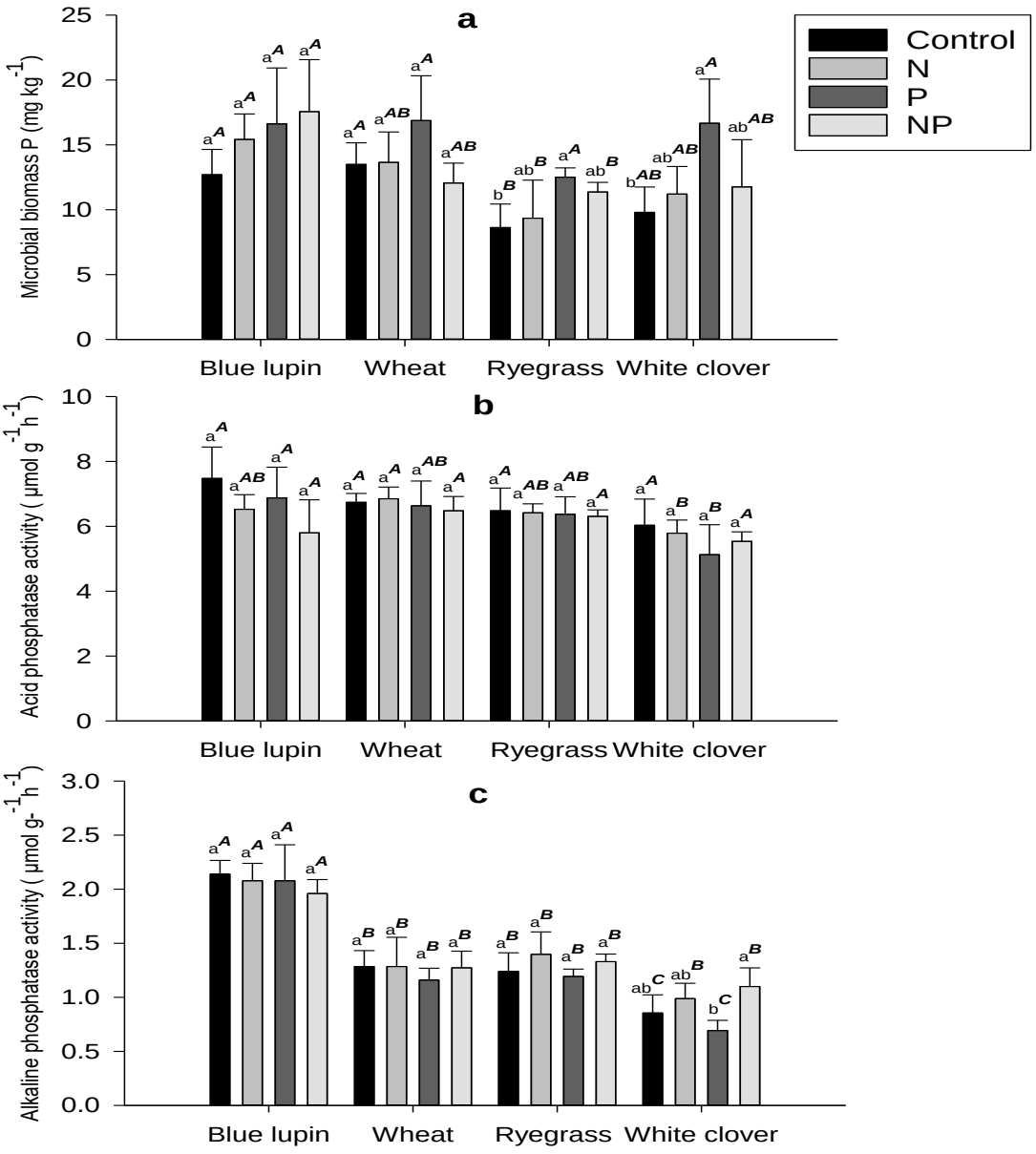


Figure 2.3 Microbial biomass P (a), acid (b) and alkaline phosphatase activity (c) in the rhizosphere of blue lupin, wheat, ryegrass, and white clover for the control (0N, 0P), N (200N, 0P), P (0N, 33P), and NP (200N, 33P) treatments. Different lowercase letters represent a significant difference (P <0.05) among nutrient treatments for the same plant. Different uppercase letters represent a significant difference (P <0.05) among plant species for the same nutrient treatment.

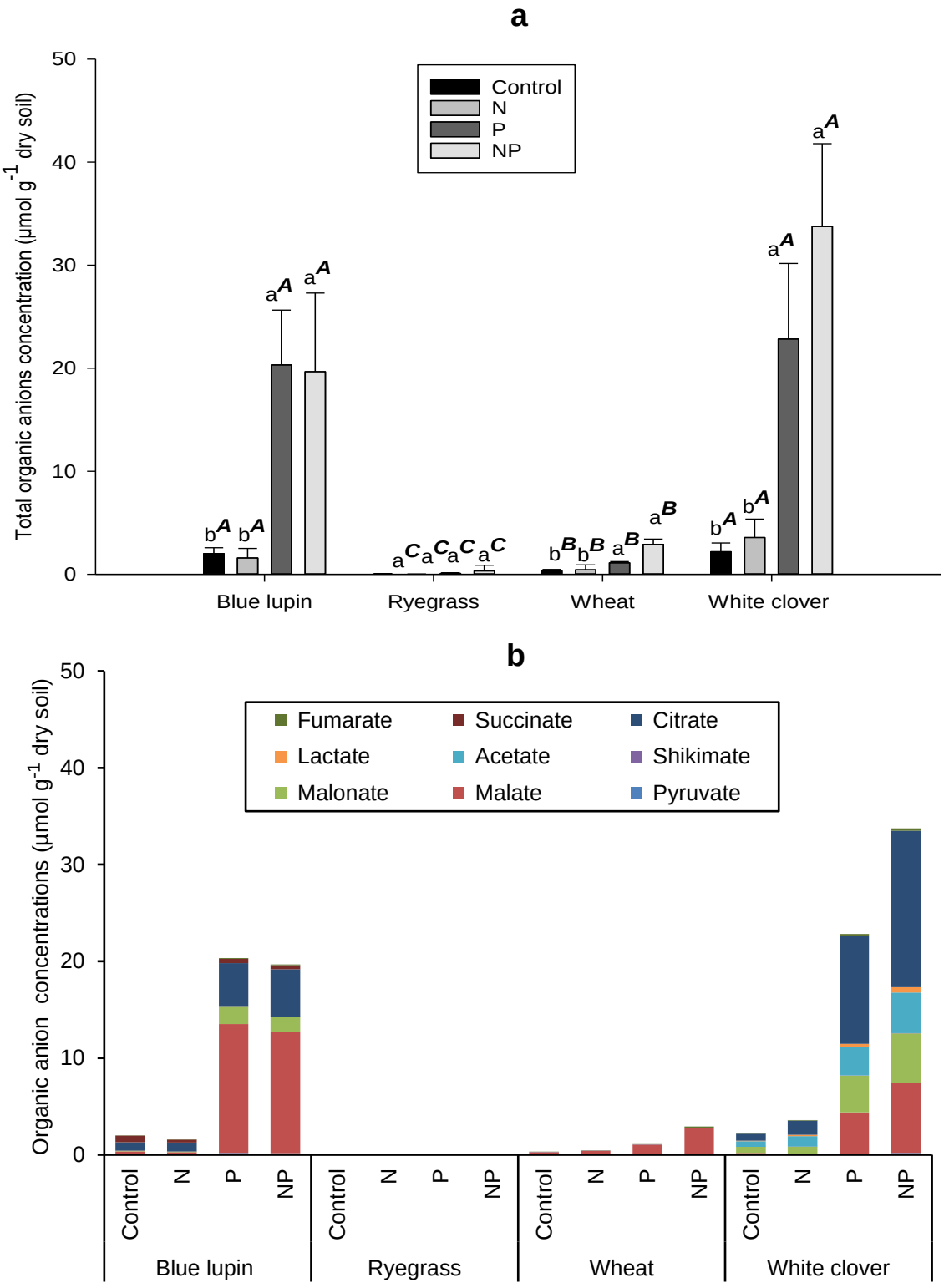


Figure 2.4 Total organic anions (a) and their composition (b) in the rhizosphere of blue lupin, wheat, ryegrass, and white clover for the control (0N, 0P), N (200N, 0P), P (0N, 33P), and NP (200N, 33P) treatments. Different lowercase letters represent a significant difference (P <0.05) among nutrient treatments for the same plant. Different uppercase letters represent a significant difference (*P* <0.05) among plant species for the same nutrient treatment.

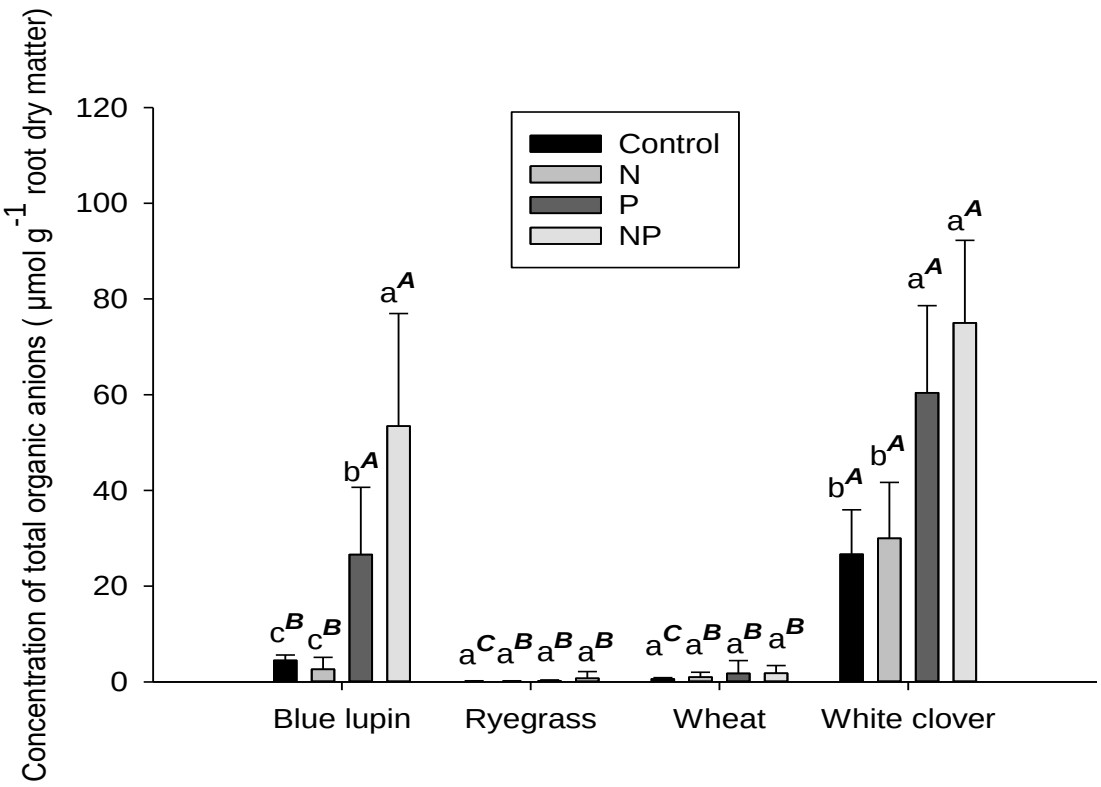


Figure 2.5 Total organic anions in the rhizosphere of blue lupin, wheat, ryegrass, and white clover for the control (0N, 0P), P (0N, 33P), N (200N, 0P), and NP (200N, 33P) treatments. Different letters represent significant difference (*P*<0.05) among treatments for the same plant and different uppercase letters represent significant difference (*P*<0.05) among plant species for the same treatment.

2.3.6 Rhizosphere P fractions

The four plant species depleted all inorganic P fractions in the control and N treatments, except the Ca-P bound fraction (HCl-Pi) that showed an accumulation. Higher depletion of moderately labile Pi (NaOH1-Pi) and stable Pi (NaOH2-Pi) was observed across plant species with an average of 8 and 7 mg kg^{-1}, respectively (Figure 2.6a, c). Interestingly, in P and NP treatments, plant species were able to deplete all inorganic P fractions but with a more pronounced depletion of moderately labile Pi (NaOH1-Pi) that showed a 4-fold increase compared to that under no P supply (Figure 2.6b, c).

The labile Po fraction ($NaHCO_3$-Po) was depleted across plant species and nutrient treatments, except for blue lupin, which accumulated this fraction in the control and the P treatment. Blue lupin significantly depleted moderately labile Po (NaOH1-Po) (around 14 mg kg^{-1} soil) under the control and P treatment, while wheat and ryegrass accumulated this fraction regardless of nutrient treatment (Figure 2.7a, b, c, and d). Without P supply, blue lupin, white clover, wheat, and ryegrass depleted stable Po (NaOH2-Po) by an average of 21, 11, 11, and 9 mg kg^{-1} soil, respectively whereas this depletion increased by 1.5-fold across plant species after P addition. No changes have been

observed in the residual P in the rhizosphere of the four plant species compared to the original soils; thus, residual P was not reported in the corresponding tables and figures. Nitrogen addition did not have any impact on P dynamics, and changes in P fractions were similar in the control and N treatment on the one hand and P and NP treatments on the other (data not shown).

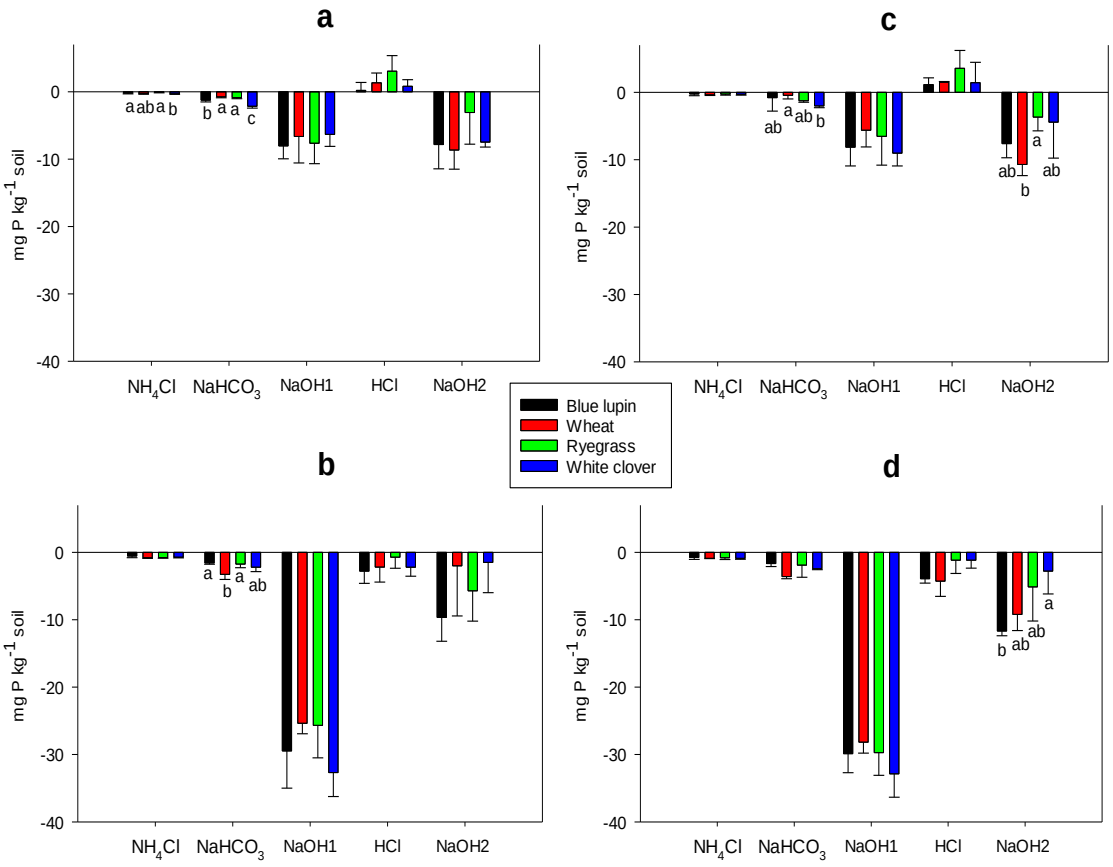


Figure 2.6 Change (depletion or accumulation) in different inorganic P fractions in the control (0N, 0P) (a), P (0N, 33P) (b), N (200N, 0P) (c), and NP (200N, 33P) (d) treatments in the rhizosphere of blue lupin, wheat, ryegrass, and white clover. Different letters represent a significant difference (*P* <0.05) among plant species for a given nutrient treatment.

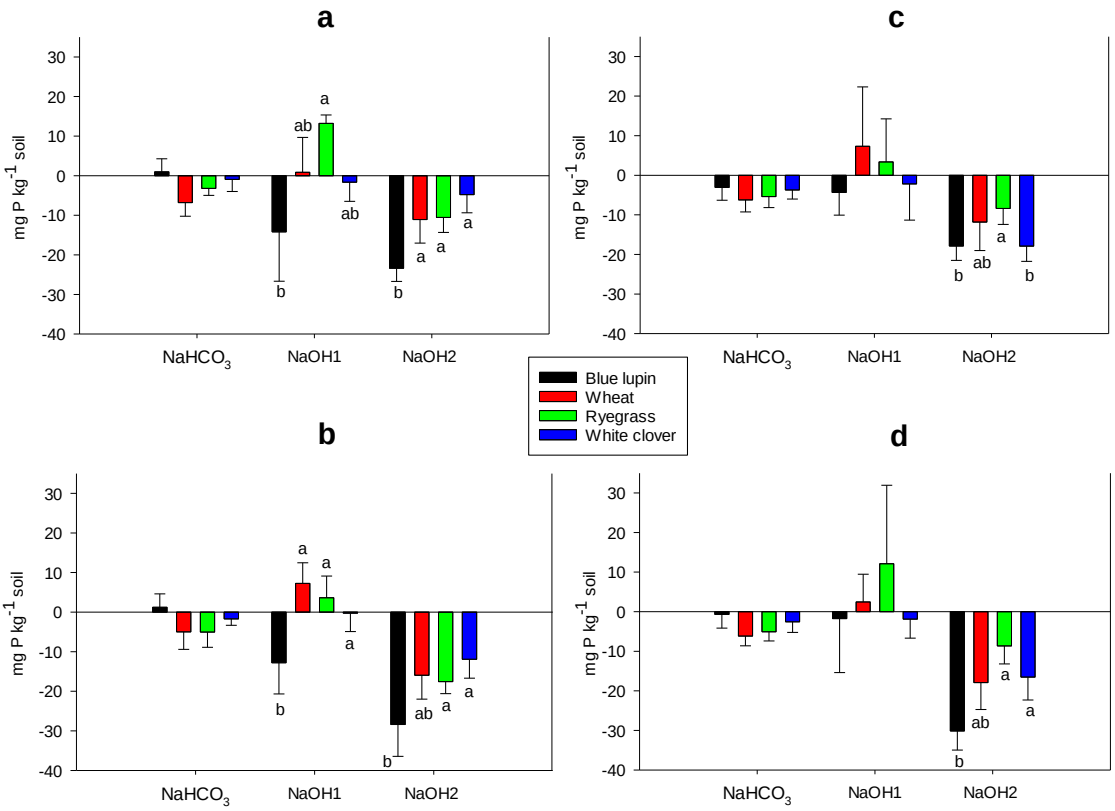


Figure 2.7 Change (depletion or accumulation) in different organic P fractions in the control (0N, 0P) (a), P (0N, 33P) (b), N (200N, 0P) (c), and NP (200N, 33P) (d) treatments in the rhizosphere of blue lupin, wheat, ryegrass and white clover. Different letters represent a significant difference (P <0.05) among plant species for a given nutrient treatment.

Table 2.3 Distribution of different soil P fractions in the four treatments at day 0 (before starting the experiment but after N and P additions) and relative accumulation compared to P treatments. The control and N treatments were reported in the same column as was for the P and NP treatments.

	P concentration (mg kg^{-1})		
	Control and N treatments	P and NP treatments	Relative accumulation
NH_4Cl-Pi	0.6 ± 0.1[1]	1.3 ± 0.2	117%
$NaHCO_3$-Pi	7.5 ± 0.2	12.1 ± 0.2	34.5%
$NaHCO_3$-Porg	56.5 ± 1.6	57.5 ± 3.4	1.8%
NaOH1-Pi	103.3 ± 2.5	144.1 ± 5.5	39.5%
NaOH1-Porg	455.9 ± 3.8	460.7 ± 7.9	1%
HCl-Pi	9.5 ± 1.8	12.9 ± 1.8	35.8%
NaOH2-Pi	59.3 ± 5.1	59.6 ± 3.5	0.5%
NaOH2-Porg	146.5 ± 4.8	155.8 ± 8.0	6.3%
Residual-P	396.7 ± 41.6	400.0 ± 35.9	0.8%

[1]Values represent the mean of eight replicates ± SE

2.4 Discussion

2.4.1 Plant response to nutrient addition

In line with our hypotheses, P addition increased plant biomass together with total P uptake regardless of plant species. Moreover, white clover exhibited the highest increase in root and shoot biomass in response to P addition, while blue lupin was the least affected by P supply but showed the highest shoot P concentration across plant species. The high P demand of white clover may explain the high response of this crop to P addition (Kidd et al. 2016; Pang et al. 2018b). With the highest shoot to root ratio, blue lupin demonstrated its aptitude for soil P acquisition as confirmed by previous studies (Egle et al. 2003; Wang et al. 2008; Funayama-Noguchi et al. 2015). In contrast to our hypothesis, N addition did not increase plant biomass nor improved total P uptake across plant species. This suggests that N was not a limiting factor for plant growth in this study. In fact, legume plants did not show any root nodulation in our experiment. The lack of response to N addition could be ascribed to the mineralisation of N from organic matter during the soil incubation we have carried out to increase soil pH before starting the experiment. Organic matter mineralisation could have increased the availability of both N and P; however, plant biomass increased in response to P addition but not N addition in this study (Gentile et al. 2012). Except for blue lupin, P addition promoted total N uptake by plant species compared to the control and N treatments, while the combined addition of N and P had a synergistic effect on total N uptake greater than the single nutrient addition. Scott et al. (2015) found that the application of N to high P fertility pasture soil reduced N leaching due to increased plant biomass utilising more N compared to low P fertility soil. Similarly, in a recent study, Francisquini Junior et al. (2020) described that P applications not only increased forage yield in a grass-legume pasture but also showed higher N use efficiency, irrespective of the solubility of P source. In the current study, P addition to P deficient soil increased plant biomass, thereby increasing plant N uptake. Moreover, P addition could increase soil microbial activity, thus triggering organic matter priming effect by soil microbes releasing higher amounts of available nitrogen. We acknowledge that the small tube volume used may have restricted plant growth in this study (Poorter et al. 2012).

2.4.2 Rhizosphere pH

Changes in rhizosphere pH have been reported in P deficient soils and in response to nutrient addition, with decreases and increases according to plant species, soil types, initial soil pH, and nutrients forms (Marschner and Römheld 1983; Hinsinger et al. 2003, 2011; Li et al. 2008a; Kreuzeder et al. 2018). In this study, rhizosphere pH was affected by plant species but was similar across nutrient treatments. Among species, legumes are known to acidify the rhizosphere soil via the release of protons to compensate for excess cations uptake (Tang et al. 1997; Hinsinger et al. 2003;

Tang and Rengel 2003). Indeed, our findings illustrated that legumes, especially blue lupin, exhibited lower rhizosphere pH compared to the other plant species. Provided that the soil was limed with 4 tonnes per hectare before starting the experiment (see Materials and Methods) and that legume plants have not shown root nodulation, we assume that the soil buffering capacity (Schaller 1987) as well as the abundance of soil nitrogen (Nuruzzaman et al. 2006; Sugihara et al. 2016) might have cancelled out the effect of nutrient addition on rhizosphere pH. Additionally, Youssef and Chino (1989) reported that differences in rhizosphere pH are more related to plant species and initial bulk soil pH than nitrogen forms and application rates. Overall, there was no clear effect of P and N additions on rhizosphere pH in this study.

2.4.3 Microbial biomass P

Phosphorus addition increased microbial biomass P across plant species and especially in ryegrass and white clover. This corroborates the results reported by Dodd et al. (2014) after adding inorganic P fertilisers to a similar soil used in our study. They concluded that an increased microbial biomass P was removing P from the soil solution. In fact, when sufficient carbon and N are present in the soil, microorganisms tend to immobilise available inorganic P in order to maintain the stoichiometric ratio of their biomass (Heuck et al. 2015; Spohn and Widdig 2017). Microbial biomass P was not improved by N addition across plant species, which is in line with the findings reported by Deng et al. (2017) in their meta-analysis. As discussed, this result emphasised that N was not limiting in our soil and that P was the only limiting nutrient for both microbial and plant growth.

2.4.4 Phosphatase activities

Acid phosphatase activity was not affected by plant species or nutrient addition in this study. Several reports have shown that P deficiency did not always imply an increase of phosphatase activity, while the addition of soluble P felt to show a repression effect on phosphatase enzymes in different agroecosystems (Adams 1992; Clarholm 1993; Starnes et al. 2008; Mat Hassan et al. 2012; Yang et al. 2015). In acidic soils, the activity of acid phosphatase enzymes is considered to be higher compared to other phosphomonoesterases (Skujins et al. 1962; Tabatabai 1994). In our study, rhizosphere pH was affected by plant species but was still acidic under the four plant species. This could explain why no differences were detected in acid phosphatase activity. Moreover, phosphatase enzymes can be adsorbed to organic matter and the soil solid phase, especially clay minerals (Tabatabai 1994; Nannipieri et al. 2011), thus complicating the interpretation of enzyme assays as they could include the active, latent, and adsorbed enzymes (Nannipieri et al. 2002). Nitrogen addition can probably increase acid phosphatase activity as N is an essential component of phosphatase enzymes (Duff et al. 1994; Olander and Vitousek 2000). However, N was not a limiting factor in the soil used in this

study; thus, plants and microorganisms may have used this excess N for the synthesis of acid phosphatase enzymes (Marklein and Houlton 2012).

In contrast to acid phosphatase activity, alkaline phosphatase activity was affected by plant species, with blue lupin exhibiting significantly higher activity in its rhizosphere. However, no obvious nutrient effect was observed across plant species for these enzymes. Alkaline phosphatase enzymes are believed to be mainly released by microorganisms (Nannipieri et al. 2011) and affected by plant species, microbial community composition, and rhizodeposition (Badri and Vivanco 2009; Wasaki et al. 2018; Finkel et al. 2019). For instance, Wasaki et al. (2018) found that the rhizosphere of P deficient white lupin plants exhibited an increase in the alkaline phosphatase activity derived from microbes together with a decrease in different organic P forms such as phytates. Moreover, alkaline phosphatase enzymes are ubiquitous in soils, but their activity is somehow influenced by the quantity and quality of rhizodeposits (Ragot et al. 2017; Wasaki et al. 2018; Wu et al. 2018; Wei et al. 2019a). Our results showed a substantial depletion of stable organic P (NaOH2-Po) under blue lupin coupled with higher alkaline phosphatase activity. Although our data showed that blue lupin had the highest alkaline phosphatase activity, microbial biomass P was not significantly different among the four plant species. These findings indicate that white lupin may have shaped its microbial niche towards a cohort of microbes dedicated to releasing alkaline phosphatase enzymes, probably via rhizodeposition (Wasaki et al. 2018; Wu et al. 2018). Nevertheless, a more detailed assessment of microbial diversity and phosphatases genes abundance and expression are required to verify this hypothesis.

2.4.5 Organic anions

Organic anion release is a physiological adaptation of plants to P deficiency (Neumann and Römheld 1999), and its contribution to enhancing P availability has been widely demonstrated (Bolan et al. 1994; Veneklaas et al. 2003; Oburger et al. 2011). Our results showed the presence of 9 organic anions in the rhizosphere of the four plants studied, with citrate, malate, and malonate representing the main organic anions released. Legumes released higher concentrations of organic anions in their rhizospheres compared to grass plants, which corroborates previous studies reporting that legumes relied on organic anions to increase their P acquisition (Earl et al. 1979; Veneklaas et al. 2003; Nuruzzaman et al. 2006; Pearse et al. 2006). Citrate, malate, and malonate have been described to be efficient at the desorption, chelation, and complexation of Fe and Al oxides to liberate P for plant uptake (Jones 1998; Hocking 2001; Gerke 2015; Wang et al. 2016b). Most of the inorganic P added in our study was found in the NaOH-P fraction, suggesting that P was bound to Fe and Al oxides (Table 2.3). Therefore, the depletion of sparingly available inorganic P (NaOH-Pi) observed in the rhizosphere of the four plants could be attributed to the release of organic anions. However, our

results showed contrasting amounts of organic anions measured in the rhizosphere of the four plant species, while no significant differences were observed in the depletion of NaOH1-Pi and NaOH2-Pi fractions among plant species. These findings suggest that organic anions were not related to P acquisition of sparingly available inorganic P (NaOH-Pi) in our study and that some other parameters might have been involved in this acquisition, such as rhizosphere pH and root morphology. Indeed, several scholars have highlighted that organic anions played a minor role in inorganic P acquisition and that plant species exhibited considerable variations in the release of organic anions even under the same genus (Jones 1998; Pearse et al. 2006, 2007). For instance, Pearse et al. (2007) found no consistent relationship between the ability to mobilise different inorganic P forms and carboxylates exudation from plant roots. Likewise, Wang et al. (2016a) pointed out that organic anion release under wheat, oat, potato, and canola did not improve P availability and was not related to shoot P uptake.

The expression of organic anions by unit of root dry matter has given similar results as by soil dry matter (Figure 2.5), which emphasised that organic anions released after P addition were not solely derived from plants but could come from other sources. Most of the studies looking at organic anion release and their influence on P availability and plant P uptake have been carried out on sand or liquid cultures. Therefore, the impact of soil organic matter and soil microorganisms on organic anion release is still not well understood (Jones 1998; Wang et al. 2016a; Oburger and Jones 2018). It has been found that inorganic P inputs to grassland soils increased microbial activity, thus triggering soil organic matter degradation and carbon mineralisation (Condron et al. 2012; Wakelin et al. 2014, 2017). Recent findings have shown that the addition of inorganic P increased microbial respiration and the desorption of organic carbon (Spohn and Schleuss 2019). Thus, the increase of organic anions found after P addition in our study could be partly ascribed to an increased priming effect on soil organic matter by soil microorganisms. Up to 50 % of soil microbes are able to mobilise soil P by duplicating different mechanisms used by plants, such as the exudation of organic anions and the release of phosphatase enzymes (Jones 1998; Schefe et al. 2008; Nannipieri et al. 2011; Richardson and Simpson 2011; Zhou et al. 2018). Plants and microorganisms are in competition for available nutrients in the soil solution, especially in low fertility conditions (Richardson 1994; Jakobsen et al. 2005; Blackwell et al. 2010). Our results showed that the addition of inorganic P increased microbial biomass P, indicating an immobilisation of inorganic P by microbes. Thus, plants and microorganisms may have increased their organic anion release to mobilise more P needed to meet their P demand (Li et al. 2018). Plant biomass increased by 3.5 and 1.2-fold in white clover and blue lupin, respectively after P addition compared to the control. This indicated that the increase in organic anion release under white clover could mainly coming from plant roots without excluding other sources such as soil microbes and organic matter. However, it is suggested that organic anion release

under blue lupin could be a combination of different sources, including soil microbes, organic matter degradation, and plant roots. Nevertheless, the relative contribution of microbes, plants, and soil organic matter to organic anion release in soils and rhizospheres needs further research (Schefe et al. 2008).

In summary, our results suggest that (1) organic anions played a minor role in the acquisition of sparingly available inorganic P and that (2) organic anions measured in the rhizosphere of legume species after P addition could be a combination of different sources such as soil microbes, microbial priming effect on soil organic matter, and release from plant roots.

2.4.6 Rhizosphere P fractions and P acquisition

Inorganic P can be adsorbed to the active mineral surfaces of Fe, Al, and Ca, thus decreasing its availability for plant uptake (Hedley et al. 1982; Hinsinger 2001; Pierzynski and McDowell 2005). Our results showed that the four plant species depleted different inorganic P fractions, including readily available (NH_4Cl-Pi), labile ($NaHCO_3$-Pi), moderately labile (NaOH1-Pi), and stable inorganic P (NaOH2-Pi). Whereas after P addition, a 4-fold increase in the depletion of moderately labile inorganic P (NaOH1-Pi) was observed, most likely due to an increased plant P uptake and microbial immobilisation. Depletion of labile Pi fractions has been reported by different authors (Wang et al. 2008; Vu et al. 2008; Mat Hassan et al. 2012; Ye et al. 2018), while Chen et al. (2003a) found that the NaOH-Pi fraction was accessible by plants and able to supply available P in a range of temperate grassland soils. In the literature, the depletion of NaOH-Pi was mainly related to the action of organic anions (Jones 1998; Oburger et al. 2011; Gerke 2015). In our study, the depletion of this fraction was similar across plant species, although different amounts of organic anions were found in the rhizosphere of the four plant species. As discussed previously, this indicates that the depletion of this fraction was not directly related to organic anions, and some other factors such as root traits and rhizosphere pH may have been involved in the depletion of this fraction. Our results showed that calcium-P (HCl-Pi) increased in the absence of P input which could be ascribed to the complexation of some inorganic P by free calcium cations present in the soil due to liming. In contrast, the depletion of this fraction after P addition may reflect an increased plant P uptake. Overall, our findings indicated that under P deficient conditions and restricted soil volumes (rhizosphere soil), plants are able to mobilise both labile and recalcitrant inorganic P fractions to meet their P demand.

Organic P can represent a high proportion of total P in soils (George et al. 2018), especially in pastures where organic residues have little turnover and accumulate (Dalal 1977; McDowell and Condron 2012; Nash et al. 2014). In our study, organic P represented more than 50 % of total P mainly comprised of NaOH-Po, thus being a potential source of P for plants uptake (Condron and Goh 1989; Condron et al. 1996; Nash et al. 2014; Wei et al. 2019a). Phosphatase enzymes are released by

plants and microorganisms to mobilise organic P via the cleavage of carbon-P bonds, which release available orthophosphates into the soil solution (Condron et al. 2005; Nannipieri et al. 2011). From all the organic P fractions, stable organic P (NaOH2-Po) was the most depleted by the four plants species, though a 1.5-fold depletion was noted after P addition for this fraction, most likely due to increased plant uptake. Although listed as stable, this is defined by resistance to extraction and clearly not to action by plants or microbes. More importantly, our findings showed that blue lupin significantly depleted this fraction compared to other plant species, which concurred with higher activity of alkaline phosphatase enzymes in the rhizosphere of this plant. White lupin has been found to deplete organic P pools due to higher alkaline phosphatase activity derived from microbes, probably due to the release of an array of carboxylases shaping its microbial community composition (Wasaki et al. 2018). In another study carried out by Wei et al. (2019), alkaline phosphatase enzymes released by a specific bacterial community in the rhizosphere of rice was linked to the depletion of organic P. Thus, we suggest that alkaline phosphatase enzymes were possibly responsible for the higher aptitude of blue lupin to deplete stable organic P in this study. Residual P changed little during our experiment, presumably due to its high recalcitrance together with the mobilisation of other P pools (Li et al. 2008a; Ye et al. 2018).

Several studies have shown that in N-limited environments, N addition in the form of fertilisers or via atmospheric deposition can contribute to the depletion of different P fractions and can exacerbate P-limitation due to increased phosphatase activity and rhizodeposition (Marklein and Houlton 2012; Zang et al. 2017; Chen et al. 2018; Fan et al. 2019). However, our study showed no effect of N addition on P dynamics and transformation. This is probably because N was not a limiting factor in our soil, and therefore it did not influence plant growth and rhizosphere processes related to P-acquisition in the four plant species used in this experiment.

Provided that in our study, rhizosphere P fractions were compared to the soil at day 0, we acknowledge that some P depletions, especially in the labile P fractions, could be partly due to wetting and drying processes, though depletions across plant species were consistent. Moreover, we estimate that the P supplied by seeds was negligible compared to P present in the soil (soil total P: 1236 mg kg^{-1}) and would not probably impact soil P dynamics (Saeed et al. 2017; Julia et al. 2018). Moreover, the weight of seeds for ryegrass and white clover were extremely small (ryegrass was 15 and 7 times smaller than white lupin and wheat, respectively, while white clover was 50 to 25 times smaller than white lupin and wheat, respectively), while the depletion of P fractions was similar among plant species. Therefore, we suggest that the number of seeds would have had minimal or no effect on soil P dynamics.

2.5 Conclusions

Plant species and nutrients availability are among key parameters governing soil P-acquisition. Our study showed that legumes and grasses reacted differently to P addition in terms of plant biomass, total P uptake, and rhizosphere processes involved in soil P-acquisition. Phosphorus addition alone or in combination with N increased plant growth, total P and N uptake, microbial biomass P, and organic anion concentrations across plant species but did not affect acid phosphatase activity and rhizosphere pH. In contrast, N addition alone had no effect on plant biomass and soil P fractions compared to the control, indicating that N was not a limiting factor for plant growth. This result was attributed to high amounts of available N present in the soil, probably driven from organic matter mineralisation. Organic anion concentrations increased after P addition, especially under legumes. This result could be mainly attributed to the release of organic anions from plant roots under white clover, while a combination of plant and microbial sources under blue lupin. sources under blue lupin it could be ascribed to a combination of sources, including activity of soil microbes either via microbial release or microbial priming effect on soil organic matter. Alkaline phosphatase activity was higher under blue lupin. Moderately labile inorganic P (NaOH1-Pi) and stable organic P (NaOH2-Po) were the most depleted fractions across plant species, with blue lupin exhibiting higher ability in mobilising the later P pool. The depletion of these two P fractions increased after P addition, most likely due to increased plant biomass. Although releasing different amounts of organic anions, the four plant species showed similar depletion of inorganic P fractions in their rhizospheres. We conclude that organic anions played a minor role in inorganic P-acquisition under the four plant species investigated in this study. The higher depletion of stable organic P observed in the rhizosphere of blue lupin could be related to alkaline phosphatase activity. Furthermore, in restricted soil volumes such as the rhizosphere, plants can mobilise different inorganic and organic P fractions regardless of their potential chemical availability and can still deplete sparingly available P fractions despite being supplied with soluble inorganic P.

Chapter 3

Impacts of Nitrogen and Phosphorus Addition and Seasonal Conditions on Plant Growth, Soil Microbial Biomass and Activity, and Phosphorus Dynamics in Nitrogen Limited Pasture Soil

3.1 Introduction

Nitrogen (N) and phosphorus (P) are the most common nutrients limiting plant primary productivity (Elser et al. 2007; Vitousek et al. 2010). In general, N:P ratio in the soil, microbial biomass, and plants is 16:1, meaning that in a situation where there are >16 moles of N to 1 mole of P, soils, microbes, or plants are P-limited, and N-limited if the ratio is <16:1 (Redfield 1934). Such limitations may have severe impacts on productivity. Consequently, N and P fertilizers are commonly applied to agroecosystems (Tilman et al. 2002; Haygarth et al. 2013); however, excess fertilizer inputs has raised many concerns about accelerated eutrophication and build-up of soil P legacy (Bouwman et al. 2017; McDowell et al. 2020; Pavinato et al. 2020).

It is well recognised that modifying stoichiometry through fertilisation can affect soil nutrient dynamics in agroecosystems (Liu et al. 2019; Dai et al. 2020; Widdig et al. 2020; Cui et al. 2021). Using P fractionation combined with ^{18}O isotopes, Bauke et al. (2018) found that continual P applications under N deficiency increased P accumulation due to lower plant production and higher P inputs compared to plant P demand. However, an adequate application of NPK increased the recycling of Ca-P pool in the subsoil, which was attributed to an increase of root growth and microbial activity under sufficient nutrient conditions in the topsoil. On the other hand, Scott et al. (2015) indicated that the application of N to high P fertility pasture soil reduced N leaching due to increased plant biomass. Similarly, Francisquini Junior et al. (2020) described that P applications not only increased forage yield in a grass-legume pasture but also showed higher N use efficiency, irrespective of the solubility of P source.

Nitrogen applications have been used as an effective way of phytoremediation of high P contaminated soils (Newman et al. 2009; van der Salm et al. 2009). Due to increased plant production in response to N inputs, increased offtake of soil P was observed (Messiga et al. 2014; Liu et al. 2019), along with a significant decrease in P losses (Dodd et al. 2014). Besides the increase in plant yield, N addition can affect soil chemical properties, microbial activity, and root traits, thereby influencing soil P dynamics. For example, N addition can decrease soil pH, thus enhancing the availability of more recalcitrant inorganic P forms (Sherman et al. 2006; Fan et al. 2019). Nitrogen is a

principal component of phosphatase enzymes involved in organic P mineralisation (Olander and Vitousek 2000; Marklein and Houlton 2012). Nitrogen supply can increase rhizodeposition with a concomitant increase in microbial activity and plant P uptake (Xiao et al. 2019; Bicharanloo et al. 2020). On the other hand, N applications can increase mycorrhizal symbiosis as well as root biomass and density, thereby expanding the soil volume explored and subjected to rhizosphere processes (Treseder 2004; Yuan and Chen 2012; Fan et al. 2019; Schleuss et al. 2020).

The effects of N addition on soil P dynamics have been shown to differ according to soil types, agroecosystems, N applied, as well as climatic conditions (Arenberg and Arai 2019; Taylor et al. 2021). Under pasture systems in particular, discrepancies have been found in soil P dynamics as affected by N fertilization. For instance, urea application to a semi-arid grassland for 11 years enhanced readily available P and promoted the transformation of refractory to less available inorganic P fractions (Wang et al. 2021a). Labile P decreased, while alkaline phosphatase activity and organic P mineralization increased with increasing N applications (mineral + organic) to N-limited semi-arid grassland in China (Cui et al. 2021). On the other hand, N addition in the form of NH_4NO_3 decreased labile Pi fractions but did not stimulate Po mineralization in a calcareous grassland in Inner Mongolia (Liu et al. 2019).

Previous studies under pasture systems have shown that N and P inputs can have a significant impact on soil P immobilization and mineralization processes as well as microbial and earthworm community composition (Sarathchandra et al. 2001; Chen et al. 2014; McLaren et al. 2020; Cui et al. 2021). Although these studies have revealed some new insights on microbial and biochemical processes linked to P cycling, they were based on soil samples taken at one single point and carried out under either N or P inputs. Therefore, the assessment of temporal changes and interactions between N and P inputs has been recommended for a more detailed understanding of the impact of nutrient inputs on soil P dynamics (Dai et al. 2020; Cui et al. 2021).

Beside P, N inputs have been increasingly applied to New Zealand pasture soils to enhance the productivity and profitability of dairy farms (Parfitt et al. 2012; Pinxterhuis and Edwards 2018). In fact, N inputs in New Zealand increased by more than 6-fold between 1990 and 2015, mainly as urea fertilizer (Stats NZ 2019). In recent years, Italian ryegrass (*Lolium multiflorum* Lam.) has shown promising results in mitigating N losses under intensively managed dairy farms, thus being considered a great alternative to the well-established grass-legume pastures in New Zealand (Woods et al. 2018; Al-Marashdeh et al. 2021). While the impact of N fertilization on N dynamics has been well studied under Italian ryegrass (*Lolium multiflorum* Lam.) in New Zealand (Malcolm et al. 2015; Woods et al. 2017; Maxwell et al. 2019), little is known in terms of P dynamics under this promising grass crop, especially in response to the single and combined addition of N and P inputs.

Past research carried out in pasture systems mainly assessed the impact of N addition on soil P availability and changes in different inorganic P fractions (Liu et al. 2019; Wang et al. 2021a), whereas experiments combining N and P additions focused on investigating organic P mobilization and the release of phosphatase enzymes (Tian et al. 2016; Schleuss et al. 2020). Therefore, scare data is available under pasture systems detailing transformations in inorganic and organic P pools along with changes in soil microbial biomass and activity as affected by N and P additions. This study aimed at determining the short-term effects of N fertilization (2 years), alone or when combined with P, on plant growth, soil microbial biomass P, phosphatase enzyme activity, as well as changes in different inorganic and organic P fractions under an intensively managed grass-pasture (Italian ryegrass (*Lolium multiflorum* Lam.)). We hypothesized that (1) P addition would increase plant biomass while inhibiting soil microbial activity through immobilization of P and a decrease of phosphatase activity; that (2) N addition would accelerate P cycling through an increase of phosphatase activity and enhance the mobilization of recalcitrant P fractions; and that (3) the combined addition of N and P would have a relative advantage in plant biomass, P uptake, and mobilisation of soil P fractions compared to single P addition.

3.2 Material and methods

3.2.1 Site description and soil characteristics

The study site was located at Lincoln University, New Zealand (latitude: 43°38'S, longitude: 172°27'E, and altitude: 9 m). The climate is warm and temperate, with a mean air temperature of 11.3 °C and a mean annual rainfall of 640 mm. The soil was a Wakanui silt loam (NZ classification: Mottled Immature Pallic; USDA classification: Udic Ustochrept) that had not been P fertilised for the last nine years and had been cultivated between 2010 and 2016 with wheat (*Triticum aestivum* L.), Italian ryegrass (*Lolium multiflorum* Lam.), kale (*Brassica oleracea spp. acephala*), and green globe turnips (*Brassica rapa*), respectively. In 2017, the study site was sprayed with glyphosate, ploughed, and sown with Italian ryegrass (*Lolium multiflorum* Lam. cv. Tabu) at 20 kg ha^{-1}. The soil characteristics before starting the experiment were sand: 34.4 %, silt: 54.5 %, clay: 11.1 %, pH: 6.3, organic matter: 4.3 %, total C: 26 g kg^{-1}, total N: 2.3 g kg^{-1}, C/N: 11.3, total P: 780 mg kg^{-1}, Olsen P: 10 mg kg^{-1}. The soil was considered low in total C, total N, and available P for New Zealand soils, according to Blakemore et al. (1982). The nutrient treatments were control (no fertilisation), P (application of 50 kg P ha^{-1} y^{-1}), N (application of 250 kg N ha^{-1} y^{-1}) and NP (application of 50 kg P ha^{-1} + 250 kg N ha^{-1} y^{-1}). Each plot was 3 x 5 m^2 with a one-meter buffer strip to avoid nutrient transfer. The experiment was set up in a randomised block design with 5 replicates (Figure 3.1). Phosphorus fertilisation (single superphosphate) was carried out in August 2018 and 2019, whereas nitrogen (urea) was split in 5 applications (50 kg N ha^{-1} each) for optimal annual yield (Sun et al. 2008) and applied in August, October, November, March, and April each year. Besides P and N, the plots received 50 kg ha^{-1} of

sulfur (elemental sulfur) in August 2018 and 2019. All fertilisers were applied manually. The plots were irrigated with sprinklers when soil moisture in the top 0-7.5 cm was below 20 % gravimetric water holding capacity. Following soil and plant sampling, plots were cut at 2-3 cm high with a mower, and plant biomass was removed.

Figure 3.1 Picture of the field trial.

3.2.2 Soil and plant sampling

Soil and plant samples were collected each season over two years, that is, October 2018 (Spring), January 2019 (Summer), March 2019 (Autumn), July 2019 (Winter), October 2019 (Spring), January 2020 (Summer), March 2020 (Autumn), and July 2020 (Winter). Soil samples were taken using a soil auger from the 0-7.5 cm horizon at 15 random locations within each plot. Shoot biomass was sampled one day later by harvesting within a 0.5 m^2 metallic frame randomly placed in each plot. Shoots were cut at two centimetres above the ground. Soil samples were sieved to pass through a 2 mm sieve and split into two portions. The first one was stored at 4 ^oC and used to determine soil moisture and soil biological and biochemical parameters within one week. The second portion was air-dried and used to carry out soil chemical analyses. Olsen P, microbial biomass P, acid and alkaline phosphatase activities, shoot biomass, shoot P concentration, and shoot N and P uptake were determined each season. Soil pH, total C and N, available N (NO_3^--N and NH_4^+-N), and soil P fractions were measured in July 2019 and July 2020.

3.2.3 Soil and plant analyses

Soil moisture was calculated after oven-drying 10 grams of moist soil at 105 ^oC for 48 hours. Soil pH was determined after shaking air-dried soil with deionised water (ratio 1:2.5) for one hour. Total N and C were measured after digestion of 0.5 grams of air-dried and ground soil in an Elementar Vario

Max CN analyser. Available N (NH_4^+-N and NO_3^--N) was assessed by extracting field-moist soil with 2 *M* KCl (ratio 1:10) for one hour and then analysing the aliquot in a total N analyser (Blakemore et al. 1987). Olsen P was determined according to Watanabe and Olsen (1965). Microbial biomass P (MBP) was determined following the fumigation-extraction method of Brookes et al. (1982) with a recovery coefficient of 40 %. Potential acid and alkaline phosphatase activities were assessed according to the procedure of Tabatabai (1994) for phosphatase assays. Potential phosphatase activities were expressed as µmol *p*-nitrophenol produced g^{-1} fresh soil h^{-1}. Hereafter, when mentioning phosphatase activities, it means potential phosphatase activities.

Phosphorus fractionation was performed as described by Condron and Newman (2011) as reported elsewhere (Gatiboni et al. 2021; Touhami et al. 2021). These consisted of the addition of a salt (1M NH_4Cl) in the first extraction to wash out excess calcium, the inclusion of a washing step using NaCl between extractions to avoid carryover of P, and the use of a second NaOH extraction after HCl to extract P firmly bound to Al and Fe oxides and P present in the microaggregates. In short, 0.5 g of air-dried soil was sequentially extracted using 10 ml of solution following the scheme outlined in Table 3.1.

Table 3.1 Scheme of sequential P fractionation indicating steps and corresponding extractant solution, the form of P extracted, and the availability of each fraction determined.

Step	Extractant solution	Form of P extracted	Availability
1	1 M NH_4Cl	Pi	Readily available
2	0.5 M $NaHCO_3$ (pH 8.5)	Pi + Po	Labile
3	0.1 M NaOH	Pi + Po	Moderately labile
4	1 M HCl	Pi	Calcium-P
5	0.1 M NaOH	Pi + Po	Stable
6	1 M H_2SO_4 + H_2O_2	Pi	Residual

Pi and Po mean inorganic P and organic P, respectively.

Each soil solution was shaken for 16 hours, centrifuged for 15 min, and the aliquot was kept in the fridge at 4 ^oC for further analyses. The final soil residue was oven-dried at 50 ^oC to determine residual P, according to Olsen and Sommers (1982). Soil extracts were analysed for Pi using two different methods to avoid organic P mineralisation and overestimation of Pi. Inorganic P in acid extracts (NH_4Cl, HCl, and residual P) was measured using the molybdenum blue method (Murphy and Riley 1962). In comparison, Pi in alkaline extracts ($NaHCO_3$, NaOH1, NaOH2) was analysed following the procedure of Dick and Tabatabai (1977). Total P in the alkaline extracts was assessed by ICP-OES according to He and Honeycutt (2005), while Po was calculated by deducting Pi from total P. Total soil P represented the sum of all nine fractions. To understand the incorporation of applied P into different P fractions, P fractionation was carried out on soil samples before starting the experiment

(July 2018) and after two months of P addition (October 2018). Moreover, P fractionation was performed on soils taken after one (July 2019) and two years (July 2020).

After harvest, plant shoots were washed with deionised water, oven-dried for two days at 65 ^{o}C, and weighed. Dried plant shoots were ground in a stainless grinder, sieved less than 1 mm, and then digested in a mixture of H_2O_2 and HNO_3 (2:1) to determine shoot P concentration using ICP-OES (Anderson 1996). To determine shoot N concentration, dried and ground plant materials were combusted following the Dumas method and the resultant N_2 gas was determined by thermal conductivity conductor in a VarioMAX Macro Elementar Analyser. Shoot N and P uptake were calculated by multiplying P and N concentration by dry shoot biomass. Shoot biomass for each growing season was calculated as the sum of the four plant samplings from October to July, while the total shoot biomass represents the sum of the two growing seasons. Similar calculations were done for shoot P and N uptake.

3.2.4 Meteorological data

Monthly rainfall for the study period was retrieved from Lincoln University weather station, whereas mean soil temperature was calculated from the data provided by four sensors buried at 0-7.5 cm in four different locations.

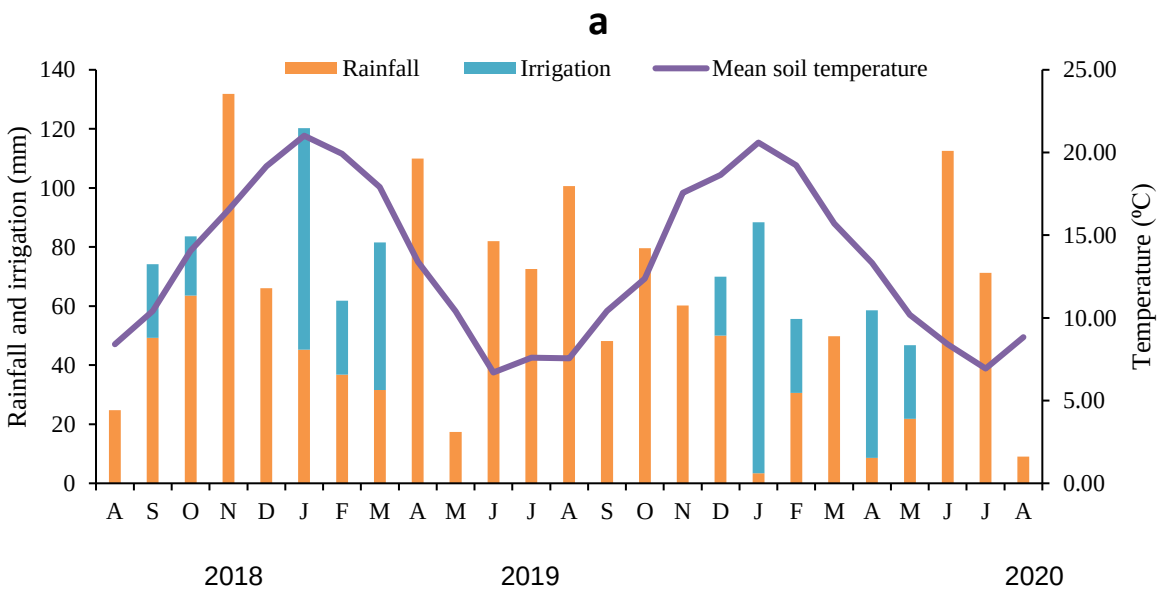
3.2.5 Statistical analysis

Data were subjected to two-way repeated measures ANOVA with nutrient treatment as a factor and sampling dates as within-subject variables. Friedman's test was used for non-normally distributed data such as Olsen P and shoot P uptake. In the presence of a significant effect, one-way ANOVA followed by the Tukey *post-hoc* test was performed to differentiate significant differences among treatment means and sampling dates separately at 5 % probability. Soil P fractions and soil chemical properties (pH, total C, and N, available N) were analysed using one-way ANOVA to test the effect of nutrient treatment and sampling date separately. When data did not meet homoscedasticity, the Kruskal-Wallis test was used instead, followed by the Games-Howell *post-hoc* test to separate means at 5 % probability. Correlation analysis was performed and expressed as Spearman's coefficients to determine relationships between soil, plant, and environmental parameters as well as P fractions. All data analyses were performed using SPSS 25.0 for Windows (SPSS, Chicago, IL, USA), while Microsoft excel and SigmaPlot 14.0 (SYSSTAT) were used for plotting the data.

3.3 Results

3.3.1 Environmental conditions

The study site received a total of 926 mm of water (731 mm rainfall, 195 mm irrigation) between August 2018 and July 2019 in comparison to 850 mm (645 mm rainfall, 205 mm irrigation) in the second growing season from August 2019 to July 2020 (Figure 3.2a). Maximum temperatures were recorded in January each year, whereas minimums were noted in June 2019 and July 2020 (Figure 3.2a). Soil moisture varied from 16 to 29 %, with lower values observed in summer and higher values in winter (Figure 3.2b). Albeit N and NP treatments showed lower soil moisture throughout the study period, no significant differences were detected in soil moisture between nutrient treatments (Figure 3.2b).



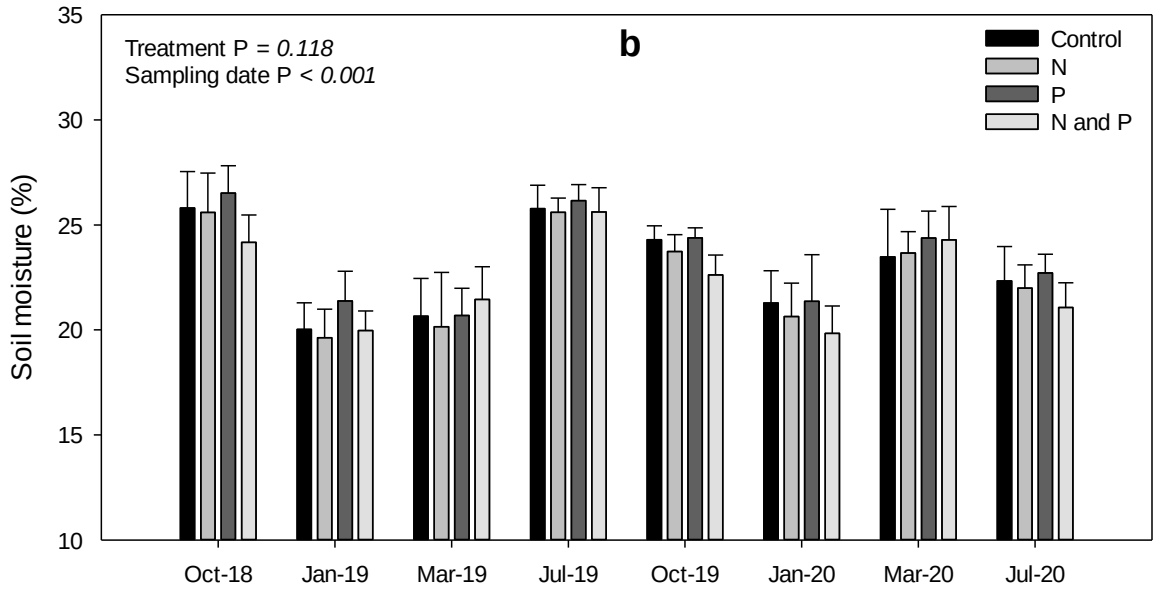


Figure 3.2 Climatic conditions (a) (monthly mean soil temperature, rainfall, and irrigation) at the trial site during the growing seasons of 2018-2019 and 2019-2020, and temporal changes in soil moisture (b) under the control, N (250 kg N $ha^{-1}y^{-1}$), P (50 kg P $ha^{-1}y^{-1}$), and NP (50 kg P + 250 kg N $ha^{-1}y^{-1}$) treatments. Soil samples were taken from 0-7.5 cm horizon under Italian ryegrass (*Lolium multiflorum* Lam.) between October 2018 and July 2020. Values are means of 5 replicates, and bars represent standard errors.

3.3.2 Shoot biomass and shoot nutrient uptake

Total shoot biomass of Italian ryegrass over two growing seasons did not significantly respond to P addition alone (P treatment) compared to the control (Table 3.2). However, an increase of 65 and 63 % was observed in the total shoot biomass under N and NP treatments, respectively, in comparison to the control. The total shoot P uptake under P, N, and NP treatments was significantly higher by 1.3-, 1.5-, and 1.7-fold compared to the control, respectively (Table 3.2). Total shoot N uptake was similar under the N and NP treatments but increased significantly by 76 % in comparison to treatments without N addition (Table 3.3). Shoot biomass, shoot P uptake, and shoot N uptake were higher in season 1 than season 2, irrespective of nutrient treatments (Table 3.2, Figure 3.3). Peaks of shoot biomass and shoot N uptake were recorded in January 2019 and January 2020 (summer), especially under N and NP treatments. In contrast, shoot P uptake was maximum in October 2018 and January 2020 (Figure 3.3). In summer, plots not receiving N inputs showed an increase in clover content between 40 to 50 % (visually estimated).

Table 3.2 Shoot biomass, shoot P uptake, shoot N uptake in season 1 (July 2018-July 2019), season 2 (October 2019 – July 2020), and the whole period of the study (total) under the control, N (250 kg N $ha^{-1}y^{-1}$), P (50 kg P $ha^{-1}y^{-1}$), and NP (50 kg P + 250 kg N $ha^{-1}y^{-1}$) treatments. Within rows, different letters indicate significant differences (P < 0.05) among nutrient treatments.

	Control	N	P	NP
		Shoot biomass (T ha^{-1})		
Season 1	5.7 ± 0.3 b	8.8 ± 0.3 a	5.9 ± 0.3 b	9.1 ± 0.4 a
Season 2	5.9 ± 0.5 b	10.3 ± 0.9 a	6.6 ± 0.3 b	9.8 ± 0.4 a
Total	11.5 ± 0.7 b	19.1 ± 1.0 a	12.5 ± 0.4 b	18.9 ± 0.6 a
		Shoot P uptake (kg ha^{-1})		
Season 1	14.3 ± 0.8 c	19.5 ± 1.1 b	18.5 ± 0.7 b	24.6 ± 1.2 a
Season 2	20.3 ± 2.7 c	33.1 ± 4.1 a	25.6 ± 1.9 b	35.4 ± 2.5 a
Total	34.7 ± 3.3 d	52.6 ± 3.0 b	44.1 ± 2.1 c	60.0 ± 2.4 a
		Shoot N uptake (kg ha^{-1})		
Season 1	109.2 ± 5.7 b	210.4 ± 7.1 a	112.7 ± 5.6 b	206.2 ± 9.1 a
Season 2	128.0 ± 13.6 b	232.3 ± 30.7 a	146.7 ± 6.7 b	224.2 ± 13.9 a
Total	237.2 ± 16.5 c	442.7 ± 31.8 a	259.4 ± 3.5 b	430.5 ± 19.7 a

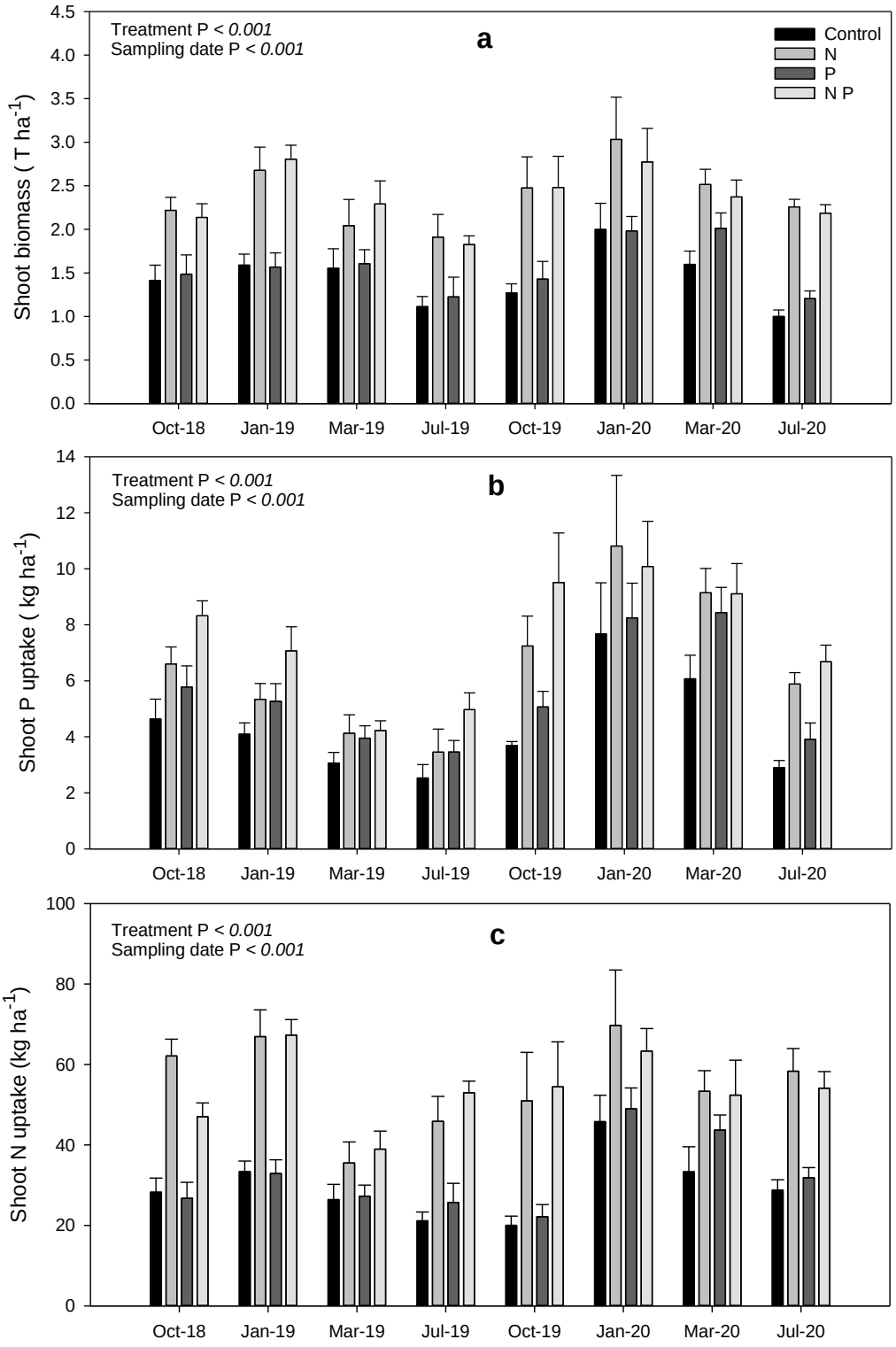


Figure 3.3 Temporal changes in shoot biomass (a), shoot P uptake (b), and shoot N uptake (c) under the control, N (250 kg N $ha^{-1}y^{-1}$), P (50 kg P $ha^{-1}y^{-1}$), and NP (50 kg P + 250 kg N $ha^{-1}y^{-1}$) treatments. Values are means of 5 replicates, and bars represent standard errors.

3.3.3 Soil properties

Soil pH showed no significant changes across nutrient treatments at the end of the first growing season (July 2019). However, a significant decrease in soil pH was observed at the end of the second growing season (July 2020) under N addition treatments (Table 3.3), though changes in soil pH were small (0.2 units). Total C and N were not affected by nutrient treatment and sampling date in this study (Table 3.3). In comparison to the control and P treatments, NO_3^--N concentrations were significantly higher under treatments with N addition in July 2019 and July 2020. However, NH_4^+-N concentrations were similar regardless of nutrient treatment (Table 3.3).

Table 3.3 Soil pH, total carbon and nitrogen (N), available N (NO_3^--N and NH_4^+-N) under the control, N (250 kg N $ha^{-1}y^{-1}$), P (50 kg P $ha^{-1}y^{-1}$), and NP (50 kg P + 250 kg N $ha^{-1}y^{-1}$) treatments. Soil samples were taken from the 0-7.5 cm horizon under Italian ryegrass (*Lolium multiflorum* Lam.) in July 2019 and July 2020. Values represent means ± standard error (n = 5). Within rows, different letters indicate significant differences (P < 0.05) among nutrient treatments.

	Control	N	P	NP
		Soil pH		
July 2019	6.3 ± 0.1	6.2 ± 0.1	6.3 ± 0.1	6.2 ± 0.1
July 2020	6.3 ± 0.1 a	6.0 ± 0.1 b	6.3 ± 0.1 a	6.0 ± 0.0 b
		Total carbon (g kg-1)		
July 2019	26.4 ± 1.5	26.0 ± 1.6	26.0 ± 1.5	26.1 ± 0.7
July 2020	26.7 ± 1.1	25.8 ± 1.6	26.5 ± 0.7	25.7 ± 0.5
		Total nitrogen (g kg^{-1})		
July 2019	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.1
July 2020	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.5 ± 0.0
		NO_3^--N (mg kg^{-1})		
July 2019	20.7 ± 2.5 b	30.6 ± 5.8 a	23.0 ± 5.2 ab	27.2 ± 4.5 ab
July 2020	13.1 ± 3.4 b	26.5 ± 4.9 a	15.4 ± 2.3 b	20.1 ± 5.1 ab
		NH_4^+-N (mg kg^{-1})		
July 2019	5.0 ± 1.6	5.0 ± 1.0	5.2 ± 0.8	4.7 ± 1.1
July 2020	5.7 ± 0.5	4.6 ± 0.9	4.9 ± 1.4	5.7 ± 2.0

Olsen P concentrations significantly increased after P addition and reached an average of 22 mg kg^{-1} under P and NP treatments compared to an initial Olsen P of 10 mg kg^{-1} (Figure 3.4a). Throughout the growing season, Olsen P concentrations declined irrespective of nutrient treatment but remained significantly higher under P and NP treatments as compared to treatments without P addition (Figure 3.4a). Olsen P concentration decreased by 32 % under N treatment compared to the control, while this decrease was 14 % under NP treatment compared to P alone. Microbial biomass P did not respond to N and P additions but was significantly different between seasons regardless of nutrient treatment (Figure 3.4b). In general, higher microbial biomass P was observed in summer, especially under N and NP treatments, though differences between nutrient treatments were not significant.

Acid and alkaline phosphatase activities significantly decreased following P addition (P and NP treatments) in October 2019 and October 2020. Significantly higher phosphatase activities were observed in summer (January 2019 and January 2020) under N and NP treatments, especially for alkaline phosphatases, compared to the control and P treatments (Fig 3.5). Acid and alkaline phosphatase activities followed similar seasonal trends, with maximum values measured in summer and minimum values in winter (Fig 3.5).

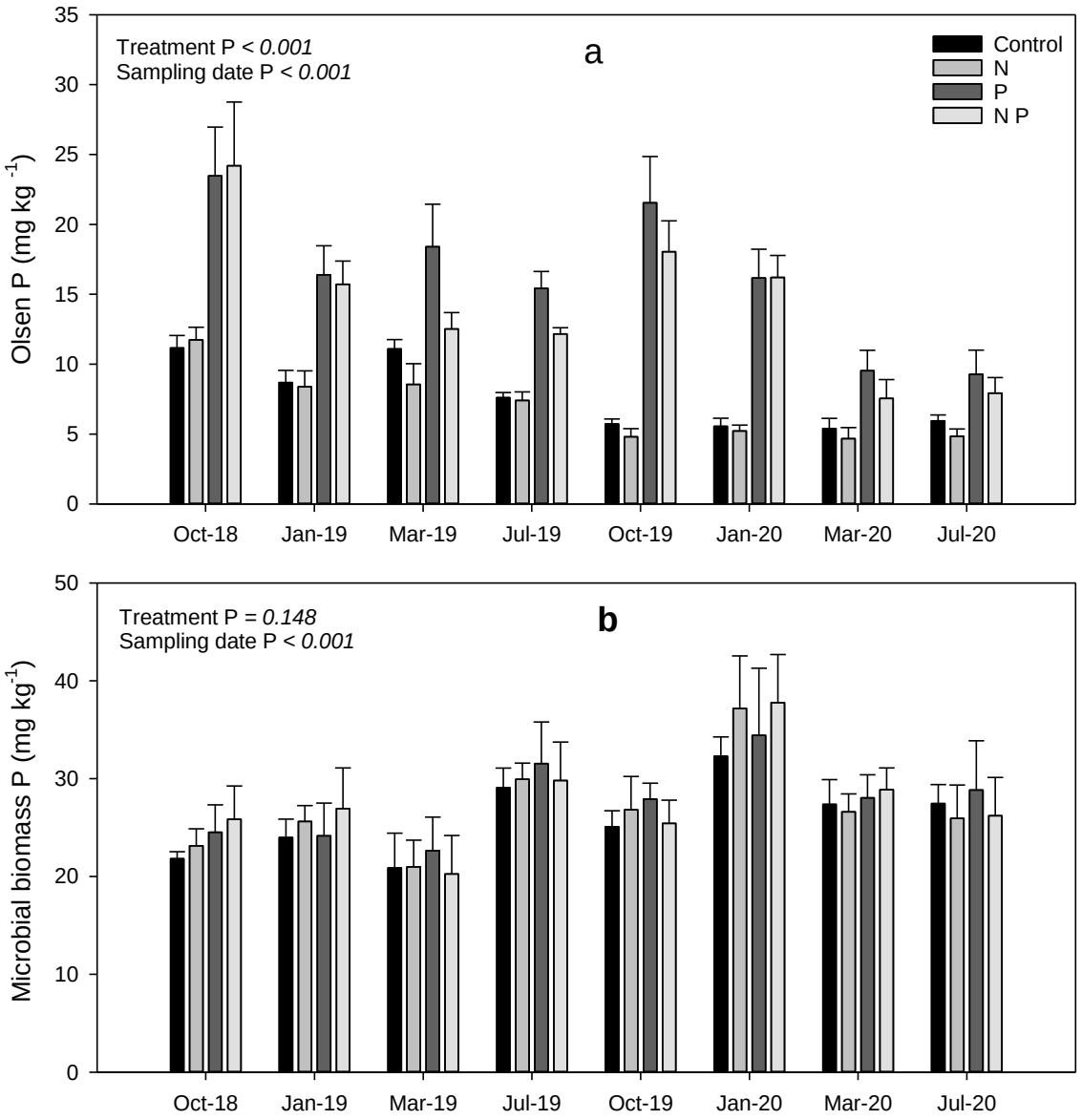


Figure 3.4 Temporal changes in Olsen P (a), and microbial biomass P (b) under the control, N (250 kg N $ha^{-1}y^{-1}$), P (50 kg P $ha^{-1}y^{-1}$), and NP (50 kg P + 250 kg N $ha^{-1}y^{-1}$) treatments. Soil samples were taken from 0-7.5 cm horizon under Italian ryegrass (*Lolium multiflorum* Lam.) between October 2018 and July 2020. Values are means of 5 replicates, and bars represent standard errors.

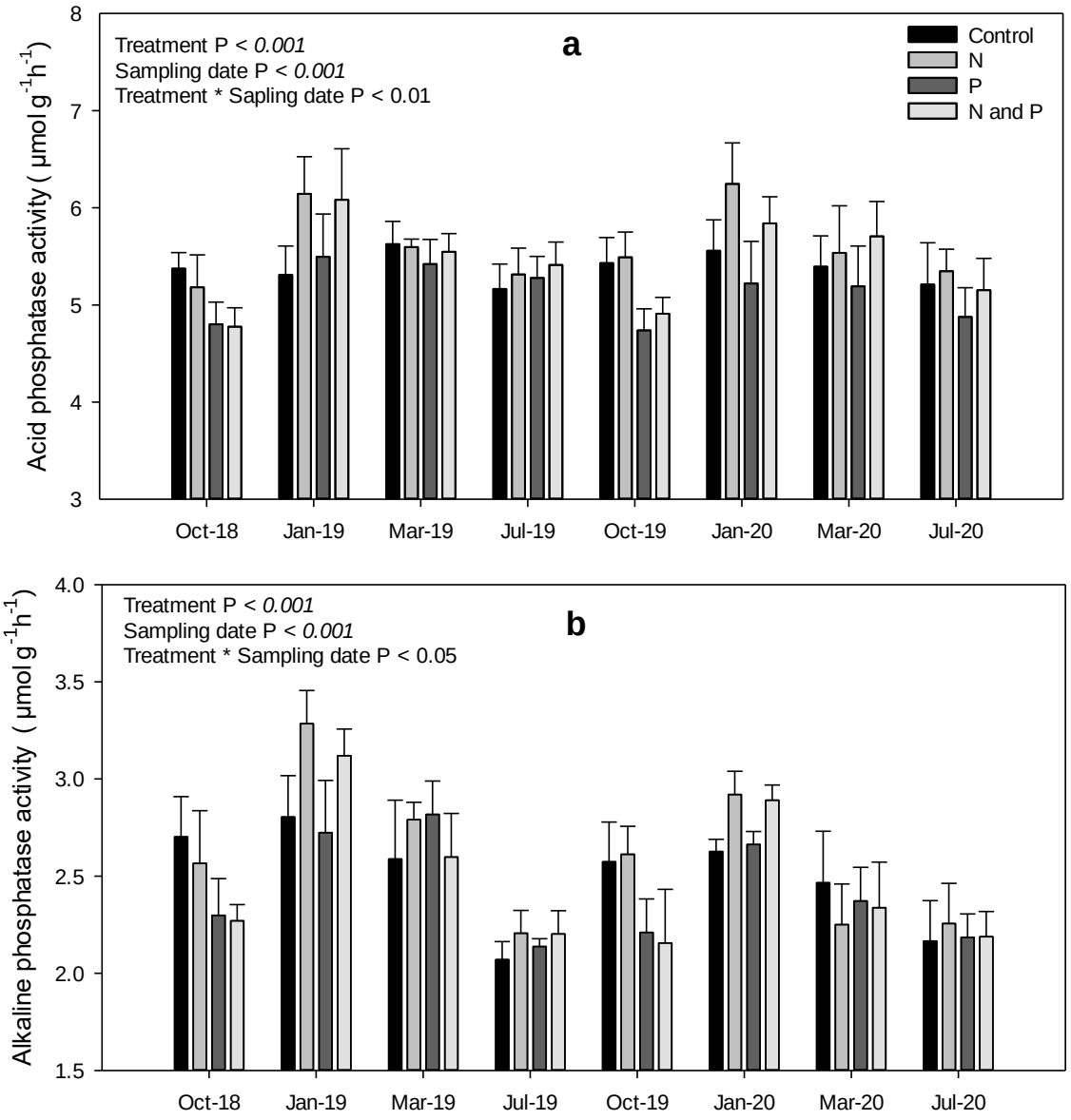


Figure 3.5 Temporal changes in acid (a) and alkaline (b) phosphatase activities under the control, N (250 kg N $ha^{-1}y^{-1}$), P (50 kg P $ha^{-1}y^{-1}$), and NP (50 kg P + 250 kg N $ha^{-1}y^{-1}$) treatments. Soil samples were taken from 0-7.5 cm horizon under Italian ryegrass (*Lolium multiflorum* Lam.) between October 2018 and July 2020. Values are means of 5 replicates, and bars represent standard errors.

3.3.4 Soil P fractions

Phosphorus fractionation data for the soil before starting the experiment (July 2018) showed that moderately labile P (NaOH1-P) was the main fraction accounting for more than 48 % of total P (Table 3.4). Residual Pi was the principal inorganic P (Pi) fraction representing 19 % of total P followed by moderately labile Pi (NaOH1-Pi), Ca-Pi (HCl-Pi), and labile Pi ($NaHCO_3$-Pi), with 17, 16, and 4 % of total P, respectively. Organic P (Po) was mainly composed of moderately labile Po (NaOH1-Po) (77 % of

total Po), whereas labile Po ($NaHCO_3$-Po) comprised 13 % of total Po. Comparison between P fractionations of the original soil (July 2018) and two months after P addition (P and NP treatments in October 2018) revealed that P fertiliser was incorporated into labile Pi ($NaHCO_3$-Pi) (16 mg kg^{-1}) but also moderately labile Pi fractions (NaOH1-Pi) (14 mg kg^{-1}) (Tables 3.4 and 3.5).

Table 3.4 Distribution of different inorganic and organic P fractions (mg kg^{-1}) before starting the experiment. Soil samples were taken from the 0 - 7.5 cm horizon under Italian ryegrass (*Lolium multiflorum* Lam.) in July 2018. Values represent means ± standard error (n = 5).

P fraction	Value	% of total P	% of total inorganic P	% of total organic P
NH_4Cl	0.4 ± 0.1	< 0.1	0.1	-
$NaHCO_3$-Pi	33.1 ± 1.3	4.4	7.4	-
$NaHCO_3$-Po	40.8 ± 2.4	5.4	-	13.2
NaOH1-Pi	126.9 ± 1.4	16.8	28.4	
NaOH1-Po	255.6 ± 14.7	31.4	-	76.9
HCl-Pi	120.2 ± 5.9	15.9	26.9	-
NaOH2-Pi	26.0 ± 1.4	3.4	5.8	-
NaOH2-Po	30.3 ± 2.8	4.0	-	9.8
Residual-Pi	140.5 ± 5.2	18.6	31.4	-
Total P [a]	775.7 ± 18.1	100.0	-	-

[a] calculated as the sum of different inorganic and organic P fractions.

Changes in soil P fractions illustrated in Table 3.5 showed that after one year of plant growth (July 2019), a significant decrease in labile Pi ($NaHCO_3$-Pi) and labile Po ($NaHCO_3$-Po) was observed across all treatments, except P treatment, while readily available Pi (NH_4Cl-Pi) significantly decreased only under P and NP treatments. At the end of the second growing season (July 2020), readily available Pi, labile Pi, and moderately labile Pi (NaOH1-Pi) were significantly depleted under the control and N treatments, while labile Po decreased significantly under N and NP treatments. After two consecutive years of plant growth, total P significantly decreased under N treatment and was unchanged under P and NP treatments. Other soil P fractions (HCl-Pi, NaOH1-Po, NaOH2-Pi, NaOH2-Po, residual-Pi) were similar regardless of nutrient treatment and sampling date.

Compared to the control, two consecutive years of N addition alone significantly decreased readily available Pi, labile Pi, labile Po, and moderately labile Pi by 55, 19, 28, and 7%, respectively. On the other hand, the combined addition of N and P significantly decreased readily available Pi, labile Pi, and labile Po by 39, 26, and 28%, respectively, whereas moderately labile Pi was not affected compared to the application of P alone (Table 3.5). Additionally, as compared to the initial soil before starting the experiment (Table 3.4), N addition alone decreased labile Pi (from 33 to 15 mg kg^{-1}), moderately labile Pi (from 127 to 105 mg kg^{-1}), and labile Po (from 41 to 21 mg kg^{-1}) (Table 3.5). Phosphorus addition alone increased soil labile Pi (from 33 to 42 mg kg^{-1}), increased moderately labile Pi (from 127 to 142 mg kg^{-1}) and depleted labile Po (41 to 32 mg kg^{-1}) (Table 3.5), compared to

the initial soil (Table 3.4). On the other hand, the combined addition of N and P had similar soil labile Pi (from 33 to 31 mg kg^{-1}), slightly increased moderately labile Pi (from 127 to 140 mg kg^{-1}) and depleted labile Po (41 to 22 mg kg^{-1}) (Table 3.5) compared to the initial soil (Table 3.4).

Table 3.5 Distribution of different inorganic and organic P fractions (mg kg^{-1}) under the control, N (250 kg N $ha^{-1}y^{-1}$), P (50 kg P $ha^{-1}y^{-1}$), and NP (50 kg P + 250 kg N $ha^{-1}y^{-1}$) treatments. Soil samples were taken from the 0-7.5 cm horizon under Italian ryegrass (*Lolium multiflorum* Lam.) in October 2018, July 2019, and July 2020. Values represent means of five replicates. Within rows, different lowercase letters indicate significant differences (P < 0.05) among nutrient treatments, while within columns, different uppercase letters indicate significant differences (P < 0.05) among sampling dates.

	Control	N	P	N P
October 2018				
NH_4Cl	0.8 bA	1.0 bA	1.8 aA	1.7 aA
$NaHCO_3$-Pi	29 bA	31 bA	49 a	48 aA
$NaHCO_3$-Po	43 A	43 A	42 A	44 A
NaOH1-Pi	126 bA	128 bA	139 ab	144 a
NaOH1-Po	248	255	252	255
HCl-Pi	131	116	119	118
NaOH2-Pi	26	28	26	25
NaOH2-Po	26	26	28	28
Residual-Pi	139	141	140	145
Total P [a]	770	769 A	797	809
July 2019				
NH_4Cl	0.8 cA	0.8 cA	1.2 aB	1.0 bB
$NaHCO_3$-Pi	26 cB	24 cB	41 a	33 bB
$NaHCO_3$-Po	30 B	31 B	34 AB	29 B
NaOH1-Pi	132 bA	129 bA	153 a	147 a
NaOH1-Po	257	253	252	255
HCl-Pi	117	125	129	127
NaOH2-Pi	27	26	27	26
NaOH2-Po	27	28	28	27
Residual-Pi	144	140	142	140
Total P [a]	760 b	756 bA	796 a	785 ab
July 2020				
NH_4Cl	0.3 cB	0.1 cB	1.2 aB	0.8 bB
$NaHCO_3$-Pi	19 cC	15 cC	42 a	31 bB
$NaHCO_3$-Po	29 aB	21 bC	31 aB	22 bC
NaOH1-Pi	114 bB	105 cB	142 a	140 a
NaOH1-Po	252	266	261	259
HCl-Pi	120	124	123	125
NaOH2-Pi	27	28	28	27
NaOH2-Po	29	28	30	27
Residual-Pi	144	140	141	142
Total P [a]	735 b	726 bB	801 a	781 a

[a] calculated as the sum of different inorganic and organic P fractions.

3.3.5 P balance

Phosphorus balance was calculated as the difference between P input, which is the total amount of mineral P fertilizer added over the period of the trial and P output representing the P removed in plant biomass by cutting. The control and N treatments had negative P balance, but N treatment decreased P balance by 51% compared to the control (Table 3.6). Phosphorus balance under P and N and P treatments was positive but N and P treatment decreased P balance by 28% compared to P treatment (Table 3.6).

Table 3.6 Phosphorus balance (difference between P inputs and P outputs) during the period of the trial (2 years) under the control, N (250 kg N $ha^{-1}y^{-1}$), P (50 kg P $ha^{-1}y^{-1}$), and NP (50 kg P + 250 kg N $ha^{-1}y^{-1}$) treatments.

Treatment	P input (kg P ha^{-1})	P output (kg P ha^{-1})	P balance
Control	0	35	-35
N	0	53	-53
P	100	44	56
N P	100	60	40

3.3.6 Relationships between soil, plant, environment parameters, and P fractions

Results of correlation analysis on all sampling dates showed that alkaline phosphatase activity was positively correlated with microbial biomass P while Olsen P was negatively correlated with acid phosphatase activity (Table 3.7). Shoot biomass was significantly correlated with acid and alkaline phosphatase activities, whereas P uptake was significantly correlated with Olsen P and acid and alkaline phosphatase activities. Shoot biomass, shoot P uptake, and phosphatase activities were negatively correlated with soil moisture (Table 3.7).

Correlation analysis conducted on soil chemical properties, soil P fractions, and total shoot biomass and P uptake revealed that total shoot biomass was positively correlated with NO_3^--N concentration but negatively correlated with labile Po. Total shoot P uptake was significantly negatively correlated with labile Po (Table 3.8).

Table 3.7 Values of Spearman correlation coefficients between plant parameters, soil properties, and environmental factors assessed from October 2018 to July 2020 (n = 160).

	MBP	Olsen P	Acid P	Alk P	Shoot biomass	Shoot P uptake	Soil moisture
MBP	1	-0.047	0.141	0.379**	0.095	-0.127	-0.029
Olsen P	-0.047	1	-0.349**	-0.090	0.063	0.405**	0.085
Acid P	0.141	-0.349**	1	0.382**	0.359**	0.201*	-0.434**
Alk P	0.379**	-0.090	0.382**	1	0.292**	0.216**	-0.145
Shoot biomass	0.095	0.063	0.359**	0.292**	1	0.760**	-0.356**
P uptake	-0.127	0.405**	0.201*	0.216**	0.760**	1	-0.366**
Soil moisture	-0.029	0.085	-0.434**	-0.145	-0.356**	-0.366**	1

* Significant difference at $P < 0.05$; ** Significant difference at $P < 0.01$; MBP, microbial biomass phosphorus; Acid P, acid phosphatase activity; Alk P, alkaline phosphatase activity

Table 3.8 Values of Spearman correlation coefficients between plant parameters, soil chemical properties, and P fractions assessed in July 2019 and 2020 (n = 40).

	Total shoot biomass	Total P concentration	Total P uptake
NH_4^+-N	-0.062	0.114	0.009
NO_3^--N	0.355*	-0.514**	0.020
NH_4Cl	-0.254	0.092	-0.163
$NaHCO_3$-Pi	-0.221	0.259	-0.059
$NaHCO_3$-Po	-0.422**	-0.241	-0.463**
NaOH1-Pi	-0.141	0.112	-0.074
NaOH1-Po	0.302	0.142	0.294
HCl-Pi	0.093	0.077	0.091
NaOH2-Pi	0.127	0.305	0.241
NaOH2-Po	0.107	0.048	0.053
Residual-Pi	-0.068	0.075	0.011

* Significant difference at $P < 0.05$; ** Significant difference at $P < 0.01$

3.4 Discussion

3.4.1 Nutrient addition impacts on plant biomass and nutrient uptake

In co-limited soils, the application of single or combined fertiliser inputs can modify the soil nutrient balance, thereby affecting plant primary productivity and, in turn, plant nutrient uptake (Deng et al. 2016; Schleuss et al. 2020; Cui et al. 2021). In contrast to our first hypothesis, our results showed that P addition alone did not increase the aboveground plant biomass of Italian ryegrass. Ikoyi et al. (2018) found that the single P application to a P-deficient soil cultivated with perennial ryegrass (*Lolium perenne* L.) had negative feedback on soil microbial activity and diversity, thus cancelling out the positive effect of P application on plant biomass. Shi et al. (2020) highlighted that low P requirements of timothy grass (*Phleum pratense* L.) were responsible for the non-response of plant

biomass to increased P applications. Edmeades et al. (2006) estimated that Olsen P of 30 mg kg^{-1} was the critical value below which pasture production will be less than 97 % of optimal production under sedimentary soils in New Zealand. However, this value was established for grass/legume pasture but not for Italian ryegrass. It is acknowledged that the P requirements of legumes are higher than grasses due to the key role of P in N fixation (Peoples et al. 1998; Sprent 1999; Lam et al. 2012). Therefore, it was suggested that soil P availability was not a limiting factor for the growth of Italian ryegrass in the current study. Nevertheless, studies determining the critical values of available soil P for Italian ryegrass under sedimentary soils in New Zealand are needed to confirm this result.

Liebig's Law of the Minimum states that growth is limited not by the total resources available but by the most limiting nutrient. In contrast to P addition, N addition alone or combined with P increased shoot biomass by 1.6-fold, emphasising that plant primary productivity in this study was limited by N availability rather than P. Furthermore, correlation analysis revealed that shoot biomass was positively correlated with NO_3^--N concentration ($P < 0.05$). Our findings partly agreed with the results reported by Schleuss et al. (2020) but stressed that plant productivity in our soil was mainly N-limited since there were no significant differences between shoot biomass under N addition alone and the combined addition of N and P. Indeed, our soil was previously under an arable cropping system characterised by high losses of C and N due to increased removal of soil nutrients, depletion of soil organic matter, and degradation of soil structure (Tiessen et al. 1982; Dalal and Mayer 1986; Haynes et al. 1991; Nguyen et al. 1995; Lobe et al. 2001).

Phosphorus addition alone significantly increased total shoot P uptake compared to the control treatments in both years, which was not seen for shoot biomass. This indicated a luxury uptake of P by Italian ryegrass. It is well established that P uptake is a function of N availability in soils (Ford et al. 2016; Gao et al. 2016; Wang et al. 2021b). Our results showed that N addition alone and in combination with P significantly increased total shoot P uptake by 19 and 37 %, respectively, compared to the single addition of P. This indicated that N addition alone increased soil P utilisation (Deng et al. 2017; Cui et al. 2021), while the combined addition of N and P had a significantly greater effect on plant P uptake than the single nutrient input (Schleuss et al. 2020). These results confirmed our second hypothesis and partly our third hypothesis. Shoot N uptake was statistically similar under N and NP treatments, indicating that P addition did not improve plant N uptake, most likely because P addition did not increase shoot biomass of Italian ryegrass in this study. This result concurred with the findings of Schleuss et al. (2020), who investigated the interactive effects of N and P additions on plant N and P uptake under N and P co-limited grassland soil in South Africa.

3.4.2 Nutrient addition impacts on soil properties

The response of soil pH to nutrient addition depends on several parameters, including the initial soil pH, the form and quantity of nutrient applied, and plant species (Marschner and Römheld 1983; Hinsinger et al. 2003; Tian and Niu 2015). In our study, no changes were observed in soil pH under P addition alone throughout the study period. However, N addition alone and in combination with P significantly decreased soil pH at the end of the second year. This is in line with the findings of Condron (1986), who found a significant decrease in soil pH (0.4 units) in the second year of N addition (200 kg N ha^{-1} yr^{-1}) to a long-term P fertilised grazed pasture. The reaction of dissolution of urea in the soil produces H^+ that can decrease soil pH (Tian and Niu 2015). However, the pH buffering capacity of the soil could have been responsible for overcoming the acidification effect of N addition in the first year, but the soil buffering capacity may have been oversaturated by H^+ in the second year (van Breemen et al. 1983). Although the decrease in soil pH was significant, it was only about 0.2 units.

Throughout the growing season, Olsen P concentration decreased regardless of nutrient treatment, mainly due to plant P uptake and P removal in plant biomass. As compared to the control and P treatments, Olsen P concentration decreased by 23 % under treatments with N addition. In N-limited soils, N addition increases net primary productivity, thereby accelerating soil P depletion (Deng et al. 2017; Schleuss et al. 2020). In a crop rotation in Germany, increasing N inputs resulted in a significant decrease in labile P pools (Wang et al. 2021b). Similarly, Cui et al. (2021) described that the depletion of labile P was proportional to the level of N addition in a semi-arid grassland. However, Schleuss et al. (2021) described that available inorganic P was not significantly different under grassland soils receiving either P or NP inputs. Their result could be explained by the similar aboveground biomass found across treatments along with P recycling through plant returns.

Nutrient stoichiometry is critical in regulating soil microbial growth and activity (Sinsabaugh et al. 2008; Maaroufi and De Long 2020; Schleuss et al. 2021). It has been shown that soil microbes can thrive under external nutrient inputs by adjusting different mechanisms involved in maintaining microbial stoichiometry, such as acquisition, partitioning, and turnover (Spohn 2016). (Randall et al. 2019) found that soil microbial biomass C, N, and P were unchanged in a grassland soil under cut and carry system regardless of P applications (0, 15, 30 kg P ha^{-1} yr^{-1}). They suggested that this result was attributed to low C and N availabilities and P adsorption to clay particles in this grassland soil. Heterotrophic soil microbes are generally C-limited, implying that their growth and activity are chiefly regulated by C availability (energy) (Griffiths et al. 2012; Heuck et al. 2015; Spohn and Schleuss 2019). Our soil was previously under an arable cropping system characterised by low labile organic C (Haynes et al. 1991; Nguyen et al. 1995; Haynes 2000). Hence, it is possible that the low

availability of C has cancelled out the effect of N and P addition on microbial biomass P due to microbial C-limitation (Xu et al. 2020b). This is supported by the increase in microbial biomass P (not significant) and alkaline phosphatase activities (significant) in summer under N and NP treatments, which may have coincided with higher release of rhizodeposits by plant roots. Soong et al. (2020) highlighted that microbial C-limitation is often overshadowed by plant nutrient limitation, yet it can feedback on soil nutrient cycling. As suggested in this study, C availability can drive microbial and biochemical pathways involved in P cycling in response to external N and P inputs. Nevertheless, an assessment of microbial biomass C, N, and P is needed in future studies to confirm this conclusion.

Phosphatase enzymes play a central role in plant P nutrition, especially under P limited conditions (Nannipieri et al. 2011). Phosphatase enzymes released by plants and microbes are responsible for the cleavage of phospho-monoester compounds into inorganic orthophosphates easily available for plant uptake (Tarafdar and Claassen 1988). It is widely acknowledged that phosphatase activity is inhibited by high P availability (Marklein and Houlton 2012). Nevertheless, several studies have shown discrepancies in the response of phosphatase enzymes to P applications, where increases, decreases, and no changes were noticed (Lemanowicz 2011; Ikoyi et al. 2018; Widdig et al. 2019; Shi et al. 2020). In this study, acid and alkaline phosphatase activities significantly decreased following the application of P fertilisers (October 2019, 2020). This indicated that soil phosphatase enzymes were sensitive to P availability (Marklein and Houlton 2012), which was further emphasised by the negative correlations ($P < 0.01$) found between acid and alkaline phosphatase activities and Olsen P concentration, confirming our first hypothesis. However, the suppression of phosphatase activities under P and NP treatments was short-lived and was not observed throughout the growing season. This suggested that inorganic P addition inhibited newly synthesised phosphatase enzymes rather than the pre-existing ones (Spiers and McGill 1979).

Nitrogen is a dominant component of phosphatase enzymes (Olander and Vitousek 2000); thus, plant and soil microbes invest more in phosphatase enzyme production when N availability is high (Marklein and Houlton 2012). However, acid and alkaline phosphatase activities were only higher in summer under N and NP treatments, which concurred with peaks of plant production. Higher shoot biomass indicates higher C inputs and, in turn, higher C release into the belowground compartment (Lu et al. 2011; Schleuss et al. 2021). Based on 35 studies using C isotope tracers, Huang et al. (2020) found that N addition increased newly synthesised C derived from plant roots. A recent study showed that the addition of 550 kg N ha^{-1} (as urea) to 5 grassland plant species, including perennial ryegrass (*Lolium perenne* L.), increased the concentration of water-extractable C released in the soil compared to the unplanted control (Leptin et al. 2021). Hence, the higher rate of rhizodeposition under N and NP treatments could stimulate microbial activity (Grayston et al. 1996), including the activity of alkaline phosphatase enzymes to mobilise organic P needed to maintain microbial

stoichiometry (Heuck et al. 2015; Spohn et al. 2015; Tian et al. 2016). Similarly, increased plant P demand in summer can trigger the release of acid phosphatase enzymes derived from plant roots to mineralise organic P (Tate et al. 1991; Scott and Condron 2003; Wasaki et al. 2018). This was further confirmed by the positive correlation found between acid ($P < 0.05$) and alkaline phosphatase activities ($P < 0.01$) and shoot biomass. Therefore, our study showed that short-term N addition caused an indirect increase in phosphatase activities through the stimulation of plant P demand and the release of rhizodeposits rather than directly affecting the production of phosphatase enzymes *per se*. This could be related to the low C availability of our soil impairing phosphatase activity despite the high availability of N. This result is consistent with the findings of Allison and Vitousek (2005) and Dodor and Tabatabai (2003) who found that phosphatase activity is chiefly controlled by C availability in soils.

We have visually observed an increase between 40 to 50 % in white clover content in the control and P treatments in summer 2019 and 2020. The increase in white clover content could be attributed to N limitation, promoting white clover growth to provide N to the system (Barthram et al. 1992; Chapman et al. 1996). Additionally, the higher competitivity of clover in terms of growth rate under higher temperature (summer) compared to the N-limited Italian ryegrass could partly explain this result (Ledgard et al. 2001; Castle et al. 2002; Chapman et al. 2017). Conversely, applications of N may have inhibited white clover growth along with the greater response of Italian ryegrass to N addition suppressing white clover by shading (Feyter et al. 1985; Ledgard et al. 1995; Schils and Snijders 2004). This increase in white clover content might have had an impact on soil properties linked to P cycling in summer. Nevertheless, long-term experiments and experiments contrasting grass only versus grass-clover pasture are recommended to provide better insights on white clover impact on soil P dynamics under N and P inputs.

3.4.3 Seasonal impacts on soil microbial properties

Microbial biomass P can represent an important source of available P to pasture plants through the year, but its transformations and turnover could be influenced by the availability of C and N (Brookes et al. 1984; Perrott and Sarathchandra 1989; Griffiths et al. 2012; Pradhan et al. 2020). Microbial biomass P was significantly affected by the sampling date regardless of nutrient treatment, indicating its essential role as sink and source of P to pastures (Perrott et al. 1992; Chen et al. 2014; Shi et al. 2020). Several studies under temperate grassland systems were successful at identifying temporal trends in soil microbial biomass P with increases in summer and decreases in spring (Tate et al. 1991; Chen et al. 2003a; Boitt et al. 2018b). These studies also reported a significant correlation between microbial biomass P and soil moisture. In the current study, soil microbial biomass P failed to show a significant correlation with soil moisture, possibly due to irrigation of this pasture system. Microbial

biomass P increased in summer, especially during the second growing season. It is well established that soil microbial biomass and activity are generally C-limited (Griffiths et al. 2012; Spohn and Kuzyakov 2013b; Heuck et al. 2015). Higher microbial biomass P observed in summer could be related to higher carbon availability due to rhizodeposition, thereby triggering P immobilisation in the microbial biomass (Perrott et al. 1992). A closer look at the microbial biomass P in summer revealed that this parameter increased under N treatments which coincided with higher plant biomass and potentially higher C released into the belowground compartment. Therefore, microbial biomass P is suggested to be influenced by a combination of temperature, plant growth, and rhizodeposition in this study.

Acid and alkaline phosphatase activities followed similar temporal trends in all nutrient treatments, with lower activities in winter and higher activities in summer, indicating that similar factors were governing organic P mineralisation regardless of nutrient inputs. Temperature is among the key parameters controlling the activity of phosphatase enzymes (Margalef et al. 2017; Zuccarini et al. 2020). It has been shown that cold temperatures can inhibit phosphatase activity, while high temperatures can stimulate it (Sofi et al. 2016; Ge et al. 2017; Sun et al. 2020). In the current study, maximum soil temperatures were recorded in summer, which concurred with higher shoot biomass as well as acid and alkaline phosphatase activities, especially under treatments with N addition. On the other hand, winter recorded lower activities of phosphatase enzymes along with lower temperatures and higher soil moisture. Data from different ecosystems, including pastures, revealed an accumulation of organic P in winter as well as a decrease in soil phosphatase activity (Harrison 1982; Sarathchandra et al. 1989; Speir and Cowling 1991; Scott and Condron 2003). Taking together, we suggest that seasonal changes in phosphatase activities were more likely controlled by a combination of factors, including environmental conditions, plant growth, and rhizodeposition (Chen et al. 2003a; Boitt et al. 2018b; Shi et al. 2020). Furthermore, in contrast to the results found in New Zealand temperate pastures, mineralisation of organic P is suggested to be maximum in summer instead of spring under this irrigated pasture system.

3.4.4 Nutrient addition impacts on soil P fractions

Soil P fractionation data revealed that almost half of total P was in the form of moderately labile P, indicating the presence of high amounts of Al and Fe oxides in the soil used in this study. This explains why the addition of inorganic P fertilizer caused an increase in both labile and moderately labile Pi fractions, which is in line with previous studies under P fertilized soils in New Zealand (Condron and Goh 1989; McDowell and Condron 2000; Touhami et al. 2020a). Readily available Pi, labile Pi, labile Po, and moderately labile Pi were the main P fractions depleted by Italian ryegrass in this study. Readily available Pi is considered a rapidly mobilizable Pi fraction (Rose et al. 2010), while

most plant species acquire their P from labile Pi (Hedley et al. 1982; Gatiboni et al. 2021). Moderately labile P is abundant in New Zealand soils and is considered as the P adsorbed to Al and Fe oxides (Maher and Thorrold 1989; Perrott and Mansell 1989; McDowell and Condron 2012). In pasture systems, several studies have indicated that labile P and moderately labile P could account for the majority of plant P uptake (Condron and Goh 1989; Chen et al. 2002, 2003a). After two consecutive years of plant growth, total P significantly decreased under N addition alone, while the extent of the decrease was not statistically significant under the control. On the other hand, total P was not significantly affected by plant growth under P and N and P treatments. This confirmed that N addition alone can accelerate soil P depletion (Wang et al. 2021a, c), whereas the addition of P can compensate for plant P uptake (Von Sperber et al. 2017).

Previous studies have shown that changes in labile P fractions as affected by N addition was partly related to plant biomass management and plant residue returns. For instance, N addition depleted recalcitrant and occluded P fractions but increased soil P availability under forest ecosystems (Sherman et al. 2006; Block et al. 2013; Fan et al. 2019). In fact, forest litter-fall plays a pivotal role in recycling inorganic P, which continually replenishes the soil available P pool. On the other hand, increased plant P demand in response to N inputs and the removal of P in plant products under intensive cropping systems decreases soil available P and enhances the depletion of sparingly available P fractions (Wang et al. 2021c). Our results showed that N addition alone significantly decreased readily available Pi and labile Pi by 75 and 19%, respectively, compared to the control treatment. This was mainly attributed to the higher shoot P uptake together with the continual removal of P in plant biomass (Boitt et al. 2018; Schleuss et al. 2020). Our results corroborated the findings of Wang et al. (2021b), where a significant decrease in resin and $NaHCO_3$-Pi fractions was observed in a crop rotation subjected to long-term N inputs in Germany. Similarly, Cui et al. (2021) described that the depletion of labile P was proportional to the level of N addition and accumulation of plant biomass in a semi-arid grassland.

Changes in moderately labile Pi in response to N addition have shown discrepancies between studies and seems to be related to soil chemical properties, including pH. Fan et al. (2019) found that long-term N addition to an acidic soil under forest ecosystem was able to mobilize moderately labile Pi by increasing the desorption of P from Al and Fe oxides due to soil acidification. Conversely, Wang et al. (2021a) noted that 11 years of urea addition to a calcareous grassland soil increased moderately labile Pi, which was linked to the complexation of free Pi by Al and Fe released from soil minerals. The soil used in this study had an acidic pH, thus depletion of moderately labile P in response to N addition is expected. Indeed, our results revealed that N treatment showed significantly higher depletion of moderately labile inorganic P compared to the control. Moderately labile inorganic P is defined as the P adsorbed to Al and Fe oxides and there is increasing evidence that organic anions

can contribute to the mobilization of this fraction via chelation, complexation, and ligan exchange processes (Wang et al. 2015b, 2016b; Wang and Lambers 2020a). A recent study showed that nitrogen supply increased rhizodeposition, available P, and plant P uptake when wheat was grown in an acidic unfertilised grassland soil (Bicharanloo et al. 2020). Therefore, it is suggested that the decrease in moderately labile Pi observed in the present study could be attributed to organic anions release rather than soil acidification, because differences in soil pH between the control and N treatment were small. Nevertheless, future research needs to quantify organic anion release under pasture systems subjected to N inputs to confirm this hypothesis.

Because of the low P use efficiency of P fertilizers and the need to increase and maintain plant productivity, P applications often exceed plant P requirements leading to soil P accumulation (Perrott et al. 1992; Condron 2003). Nitrogen addition has been suggested as one of the effective strategies to increase plant production and reduce soil P legacy (Perring et al. 2009; Dodd et al. 2014). Investigating a calcareous soil under meadow steep, Liu et al. (2019) described a decrease in labile and moderately labile inorganic P fractions under the combined N and P additions compared to P addition alone. Liu et al. (2019) ascribed the mobilization of these fractions to increased plant biomass as result of N fertilization. In the present study, the combined addition of N and P significantly decreased readily available and labile Pi by 39 and 26%, respectively, compared to the application of P alone. Furthermore, although plant biomass increased by 1.6-fold and P balance decreased by 28% under N and P treatment compared to P treatment, sparingly available Pi was similar under both treatments. The non-response of Italian ryegrass to P addition and the presence of high amounts of free Al and Fe oxides in this soil not only promoted the accumulation of P in the moderately labile Pi fractions but also prevented its mobilization through N fertilization. Therefore, it is suggested that when P applications exceed plant P requirements, especially under soils with high free Al and Fe oxides, short-term N fertilization can mobilize labile P pools (readily available and labile Pi and labile Po) but not sparingly P fractions (moderately labile Pi). Due to the short time scale of this study and the non-response of Italian ryegrass to P addition, long-term studies under N and P co-limited grassland soils are warranted to quantify if more recalcitrant P pools would be depleted. Or to quantify potential changes in available and sparingly available soil P pools.

It is well established that N fertilization enhances organic P mineralization in a range of ecosystems (Heuck et al. 2018; Cui et al. 2021; Wang et al. 2021c), however there is a lack of data on different organic P pools depleted under N fertilized pasture systems. Past research on the topic indicates that long-term N addition inhibits phosphatase enzyme activity and biological P cycling (Tian et al. 2016), while short-term N supply promotes organic P mineralization in pasture soils (Cui et al. 2021). In this

short-term study, P fractionation data revealed that labile Po was depleted by Italian ryegrass regardless of nutrient treatment. However, the extent of this depletion was 28% higher under treatments with N addition compared to the control. Plants tend to mobilize Po to meet increased P demand (Ford et al. 2016; Schleuss et al. 2020). Furthermore, external N inputs can modify the soil N:P ratio, thereby promoting higher organic P mineralisation by soil microbes to maintain microbial stoichiometry (Heuck et al. 2018). As previously discussed, shoot biomass and shoot P uptake were significantly higher by an average of 1.6-fold under N addition treatments compared to the control. These treatments also exhibited significantly higher acid and alkaline phosphatase activities in summer. Results from correlation analysis showed a positive correlation between shoot biomass and acid ($P < 0.05$) and alkaline phosphatase activities ($P < 0.01$) and a negative correlation between shoot biomass and labile Po ($P < 0.01$). Therefore, N addition in this study increased plant growth, which in turn enhanced biological P cycling via the mobilization of labile Po through phosphatase enzymes (Marklein and Houlton 2012; Heuck et al. 2018; Schleuss et al. 2020; Cui et al. 2021).

3.5 Conclusions

The results of this study revealed that although P addition increased bioavailable P and plant P uptake, they had no significant impact on shoot biomass. On the contrary, N addition, both alone and in combination with P, increased shoot biomass by an average of 1.6-fold compared to the control. This emphasised that plant primary productivity was limited by N availability rather than P. The combined addition of N and P had a significantly greater effect on shoot P uptake than the single input of N or P. In summer, N addition treatments increased acid and alkaline phosphatase activities, which was related to increased plant P demand, alleviation of C-limitation through rhizodeposition, and higher temperatures. Similar results were found for microbial biomass P, though differences were not significant between nutrient treatments. Furthermore, low C availability may have hindered the response of microbial biomass P to N and P additions. These results suggest that C availability could feedback on soil P cycling by impacting microbial and biochemical pathways. Therefore, C availability should be considered when assessing the impact of anthropogenic nutrient inputs on P cycling. Under this intensively managed grass pasture system, N addition alone accelerated P cycling by decreasing labile Pi and depleting sparingly available P fractions such as labile Po and moderately labile Pi. On the other hand, the combined addition of N and P depleted readily available and labile P fractions as well as labile Po but failed to mobilise the moderately labile Pi fraction. Therefore, knowing that most of P in New Zealand soils is adsorbed to Al and Fe oxides and present in organic form, N fertilisation under N-limited pasture soils could be a good strategy to mobilize these P pools. Nevertheless, P inputs combined with N to pasture systems need to be tailored according to plant needs and soil fertility status to avoid soil P accumulation. Due to the short time scale of this study

and the non-response of Italian ryegrass to P addition, long-term studies under N and P co-limited grassland soils are warranted to quantify if more recalcitrant P pools would be depleted under N and P additions. Moreover, optimum N:P supply ratios need to be further established for better plant, soil, and water quality under pasture systems.

Chapter 4

Impacts of Long-Term Phosphorus Fertiliser Inputs and Seasonal Conditions on Soil Phosphorus Dynamics and Phosphatase Enzyme Activity under Grazed Pasture

4.1 Introduction

Inputs of phosphorus (P) are necessary to increase the productivity of most agroecosystems (Vance et al. 2003; Haygarth et al. 2013). In addition to P removal in produce and drainage, ongoing immobilisation of added P via adsorption onto soil mineral surfaces and organic matter means that continued inputs of P in the form of mineral fertiliser and/or manure are required to maintain production at desired levels (Condron 2003; Richardson and Simpson 2011). These practices have resulted in the accumulation of significant quantities of P in many agroecosystems, which is commonly referred to as "legacy P" (Rowe et al. 2016; Pavinato et al. 2020). This accumulated P has been shown to increase the risk and occurrence of elevated P loss in drainage which has been linked to enhanced eutrophication (Sharpley et al. 2001; McDowell et al. 2003). Accordingly, to maintain or boost production while minimising P losses to water, there is an urgent need to improve P use efficiency, which in turn requires an improved understanding of the impact of P inputs on soil P dynamics and bioavailability in different agroecosystems (Frossard et al. 2000; Condron 2003).

Soil microorganisms not only compete with plants for P (Marschner et al. 2011) but also control biological P cycling via an interplay between immobilisation and mineralisation processes (Richardson et al. 2009; Ma et al. 2020). Soil microbial biomass P represented between 5 and 24 % of total P in pasture soils in England (Brookes et al. 1984), while it ranged between 0.9 to 11.7 % of total P under established pastures in New Zealand (Perrott and Sarathchandra 1989). Through annual turnover, microbial biomass P can contribute as much as 100 mg P kg^{-1} soil in managed ecosystems, emphasising its central role in supplying available P to plants (Oberson and Joner 2005; Richardson and Simpson 2011). Management practices such as the quantity and nature of P inputs, crop species, and grazing intensity have been found to influence the availability of carbon (C), nitrogen (N), and P (Condron and Goh 1989; Chen et al. 2004; Wei et al. 2017; Coonan et al. 2019), thereby affecting soil microbial biomass dynamics (Griffiths et al. 2012). However, the impact of long-term P fertilisation on microbial biomass P transformations under grazed pastures is still not fully understood.

Organic P is considered a primary source of P for plants, especially under P-limited conditions (Magid et al. 1996; Condron et al. 2005). Acid and alkaline phosphatase cleave organically P-bound to release

free orthophosphates for plant roots (Dick and Tabatabai 1984; Nannipieri et al. 2011). It is well established that phosphatase activity is linked to P availability in soils with lower activity observed in P fertilised ecosystems (Treseder and Vitousek 2001; Yadav and Tarafdar 2001; Nuruzzaman et al. 2006). Nevertheless, the response of phosphatase enzymes to P inputs has revealed contrasting results urging further research to understand why (Saha et al. 2008; Zhang et al. 2014a; Ikoyi et al. 2018). For instance, Ikoyi et al. (2018) found that the single P application to a P-deficient soil cultivated with ryegrass (*Lolium perenne* L.) increased acid and alkaline phosphatase activities, whereas Shi et al. (2020) pointed out no change in alkaline phosphatase activity in response to short-term P application to timothy (*Phleum pratense* L.).

A growing body of literature has shown that acid phosphatase enzymes are released by plant roots (Speir and Cowling 1991; Colvan et al. 2001), whereas alkaline phosphatases are mainly ascribed to the activity of soil microorganisms (Sakurai et al. 2008; Spohn and Kuzyakov 2013a). Additionally, it has been found that plant (acid) and microbial (alkaline) phosphatase activities are driven by different nutrient demand (Spohn and Kuzyakov 2013b; Spohn et al. 2015). However, evidence of this differentiation in origin and nutrient demand between acid and alkaline phosphatase enzymes has only been shown under pot experiments, while little proof is driven from field conditions.

Seasonal changes in environmental conditions such as temperature and rainfall (soil moisture) can influence P dynamics via their impact on soil microbial activity and plant growth (Mandal et al. 2007; Chen et al. 2008; Shi et al. 2013). Zhang et al. (2020) found that drought decreased microbial biomass P and acid phosphatase activity in a warm temperate forest. On the other hand, (Sun et al. 2020) showed that high rainfall decreased organic P mineralisation by inhibiting the activity of alkaline phosphatase enzymes in a tropical forest. Data from temperate pastoral and silvopastoral systems showed that higher plant growth over the spring season coincided with greater rates of organic P mineralisation (Tate et al. 1991; Scott and Condron 2003). In a recent study, Shi et al. (2020) found that short-term P fertlisation had a limited effect on temporal variations of soil microbial biomass P and alkaline phosphatase activity in a grassland soil. However, seasonal changes in these soil parameters under long-term P inputs are still under speculation.

The Winchmore P fertiliser trial was initiated in 1952 and is the longest running replicated grazed pasture experiment in the world (McDowell et al. 2021). The initial objective of this trial was to determine the pasture response to different rates of mineral P fertiliser (single superphosphate) under flood irrigation. Over the years, the Winchmore fertiliser trial has become a valuable research resource for investigating many soil chemical properties and processes, including P, sulphur, and cadmium (Condron and Goh 1989; Nguyen and Goh 1990; Gray et al. 2020). The trial has been used to investigate the effects of P inputs and pasture productivity on soil C dynamics and earthworm

ecology (Fraser et al. 1994; Condron et al. 2012; Wakelin et al. 2017), although little is known about the corresponding impacts on the biological and biochemical processes that control soil P dynamics. The primary objective of this study was to assess and quantify the cumulative impacts of long-term inputs of different rates of P fertiliser on key soil parameters linked to biological P cycling, including Olsen P, microbial biomass P, and phosphatase activities. A second objective was to investigate the impact of differences in soil P status from previous P inputs on short-term seasonal changes in these parameters. We hypothesised that while Olsen P and microbial biomass P would be significantly impacted by relative P status, phosphatase enzyme activities would be more closely linked to C and P availabilities. We also hypothesised that seasonal changes in soil biology and biochemistry related to P cycling would be influenced by changes in environmental conditions rather than P status.

4.2 Materials and methods

4.2.1 Description of the trial

The field study is situated at Winchmore, New Zealand (latitude: 43.787°S, longitude: 171.795°E, and altitude: 160 m) (Figure 4.1). Full details of the trial and associated physical and chemical data are given in McDowell et al. (2021). Briefly, the mean air temperature in this area is 11.1 ^{o}C while the average rainfall is 730 mm, with generally January being the warmest month and July the month with the most rainfall. The soil is a Lismore stony silt loam (Orthic Brown, New Zealand; Dystric Cambisols, World Reference Base for Soils Resources). In 1949, the site was sown with a pasture mixture dominated by ryegrass and white clover, and in 1952, five P fertiliser treatments were imposed replicated 4 times in a randomised block design. Each replicate plot (≈ 0.09 ha) was fenced and grazed by a separate flock of sheep in spring, summer, and autumn (no grazing during the winter period) to avoid nutrient transfer (Figure 4.1). The number of sheep per plot was rationalised based on pasture production and pasture utilisation (80 %) for each treatment. For the present study, we focused on three treatments, namely the nil or control (no fertiliser), 188P (188 kg single super phosphate (SSP) ha^{-1} yr^{-1}), and 376P (376 kg SSP ha^{-1} yr^{-1}). The fertiliser was applied by top-dressing in August-September each year (late winter), and lime was added on 3 occasions to maintain soil pH at or above 6. Between 1952 and 2018, the trial was irrigated by a border-dyke system, whereby 100 mm was applied to each replicate plot when soil moisture reached 15-20 % (0-10 cm). In 2018, the system was converted to an overhead spray irrigation system, and an average of 10 mm was applied every 3 to 5 days to ensure that ≈ 90 mm of rain and irrigation water was available monthly for pasture growth.

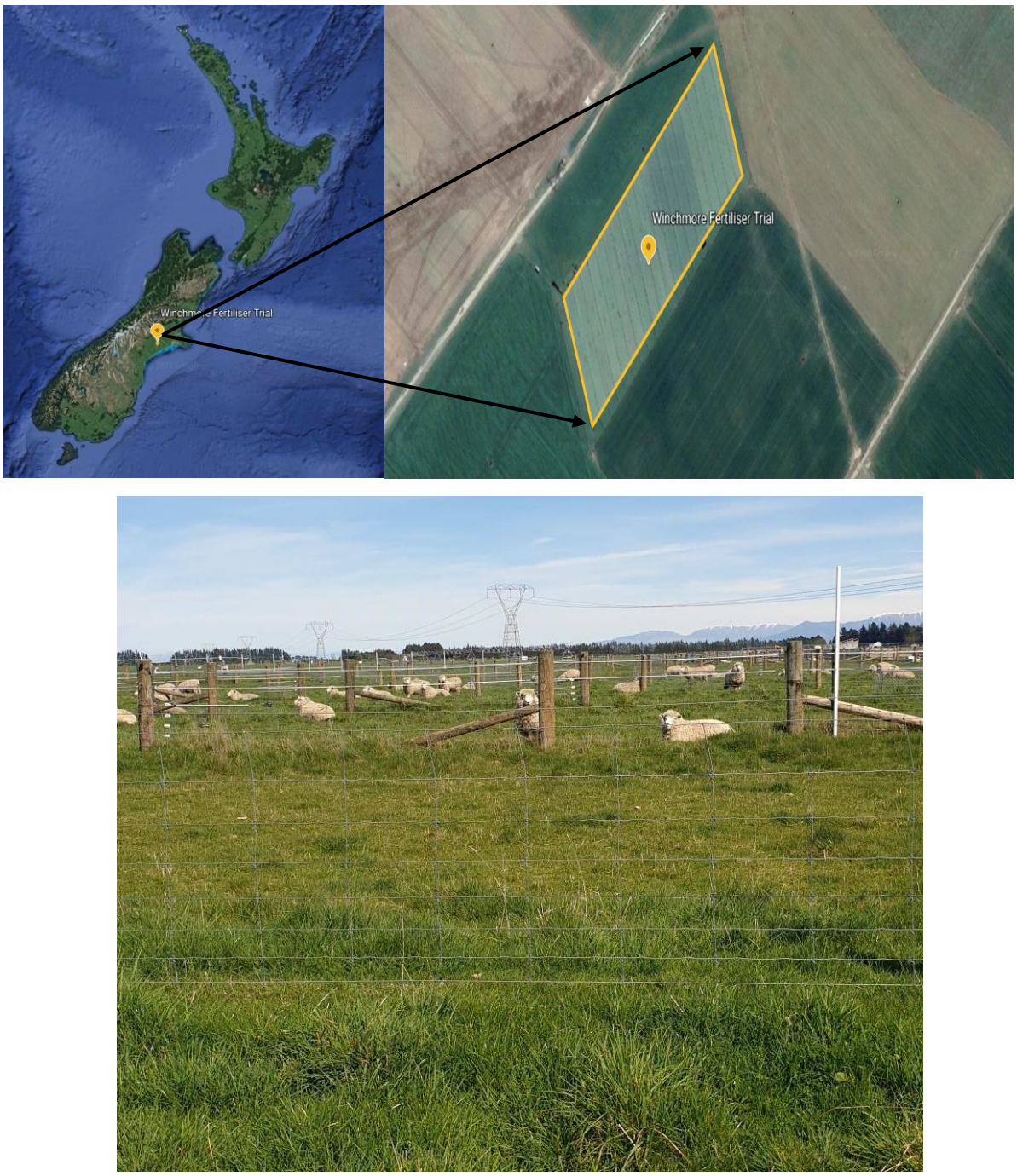


Figure 4.1 Location of the Winchmore fertiliser trial (top left), design of the trial showing the 20 plots of the trial (5 treatments with four replicates each) (top right), and picture of sheep grazing one of the treatments (bottom).

4.2.2 Meteorological data

Meteorological data (monthly rainfall and mean soil temperature) for the period of the study have been retrieved from the National Institute of Water and Atmospheric Research (NIWA) website https://cliflo.niwa.co.nz for the Winchmore weather station (Number: 6476, latitude: 43.79346˚S, longitude: 171.79512˚E). The quantities of water supplied to the trial site via central pivot have been provided by The Fertiliser Association of New Zealand. Soil temperature for one week before sampling date was used for calculations. Meteorological data are presented in Figure 4.2.

4.2.3 Soil sampling

The Winchmore fertiliser trial was sampled in October (spring), January (summer), March (autumn), and July (winter) over 2 years, starting from October 2018 till July 2020. Plots were sampled by taking 20 soil cores from the top 0-7.5 cm with a 2.5 cm diameter corer in a zigzag pattern. Care was taken to sample the same part of the plot each time while avoiding sheep camps (Nguyen and Goh 1992a). Stones and coarse plant materials were removed from the soil samples, and the soil sieved less than 2 mm. Soil samples were then kept in the fridge at 4 °C for 48 hours to allow for microbial activity to stabilise. Soil biological and biochemical properties were analysed within a week from sampling. A subsample was taken to determine the gravimetric soil moisture by oven drying the soil at 105 ^{o}C until constant weight. The remaining soil was air-dried at room temperature and used for the determination of Olsen P.

4.2.4 Soil analyses

Bioavailable P was determined by measuring Olsen P according to Watanabe and Olsen (1965), and inorganic P was analysed using the molybdenum blue method (Murphy and Riley 1962). Microbial biomass P was determined according to the fumigation-extraction method of Brookes et al. (1982) with the recommendations of Morel et al. (1996). A coefficient of recovery (40 %) was used to calculate microbial biomass P. Potential acid and alkaline phosphatase activities were assessed following the procedure of Tabatabai (1994). Potential phosphatase activities were reported as µmol *p*-nitrophenol produced g^{-1} fresh soil h^{-1}.

4.2.5 Statistical analysis

Acid and alkaline phosphatase activities and soil moisture were subjected to two-way repeated measures analysis of variance (ANOVA) to test the effects of P treatment, sampling date, and their interaction. Olsen P and microbial biomass P did not meet the assumption of data normality to carry out the two-way ANOVA, even after transformation; therefore, a non-parametric test (Friedman's test) was performed instead. In the presence of a significant effect, one-way ANOVA was used to test the effect of P treatment and sampling date separately. The *post-hoc* Tukey test was performed to separate group means. The level of significance was set at 5 % probability. Pearson's correlation analysis was carried out to determine relationships between soil biological and biochemical properties and environmental conditions (soil moisture and soil temperature). Spearman's correlation was performed whenever non-normal variables (Olsen P and microbial biomass P) were involved. All the above analyses were performed using SPSS version 25.0 (SPSS, Chicago, IL, USA), while the data were plotted using Microsoft Excel and SigmaPlot software version 14.0 (Systat Software, CA, USA).

4.3 Results

4.3.1 Environmental conditions

Between August 2018 to July 2019, the trial received a total of 1159 mm of water (888 mm rainfall, 271 mm irrigation) compared to 921 mm (630 mm rainfall, 291 mm irrigation) between August 2019 and July 2020 (Figure 4.2). Higher soil temperatures were recorded in summer (January-February), whereas lower soil temperatures were noted in winter (June-July) in both 2019 and 2020 (Figure 4.2). Soil moisture ranged from 24 to 47 % and exhibited lower values during summer-early autumn (Figure 4.3a, Table 4.1). Moreover, throughout the study period, soil water content was lower under the 188P and 376P treatments compared to the control, especially in spring and summer.

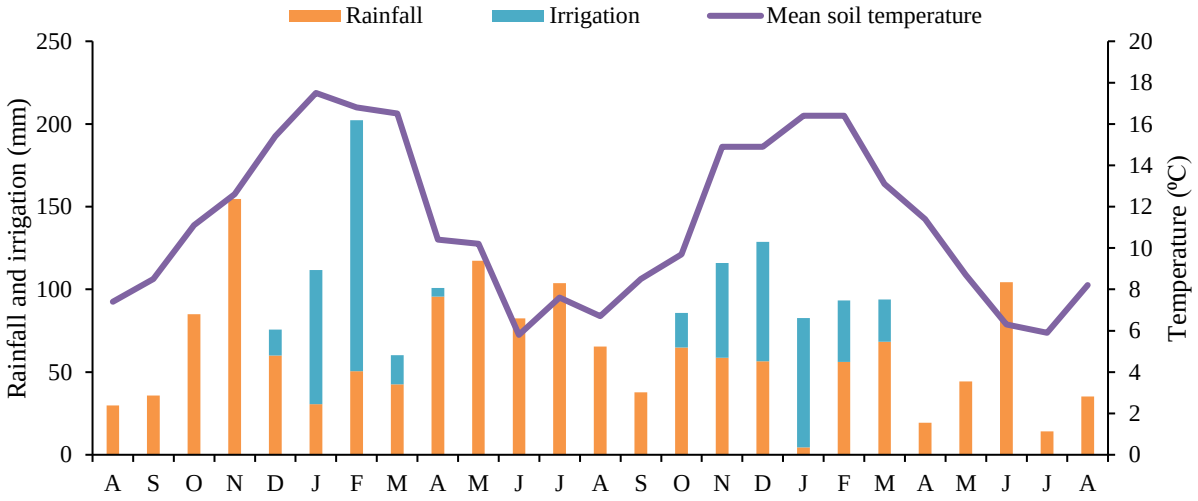


Figure 4.2 Monthly mean soil temperature, rainfall, and irrigation by pivot at the Winchmore trial from August 2018 to August 2020.

4.3.2 Olsen P

Long-term P applications significantly increased Olsen P concentrations compared to the unfertilised control. Differences in Olsen P concentrations between 376P and 188P treatments were also significant (Figure 4.3b). Concentrations of Olsen P averaged 63.1, 17.2, and 4.5 mg kg^{-1} under 376P, 188P, and the control, respectively (Table 4.3b). Over the study period, Olsen P fluctuated in the fertilised treatments, especially under 376P, while no significant differences between sampling dates were noted for the control treatment.

4.3.3 Microbial biomass P

In the 376P and 188P treatments, maximum concentrations of microbial biomass P were recorded in summer and winter (January and July), while minimum concentrations were noted in spring and autumn (October and March) across the study period. Microbial biomass P was significantly higher under 188P and 376P compared to the control treatment, especially in summer and winter (Figure 4.3c). Differences in MBP were not significant between 188P and 376P treatments during the study period. Overall averages of microbial biomass P were 50.5, 50.6, and 39.3 mg kg^{-1} for 376P, 188P, and the control, respectively (Table 4.1). Significant seasonal variations in microbial biomass P were observed under 188P and 376P, whereas the control treatment showed minor but not significant seasonal variations (Fig. 4.3c).

To evaluate the importance of microbial biomass P in soil P bioavailability and transformations, ratios of biomass P to total P and biomass P to Olsen P were calculated. Results showed that 8.5 % of total P was present as biomass P in the control, while biomass P accounted for 7.1 and 4.6 % of total P under 188P and 376P, respectively (Table 4.2). On the other hand, the ratio of biomass P to Olsen P was on average 9.0 for the control and decreased to 2.9 and 0.8 for 188P and 376P, respectively (Table 4.1).

4.3.4 Phosphatase activities

The data showed that acid and alkaline phosphatase activities were significantly affected by P treatments. In fact, acid phosphatase activity in the control was higher than P fertilised treatments (188P and 376P) with an average of 14 % over the study period (Table 4.1, Figure 4.4a). In contrast, alkaline phosphatase activity was 14 and 22 % higher under 188P and 376P, respectively, compared to the control treatment (Table 4.1, Figure 4.4b). Regardless of P status, acid and alkaline phosphatase activities exhibited similar seasonal patterns with elevated activity in summer and lower activity in winter (Figure 4.4a, b).

4.3.5 Relationship between Olsen P, microbial biomass P, phosphatase activities, and environmental conditions

Correlation analysis showed that microbial biomass P was significantly and positively correlated with alkaline phosphatase activity ($P < 0.01$), whereas there was a significant negative correlation between Olsen P and acid phosphatase activity in this study ($P < 0.01$) (Table 4.3). On the other hand, microbial biomass P and Olsen P were poorly correlated with environmental conditions (soil moisture and soil temperature) in this irrigated grazed pasture system. However, acid and alkaline phosphatase activities were significantly impacted by soil temperature and soil moisture ($P < 0.01$). In

fact, high soil temperature enhanced phosphatase activity, while high soil moisture had a repressive effect on phosphatase enzymes in this study (Table 4.3).

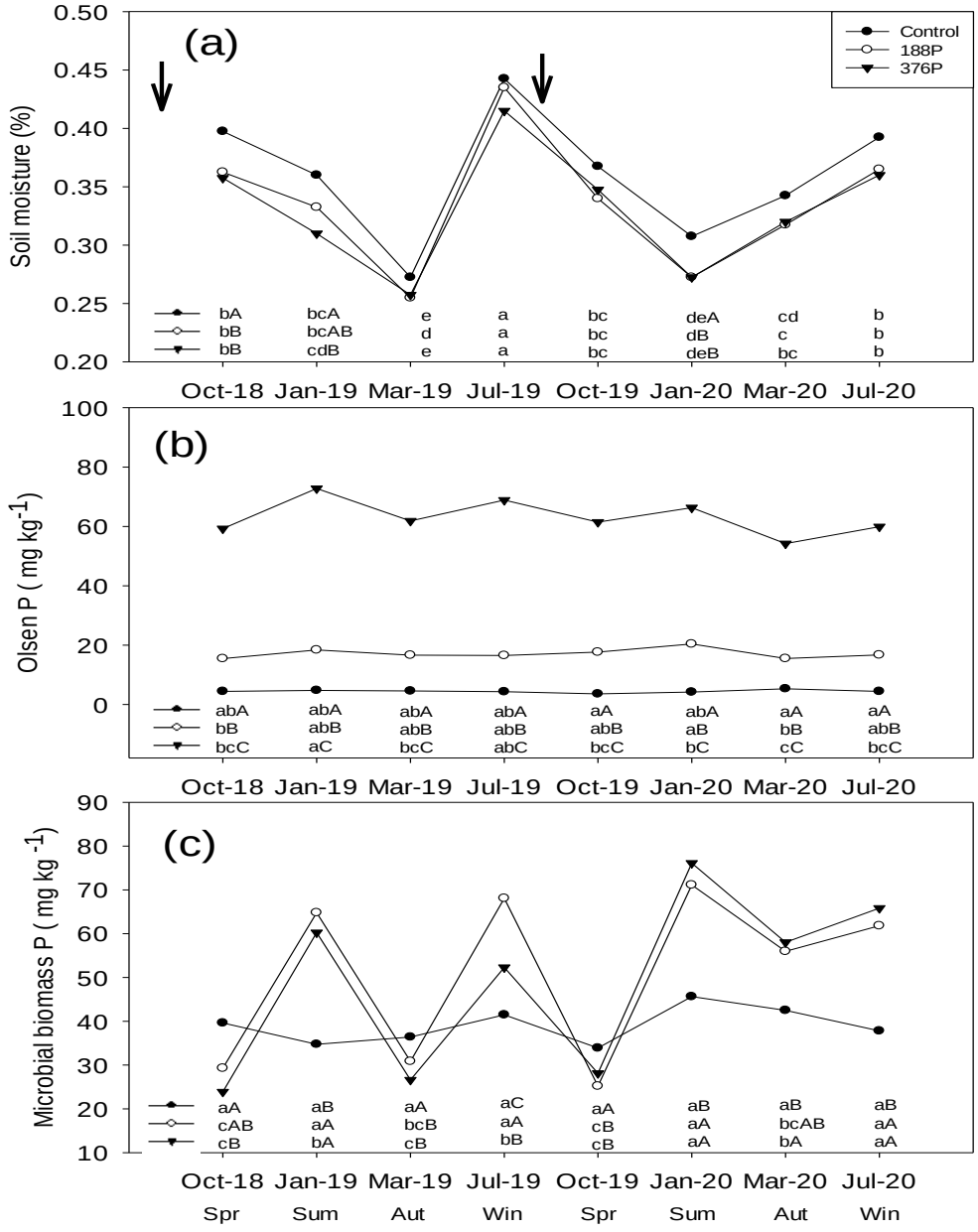


Figure 4.3 Temporal changes in soil moisture (a), Olsen P (b), and microbial biomass P (c) in the control, 188P, and 376P treatments. Soil samples were taken from 0-7.5 cm of the Winchmore fertiliser trial from October 2018 to July 2020. Values are means of 4 replicates. Error bars were omitted for clarity and lowercase and uppercase letters were used to show significance. Within columns, different lowercase letters indicate significant differences ($P < 0.05$) between sampling dates for a given P treatment, while within rows different uppercase letters indicate significant differences ($P < 0.05$) between P treatments for a given sampling date. Arrows in bold indicate times of P applications that is, late August 2018 and 2019.

Table 4.1 Range and means of Olsen P (mg kg^{-1}), microbial biomass P (MBP) (mg kg^{-1}), ratio MBP: Olsen P, acid phosphatase (acid P) (µmol g^{-1} h^{-1}), alkaline phosphatase (alkaline P) (µmol g^{-1} h^{-1}), and soil moisture (%) measured in the control, 188P, and 376P treatments. Soil samples were taken from 0-7.5 cm of the Winchmore fertiliser trial from October 2018 to July 2020.

	Control		188P		376P	
	Range	Mean	Range	Mean	Range	Mean
Olsen P	3.26 – 5.80	4.46	13.60 – 23.39	17.15	50.63 – 76.52	63.09
MBP	25.51 – 58.84	39.32	22.27 – 83.30	50.57	20.72 – 86.58	50.51
MBP: Olsen P	4.81–12.29	9.05	1.14 – 4.70	2.95	0.36 – 1.48	0.80
Acid P	9.46 – 13.87	11.56	8.29 – 12.64	10.19	7.86 – 11.96	10.10
Alkaline P	3.32 – 6.27	4.69	3.70 – 7.60	5.34	3.91 – 7.66	5.71
Soil moisture	26 - 47	36	24 - 46	33.5	24 – 43	33

Table 4.2 Plant production (kg ha^{-1}), clover content (%), grass content (%), weed content (%), excretal P (kg ha^{-1} yr^{-1}), plant residues (kg ha^{-1}), total P (mg kg^{-1}), hot water extractable carbon (mg kg^{-1}), and ratio of microbial biomass P (MBP)/total P for the control, 188P, and 376P treatments.

	Plant production [a]	Excretal P [b]	Clover content [c]	Grass content [c]	Weed content [c]	Plant residues [d]	Total P [e]	Hot water extractable carbon [e]	MBP:total P [f]
Control	5289	0.7	< 8 %	72 % [g]	20 %	7.3	464.5	1685.5	8.5
188P	11428	2.7	>13 %	75 % [g]	12 %	13.6	716.75	1938.0	7.1
376P	12242	2.9	>13 %	75 % [g]	12 %	12.6	1102.5	2035.3	4.6

[a] From Smith et al. (2012).
[b] From Tian et al. (2019), estimated based on stocking rates according to Nguyen and Goh (1992b) and Simpson et al. (2011).
[c] From Fraser et al. (2011).
[d] From Tian et al. (2019), calculated from Scott et al. (2012) and Smith et al. (2012).
[e] From Wakelin et al. (2017).
[f] Calculated using our data for microbial biomass P (MBP) and total P from Wakelin et al. (2017).
[g] In the control treatment, the grass content comprised 55 % of ryegrass and 45 % of unsown grasses, while in the 188P and 376P treatments, ryegrass represented between 90 to 95 % of the total grass content (Fraser et al. 2011).

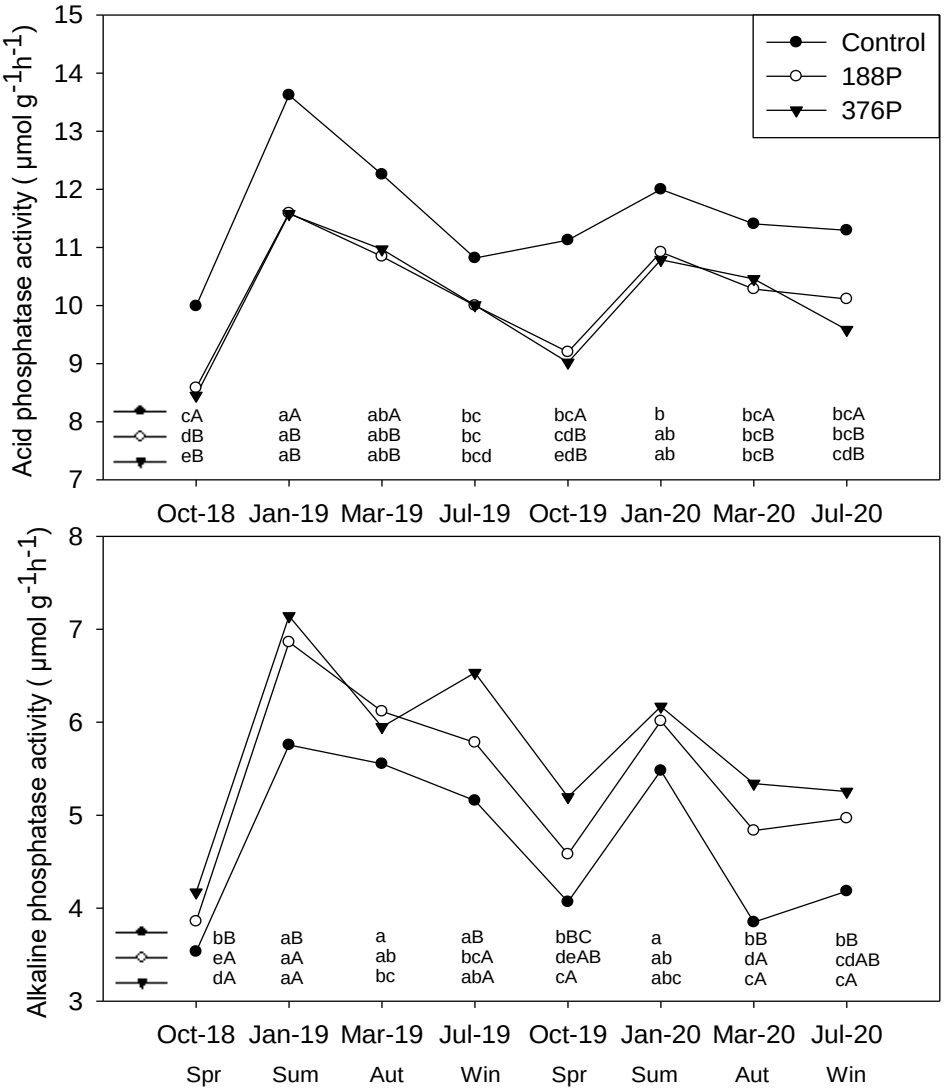


Figure 4.4 Temporal changes in acid (a) and alkaline phosphatase activities (b) in the control, 188P, and 376P treatments. Soil samples were taken from 0-7.5 cm of the Winchmore fertiliser trial from October 2018 to July 2020. Values are means of 4 replicates. Error bars were omitted for clarity and lowercase and uppercase letters were used to show significance. Within columns, different lowercase letters indicate significant differences ($P < 0.05$) between sampling dates for a given P treatment, while within rows different uppercase letters indicate significant differences ($P < 0.05$) between P treatments for a given sampling date.

Table 4.3 Values of correlation analysis (Pearson's and Spearman's) between soil biological and biochemical properties and environmental factors (n = 96)

	Olsen P	MBP	Acid P	Alk P	Soil moisture	Soil temperature
Olsen P	1	0.206*	-0.362**	0.482**	-0.196	0.067
MBP	0.206*	1	0.176	0.389**	-0.017	-0.047
Acid P	-0.362**	0.176	1	0.382**	-0.206*	0.529**
Alk P	0.482**	0.389**	0.382**	1	-0.304**	0.459**
Soil moisture	-0.196	-0.017	-0.206*	-0.304**	1	-0.735**
Soil temperature	0.067	-0.047	0.529**	0.459**	-0.735**	1

* Significant difference at $P < 0.05$; ** Significant difference at $p < 0.01$; MBP, microbial biomass phosphorus; Acid P, acid phosphatase activity; Alk P, alkaline phosphatase activity.

4.4 Discussion

4.4.1 Long-term P fertiliser impacts

Phosphorus fertilisation in agroecosystems has been recommended to enhance soil P availability and increase primary productivity (Simpson et al. 2011; Haygarth et al. 2013). However, long-term P applications in these ecosystems have led to an accumulation of inorganic P, thus increasing the risk of eutrophication due to P losses (Sharpley et al. 2001; McDowell et al. 2003). As expected, long-term P inputs showed higher concentrations of Olsen P under 376P, followed by 188P and the control. The average concentrations of Olsen P over the entire study period were 63.1, 17.2 and 4.5 mg kg^{-1} for 376P, 188P, and the control, respectively. Previous studies combining data of pasture production and bioavailable P have pointed out that Olsen P under the 376P treatment was considerably above the agronomic optimum, presenting a serious risk of P losses, especially under irrigation (Nguyen and Goh 1992b; Edmeades et al. 2006).

Microbial P immobilisation occurs in P deficient conditions as well as when P is readily available in the soil solution (Bünemann et al. 2012; Spohn and Widdig 2017; Wei et al. 2019a). Besides P, soil microbial biomass is limited by the availability of C and N (Griffiths et al. 2012; Heuck et al. 2015). Over the whole study period, microbial biomass P was increased by the long-term P applications (average: 50 mg kg^{-1}) compared to the control (average: 39 mg kg^{-1}). This is in line with previous studies in pasture soils (Tate et al. 1991; Dodd et al. 2014; Shi et al. 2020). However, microbial biomass P under 188P and 376P was not significantly different as expected. In fact, plant production, excretal P, and the proportion of white clover were quite similar under these two treatments (see Table 4.2) (Nguyen and Goh 1992b; Fraser et al. 2011; Scott et al. 2012; Tian et al. 2019). Moreover, Nguyen and Goh (1992a) and Wakelin et al. (2017) found that total N and available C were not

significantly different between 188P and 376P treatments. Therefore, similar availability of C and N has probably generated similar P immobilisation by the microbial biomass despite P availability was different under 188P and 376P treatments. In fact, it has been shown that C:N:P stoichiometry is a key factor regulating microbial biomass transformations and dynamics in different ecosystems (Griffiths et al. 2012; Maaroufi and De Long 2020; Luo et al. 2020).

The contribution of microbial P to total P can vary between 0.5 to 40 % according to cropping systems, soil organic C, and nutrient availability (Brookes et al. 1984; Chen et al. 2003a; Oberson and Joner 2005). Moreover, it is well established that microbial P is a dynamic P pool able to supply available P to plants (Achat et al. 2010; Richardson and Simpson 2011). Our data showed that 8.5 % of total P was in the microbial form under the control treatment in contrast to 7.1 and 4.6 % in 188P and 376P, respectively. This suggests that microbial biomass P played an essential role in maintaining P availability under the control treatment. This is further emphasised by the fact that the microbial biomass P to Olsen P ratio under the control was 3- and 11-fold higher compared to 188P and 376P, respectively.

Phosphatase enzymes play a crucial role in the organic P mineralisation process, thereby regulating soil P dynamics (Tarafdar and Jungk 1987; Tabatabai 1994; Nannipieri et al. 2011). Our results showed that acid and alkaline phosphatase activities exhibited opposite trends in response to contrasting long-term P inputs. While acid phosphatase activity was inhibited by P applications, alkaline phosphatase enzymes exhibited higher activity under P fertilised treatments in comparison with the control. Our results compared well with other studies showing that P applications decreased acid phosphatase activity (Nuruzzaman et al. 2006; Lemanowicz 2011). However, a closer look at the response of alkaline phosphatase activity to P applications in the literature reveals contradictory results (Shi et al. 2013; Tan et al. 2013; Ikoyi et al. 2018). While P applications decreased alkaline phosphatase enzymes in intensive cropping systems (Saha et al. 2008; Chen et al. 2019b; Liu et al. 2020), organically managed agroecosystems showed higher alkaline phosphatase activity (Sakurai et al. 2008; Liu et al. 2010; Fraser et al. 2015; Chen et al. 2017). This suggests that alkaline phosphatase activity is impacted by soil organic matter inputs and C turnover (Allison and Vitousek 2005; Demoling et al. 2007).

Plant biomass under the 188P and 376P was 2.2 and 2.4-fold higher than the control (Table 4.2) (Smith et al. 2012). Plant and root cells contain large amount of organic P in the form of phospholipids, nucleic acids, and phospho-esters (Berg and Joern 2006; Alamgir et al. 2012; Noack et al. 2012). Moreover, in grazing systems, dung can release a considerable amount of P with up to 15 % as organic P (McDowell and Stewart 2005; Arnuti et al. 2020). Higher plant residues and excretal P in P fertilised treatments (Table 4.2) indicate higher organic P returns to the soil under these

treatments in comparison with the control (Simpson et al. 2011; Stutter et al. 2015). Additionally, soil P bioavailability was high under P fertilised treatments inducing C-limitation in soil microbial growth (Griffiths et al. 2012). Therefore, soil microorganisms needed to dephosphorylate organic P in order to satisfy their C demand, thereby exhibiting higher alkaline phosphatase activity (Spohn and Kuzyakov 2013a; Spohn et al. 2015). In fact, Wakelin et al. (2017) found that C cycling was faster under P fertilised treatments because soil microbes are driven by C demand rather than P (Spohn and Kuzyakov 2013b; Heuck et al. 2015). Furthermore, our data showed a significant and positive correlation between microbial biomass P and alkaline phosphatase activity ($P < 0.01$). Taking together, our results provide evidence from long-term field trial of the microbial origin of alkaline phosphatase enzymes as well as their dependence on C availability.

It has been suggested that acid phosphatase activity is mostly derived from plant roots to mobilise organic P (McLachlan 1980; Dick et al. 1983; Yadav and Tarafdar 2001). Moreover, root phosphatase enzymes have been described to be more sensitive to P applications, thus being repressed by high P availability in soils (Tadano and Sakai 1991; Adams and Pate 1992). This probably explains the significantly lower acid phosphatase activity found under 188P and 376P in comparison with the control. Our findings are further supported by the negative and significant correlation found between acid phosphatase activity and Olsen P ($P < 0.01$). These results corroborate the findings by Colvan et al. (2001) in grassland soils.

The activity of acid and alkaline phosphatase enzymes can be affected by soil pH (Dick et al. 2000). In the current study, soil pH (0-7.5 cm) was significantly lower under the P fertilised treatments compared to the control in July 2019 and 2020 (Data not shown), corroborating the results of (Wakelin et al. 2017) in the same trial. Lower pH under 188P and 376P could be ascribed to soil acidification due to the application of superphosphate fertiliser (Horsnell 1985), together with an abundance of white clover (known for soil acidification via N_2 fixation) under these treatments (Tang and Rengel 2003; Fraser et al. 2011). This result suggests that acid phosphatase activity would be higher under the P fertilised treatments. However, our results showed that acid phosphatase activity was higher under the control, whereas alkaline phosphatase activity was higher under P fertilised treatments. Therefore, differences in acid and alkaline phosphatase activities were not related to changes in soil pH in response to long-term P inputs in this grazed pasture.

Long-term P inputs have been shown to shape the soil microbial community, thereby impacting soil phosphatase activity (Tan et al. 2013; Chen et al. 2019b; Liu et al. 2020). Our results indicated that long-term P fertilisation had a profound effect on soil phosphatase activities, while previous studies in the same site have revealed significant changes in microbial taxa linked to soil P cycling in response to long-term P inputs (Mander et al. 2012; Wakelin et al. 2012). The recent use of metagenomics has

enhanced our understanding of soil ecology in relation to biogeochemical processes involved in P cycling (Ragot et al. 2015; Gaiero et al. 2018; Liu et al. 2021). Such approaches may prove useful in unravelling the contribution of different microbial taxa to acid and alkaline phosphatase activity at the Winchmore site.

4.4.2 Impacts of seasonal conditions

Seasonal variations in soil microbial biomass and activity are central to regulating P transformations and bioavailability to plants (Tate et al. 1991; Chen et al. 2003a; Shi et al. 2013). Bioavailable P concentration in soils at any point of time is controlled by an interaction between biotic and abiotic factors, including fertiliser applications, adsorption-precipitation processes, organic P mineralisation, environmental conditions, plant P demand, and soil microbial and fauna activities (Cole et al. 1977a; Perrott et al. 1992; Frossard et al. 2000; Pierzynski and McDowell 2005; Chapuis-Lardy et al. 2011). Our data showed that the control treatment had a relatively stable Olsen P concentration throughout the study period, whereas seasonal variations were observed under P fertilised treatments, especially 376P. Similar results were reported by Tate et al. (1991b) and Perrott et al. (1990) when comparing bioavailable P in low and high P pasture soils in New Zealand. Higher P application rates and total P under P fertilised treatments, especially 376P compared to the control, can explain the higher variations of Olsen P concentrations during the study period. Furthermore, higher plant P demand, plant returns, and microbial and earthworm activities under P fertilised treatments are likely to contribute to the seasonal variations observed (Nguyen and Goh 1992b; Smith et al. 2012; Tian et al. 2019). Investigating the same trial used in this study, Wakelin et al. (2017) found that microbial activity and carbon turnover were higher under P fertilised treatments compared to the control. Fraser et al. (1994) showed that the population and biomass of earthworms were significantly higher under P fertilised treatments, especially 376P, compared to the control due to better quality of plant returns (higher P and N content) under these treatments.

Our results showed that seasonal changes in microbial biomass P were more pronounced under 188P and 376P compared to the control treatment, indicating much faster P cycling via the microbial biomass under high versus low P soils (Tate et al. 1991; Perrott et al. 1992). On the other hand, relatively stable microbial biomass P under the control treatment was essential to maintaining constant Olsen P concentration under this treatment. Microbial biomass P in 188P and 376P followed exactly similar temporal trend indicating that similar conditions have driven microbial biomass P in these treatments. As previously discussed, plant biomass, plant returns, soil microbial activity, and plant community composition were similar under P fertilised treatments. Microbial biomass P was higher in summer, probably because warmer conditions and adequate soil moisture stimulated plant growth and enhanced inputs of root exudates into the soil (Chen et al. 2003a; Shi et al. 2020). Plant

rhizodeposits would have been higher under P fertilised treatments due to higher plant biomass compared to the control. These conditions might have then promoted microbial P immobilisation. Perrott et al. (1992) found higher microbial biomass P in summer under a temperate pasture. They indicated that higher temperature and summer rainfall promoted pasture growth with a concomitant increase in root exudates, which enhanced bacterial growth. Microbial biomass P was also higher in winter in our study. Under pasture systems, an accumulation of microbial and organic P has been described in winter (Tate et al. 1991; Perrott et al. 1992; Chen et al. 2003a; Scott and Condron 2003). The storage of labile organic P and P in the microbial biomass has been suggested to be driven by low temperatures leading to lower microbial activity and plant P demand as well as potential changes in the microbial community composition (Sarathchandra et al. 1989; Perrott et al. 1990, 1992). In a pot experiment with no water limitation, Sarathchandra et al. (1989) simulated winter conditions (lower temperature) in soil cores from a highly productive pastoral system and investigated biological and biochemical soil properties. They found that C, N, and P accumulated in the microbial biomass in winter and related that to the shift in microbial population towards more fungi able to degrade root residues accumulated in winter. In a seasonal study, Chen et al. (2003a) found higher microbial biomass P during winter in a grassland soil, which concurred with higher total organic C and total N and P. They suggested that increased microbial biomass P in winter was potentially derived from grass root litter returns. In the current study, plant biomass under P fertilised treatments was on average 2.3-fold higher than the control, implying that herbage returns and root residues were likely higher under these treatments compared to the control. Hence, higher root residues may have increased the population of fungi, thereby promoting higher microbial P immobilisation under P fertilised treatments compared to the control. Nevertheless, an assessment of soil microbial diversity across P treatments and seasons is required to confirm this hypothesis. Decrease in microbial biomass P noticed in spring and autumn could be explained by the adequate environmental conditions and higher pasture P demand, thereby promoting microbial P release to meet plant uptake (Perrott et al. 1992). In fact, plant production at the Winchmore fertiliser trial has been found to be higher in spring, summer, and autumn (Smith et al. 2012). Correlation analysis results revealed that microbial biomass P was weakly correlated with soil temperature and soil moisture, suggesting that a complex array of factors regulated microbial P release in this grazed pasture system, including environmental conditions, plant P demand, plant returns, microbial activity, and microbial community composition.

Organic P mineralisation has been suggested to be driven by soil moisture and temperature, which affect soil microbial activity and plant growth (Tate et al. 1991; Fabre et al. 1996; Chen et al. 2003a). In our study, acid and alkaline phosphatase activities showed similar seasonal trends across P treatments, suggesting that environmental conditions controlled organic P mineralisation

irrespective of soil P status. In their global meta-analysis, Margalef et al. (2017) pointed out that soil temperature was among the main factors driving phosphatase activity across different biomes. Ge et al. (2017) showed that increasing temperature enhanced the activity of phosphatase enzymes in the rhizosphere of rice. Wu et al. (2015) found that warming accelerated C turnover, shifted microbial community composition, and increased phosphatase activity in a meadow. In this study, higher soil temperature during the spring-summer period suggested an increased phosphatase activity and accelerated organic P mineralisation (Chen et al. 2003a). Indeed, a significant and positive correlation was found between the activity of acid and alkaline phosphatase enzymes and soil temperature. These findings agree with previous studies in temperate pastoral and silvopastoral systems showing depletion of organic P in the spring-summer period to meet increased plant P demand (Tate et al. 1991; Scott and Condron 2003).

Soil moisture also plays a crucial role in modulating soil phosphatase activity (Margalef et al. 2017). Recent data from forest ecosystems have shown that drought decreased acid phosphatase activity (Zhang et al. 2020), whereas high rainfall decreased organic P mineralisation by inhibiting the activity of alkaline phosphatase enzymes (Sun et al. 2020). Previous studies under pastoral and silvopastoral systems have also shown a build-up of organic P during winter, which was linked to lower microbial activity and plant growth (Harrison 1979; Perrott et al. 1992; Scott and Condron 2003; Sun et al. 2020). Our results showed an inverse relationship between soil moisture and the activity of acid and alkaline phosphatase enzymes, with minimum activity observed in winter. This was consistent with the findings of Speir and Cowling (1991) and Chen et al. (2003a) where soil phosphatase activity was lower in winter compared to summer and autumn. In this irrigated pasture system investigated in the current study, adequate soil moisture and high soil temperature in summer enhanced soil microbial activity and promoted plant growth, thereby showing higher phosphatase activities across all P treatments (Chen et al. 2003a, 2008). In contrast, excessive water and cold soil temperatures during winter reduced plant growth and may have decreased the activity of phosphatase enzymes (Harrison 1979; Sun et al. 2020). Therefore, our results confirmed that environmental conditions (soil moisture and temperature) drive soil organic P mineralisation regardless of soil P status through their impact on plant P demand and soil microbial activity. Moreover, opposite to the greatest rate of organic P mineralisation observed in temperate pastures in New Zealand, maximum organic P mineralisation took place in summer in this irrigated pasture system.

4.5 Conclusions

The findings of this study showed that long-term P inputs had a significant impact on soil microbial biomass and activity related to P cycling under grazed pasture. This was directly attributed to P availability and indirectly linked to net primary productivity, organic matter turnover, and plant

community composition. As expected, Olsen P significantly increased in response to long-term P inputs while microbial biomass P was similar under treatments receiving P fertilisers (188P and 376P). The latter result was attributed to microbial biomass need to maintain nutrient stoichiometry. Moreover, long-term P inputs increased alkaline phosphatase activity but decreased acid phosphatase. Results also showed that alkaline phosphatase activity was significantly correlated with microbial biomass P and driven by C demand, while acid phosphatase activity was associated with plant roots and repressed by P availability. These findings supported the differentiation in origin and nutrient demand between acid and alkaline phosphatase enzymes. Nevertheless, further investigations using metagenomics and phosphatase genes are needed at the Winchmore fertiliser trial to disentangle the contribution of different microbial taxa to acid and alkaline phosphatase activities. Seasonal changes in Olsen P reflected changes in plant P demand and microbial and earthworm activities linked to plant growth and plant litter quality. Microbial biomass P played a central role in maintaining bioavailable P, especially for the unfertilised control. Significant seasonal variations in microbial biomass P occurred under P fertilised treatments compared to the control. Under these treatments, microbial P immobilisation occurred in summer and winter, while microbial P release was observed in spring and autumn. Mechanisms related to the seasonal patterns in microbial biomass P include 1) higher temperature and increased plant biomass and root exudates in summer, 2) higher root residues, lower temperatures, and shift in the microbial population in winter, 3) increased plant P demand and microbial P release in spring and autumn. Similar seasonal patterns were observed for acid and alkaline phosphatase activities across all P treatments, indicating that organic P mineralisation was driven by environmental conditions regardless of soil P status. The results from this study highlighted the role of long-term experiments in advancing our understanding of soil P cycling and related processes in response to management practices.

Chapter 5

Impacts of Elevated Atmospheric Carbon Dioxide and Low Phosphorus Availability on Plant Growth, Rhizosphere Properties, and Soil Phosphorus Fractions under Legumes and Grasses

5.1 Introduction

Since the industrial revolution, elevated atmospheric CO_2 (eCO_2) has been a significant driver of climate change, with concentrations increasing from 270 ppm to more than 400 ppm currently and projected to reach 550 ppm by 2050 (IPCC 2014). Increased CO_2 is well documented to enhance plant growth through its effect on photosynthesis (Ainsworth and Long 2005), thereby accelerating soil nutrient dynamics to meet increased plant demand (Lobell and Gourdji 2012; Pandey et al. 2015a; Pang et al. 2018b; Bhattacharya 2019). This acceleration of nutrient cycling is of concern, especially for non-renewable resources such as phosphorus (P) (Cordell and White 2015). Plant responses to eCO_2 in deficient and sufficient P environments have been widely studied (Lam et al. 2012; Deng et al. 2015; Pandey et al. 2015a; Watts-Williams et al. 2019). However, little attention has been given to the study of soil P transformations and dynamics under eCO_2 and low P conditions. Furthermore, some recent reports have emphasised that the investigation of root morphology and functionality in response to eCO_2 is more important than the study of biomass partitioning and plant P uptake alone, especially under P deficient environments (Bhattacharya 2019; Jiang et al. 2020). Low P availability is widespread across terrestrial ecosystems (Vitousek et al. 2010; MacDonald et al. 2011; Hou et al. 2020; Du et al. 2020), and CO_2 levels are predicted to continue increasing in the future (Myers et al. 2017; Soares et al. 2019). Therefore, it is of great interest to determine the response of different plant species to the interactive effects of eCO_2 and low P availability on soil P cycling. This understanding is essential to advance our knowledge on soil-plant interactions in response to future climate change scenarios and critical to the management of plants and fertilisation practices needed to sustain crop production in a context of global change.

Phosphorus is considered the second most limiting nutrient, behind nitrogen, for plant production and is a finite resource that must be managed very wisely to meet increasing food demand by the world population (Raghothama 2005; Cordell et al. 2009; Bhattacharya 2019). In P-limited conditions, plants have evolved a plethora of mechanisms to increase P acquisition, especially at the soil-plant interface (Marschner 1995; Raghothama 1999; Richardson et al. 2009). The release of organic anions

and phosphatase enzymes together with soil acidification and mycorrhizal symbiosis are the main mechanisms by which plants and microbes mobilise different inorganic and organic P forms (Marschener 1998; Jones 1998; Hinsinger 2001; Nannipieri et al. 2011; Wang and Lambers 2020b). Legumes and grasses have shown different adaptations in response to low P availability (Pearse et al. 2006; Pandey et al. 2015a; Sandaña and Pinochet 2016; Pang et al. 2018b). For instance, white lupin and chickpea release organic anions such as citrate, malate, and malonate to desorb recalcitrant P pools (Veneklaas et al. 2003; Lambers et al. 2013; Pang et al. 2018a). In contrast, the increase of root surface area via the production of finer roots and long root hairs together with the symbiosis with mycorrhizal fungi have been identified among the main morphological adaptations used by grasses to access soil P and increase P use efficiency (Singh Gahoonia and Nielsen 2004; Wang et al. 2010a; Pandey et al. 2015a; Sandaña and Pinochet 2016). Nevertheless, some new insights have revealed that cereals such *Triticum durum* and *Triticum aestivum* can rely on a combination of root morphological and physiological traits to increase soil P availability, particularly under P deficiency and eCO_2 environment (Pandey et al. 2018; Lal et al. 2019).

Elevated CO_2 is generally believed to increase P availability and accelerate P cycling due to enhanced rhizodeposition, increased phosphatase activity, and improved root mycorrhisation (Pandey et al. 2015b; Jin et al. 2015; Bhattacharya 2019). However, previous studies have reported contrasting results on soil P availability and dynamics in response to eCO_2 in a range of ecosystems (Khan et al. 2008; Jin et al. 2013, 2015; Zhang et al. 2014b; Bhattacharyya et al. 2014; Tfaily et al. 2018). For example, Touhami et al. (2020b) found a decrease in available P under eCO_2 resulting from increased microbial activity and organic P accumulation. On the other hand, Huang et al. (2014) and Khan et al. (2008) pointed out that forest plants subjected to long-term CO_2 enrichment enhanced soil P availability, mainly due to mobilisation of recalcitrant and stable P pools such as residual P. The discrepancy between these studies could be ascribed to differences in plants species, soil types, CO_2 enrichment designs, experiment durations, and the availability of other nutrients such as nitrogen (Edwards et al. 2005; Khan et al. 2008; Jin et al. 2017; Touhami et al. 2020b). Additionally, eCO_2 had shown to induce no consistent patterns in terms of plant physiological adaptations (release of phosphatase enzymes and organic anions) to P deficiency. For instance, eCO_2 and low P availability increased phosphatase activity in wheat (Barrett et al. 1998), rice (Bhattacharyya et al. 2014), and *Arabidopsis thaliana* (Niu et al. 2013a). However, other studies have described a repressive or no effect of eCO_2 on phosphatase enzymes (Almeida et al. 1999; Wasaki et al. 2003; Norisada et al. 2006). While several experiments have shown an increased release of organic anions under plant species subjected to eCO_2 (Delucia et al. 1997; Campbell and Sage 2002; Haase et al. 2007; Johansson et al. 2009; Bhattacharyya et al. 2014), Jin et al. (2015) pointed out that this higher release is not sufficient to claim more solubilisation of P unless this environment stimulates the release of more

efficient organic anions in mobilising P from sparingly available forms. Moreover, the role of organic anions in real soil conditions is still not well understood (Jones 1998; Valentinuzzi et al. 2015; Oburger and Jones 2018). Therefore, the assessment of organic anions in P deficient soils and eCO_2 conditions is recommended to close this knowledge gap (Pang et al. 2018b; O'Sullivan et al. 2020).

Grasses and legumes are essential components of human and animal diet, besides their ecological relevance to agroecosystems and their critical role in soil fertility (Jones and Lazenby 1988; Cordain 1999; Lambers et al. 2013; Pandey et al. 2015a; Pang et al. 2018b). In New Zealand, wheat represents the main arable crop, while ryegrass is considered the dominant grass species sown in pasture systems (Charlton and Stewart 1999; Lambin and Geist 2006). Legumes such as blue lupin (narrow-leafed lupin) constitute a high-quality feed for animals and are well known for their physiological adaptations to P deficiency (Wang et al. 2008; Chen et al. 2013). While some information is available on soil P dynamics for wheat under eCO_2 (Manoj-Kumar et al. 2011; Jin et al. 2013, 2014), the response of ryegrass and blue lupin to eCO_2 when grown in a low P soil and their impact on soil P fractions is still unknown. Therefore, this study aimed at determining the combined effects of eCO_2 and low P availability on plant growth and rhizosphere properties together with changes in soil P fractions under three plant species characterised by divergent root morphology and secretory ability (phosphatase enzymes and organic anion release). We hypothesised that (1) eCO_2 will increase plant growth and P uptake, which will deplete available soil P; and (2) under eCO_2 and P deficiency, plants species will be able to access more recalcitrant P pools due to increased rhizosphere acidification and release of organic anions and phosphatase enzymes.

5.2 Material and methods

5.2.1 Soil characteristics and sampling

Soil samples were taken in May 2019 from the 0-20 cm layer of soil sown with Italian ryegrass for the last two years. The soil has not received any P fertilisation over the previous nine years and was previously planted with several crops from 2011 to 2017, including wheat, Italian ryegrass, kale, and green globe turnips, respectively. The soil was a Wakanui silt loam (NZ classification: Mottled Immature Pallic; USDA classification: Udic Ustochrept) from the Field Research Centre at Lincoln University. The soil samples were air-dried and sieved ≤ 2 mm to remove any plant residues or stones. One composite sample was sent to Hill Laboratories (Christchurch, New Zealand) for the assessment of basic physicochemical properties of the soil. The soil had an Olsen P of 7 mg kg^{-1} and was considered P deficient, according to Blakemore et al. (1982). Table 5.1 summarises the other properties of the soil used in this experiment.

Table 5.1 The basic physiochemical properties of the Iversen soil used in this study.

Soil texture (%)			pH (H_2O)	Total C	Total N	Total P	Available sulphur	Exchangeable potassium	Exchangeable calcium
Sand	Silt	Clay		g C kg^{-1} soil	g N kg^{-1} soil	mg P kg^{-1} soil	mg S kg^{-1} soil	mg K kg^{-1} soil	mg Ca kg^{-1} soil
34.4	54.5	11.1	6.3	23	2.3	742	3	340	1580

5.2.2 Experimental design

The experiment was carried out in the New Zealand Biotron facility at Lincoln University between May and June 2019. More details about the Biotron facility are presented in Shi et al. (2011). For the purpose of this study, two different cabinets were used. The first one was maintained at ambient CO_2 (aCO_2) (390 ± 20 ppm), while the second was set at eCO_2 (700 ± 35 ppm). The CO_2 injection was controlled by a solenoid based on feedback from a CO_2 analyser WMA-4 (PP Systems, USA). The light was kept for 16 hours with a PAR of ≈ 500 µmol m^{-2} s^{-1} at the pot surface, while the relative humidity was kept at 65 %. The temperature was set at 20 °C during the photoperiod and 15 °C at night and monitored using Thermistor temperature probes (Servotech Instrumentation Ltd, New Zealand). In this study, PVC tubes were used as pots, as reported by Touhami et al. (2020a). The tubes had the following dimensions: 9 cm high and 5 cm wide and were packed with 120 g of air-dry soil. All the soil in the tube was considered as rhizosphere soil due to the high root density. The rationale behind using a small volume of soil was to simulate rhizosphere conditions and encourage maximum P depletion; however, this could reduce plant growth. Apart from P, 225 mg kg^{-1} of potassium as K_2SO_4 and 35 mg kg^{-1} of magnesium as $MgCl_2.6H_2O$ were mixed dry thoroughly with the soil and put in the tubes. Three plant species were used in this study, namely wheat (*Triticum aestivum* L., cv. Graham), perennial ryegrass (*Lolium perenne* L., cv. Samson), and blue lupin (*Lupinus angustifolius* L., cv. Fest), while unplanted tubes were used as bulk soil controls (Figure 5.1). Before sowing, 6 seeds of wheat and blue lupin and 12 of ryegrass were soaked in 2 % sodium hypochlorite solution for 20 min with continual stirring, washed three times with deionised water, and pre-germinated in the dark between two wet filer papers. After emergence, seedlings were thinned to 4 plants per tube for wheat and blue lupin and 8 for ryegrass. Nitrogen was applied to limit N_2 fixation by blue lupin as well as enhance soil nitrogen to overcome any possible nitrogen limitation for plant growth. A nitrogen solution was added to each tube three times at a one-week interval starting from the second week after planting. The total nitrogen supply was 165 mg kg^{-1} in the form of urea. A micronutrient solution containing 3 mg kg^{-1} Mn ($MnCl_2,4H_2O$), 2 mg kg^{-1} Zn ($ZnCl_2$), 2 mg kg^{-1} Cu ($CuCl_2,2H_2O$), 3 mg kg^{-1} B (H_3BO_3), and 0.2 mg kg^{-1} Mo ($Na_2MoO_4,2H_2O$) was added to each tube at the

end of the second week after planting. Irrigation was monitored by weighing, and the tubes were irrigated every other day. Soil moisture was kept at 75 % of field capacity throughout the experiment, and the irrigation was stopped two days before sampling to allow for optimal soil sampling. The tubes were placed in a completely randomised design with four replicates and randomly reallocated weekly within each growth chamber to allow for even light distribution. After 45 days, plant materials and rhizosphere soil were sampled for chemical, biological, and biochemical measurements.

Figure 5.1 Design of the experiment inside one of the CO_2 cabinets in the Biotron Facility at Lincoln University.

5.2.3 Soil and plant analyses

At the end of six weeks, plants were removed from the tubes, and the roots were gently shaken and brushed off to remove the rhizosphere soil (all the soil in the tube was considered as rhizosphere soil as described above). The rhizosphere soil samples were sieved ≤ 2 mm and divided into two parts. The first one was stored at 4 °C for microbial and biochemical parameters, whereas the second portion was air-dried to serve for chemical measurements. For organic anion collection, the roots with the remaining adhering soil (rhizosphere soil) were gently immersed in a trap solution (0.2 mM $CaCl_2$) and lightly shaken for two minutes (volumes varied from 50 to 100 mL according to root biomass) (Pearse et al. 2007; Wang et al. 2016a, 2017b; Touhami et al. 2020a). After this step, shoots and roots were rinsed with deionised water and oven dried at 65 °C until a constant weight was reached for biomass measurements. Plant materials were then ground in a stainless-steel grinder to pass through a 1 mm sieve for P analyses. The concentration of P in shoots and roots was determined using inductively coupled plasma optical emission spectroscopy (ICP-OES) after digesting the plant samples with a mixture of HNO_3 and H_2O_2 (Wu et al. 1997). Phosphorus concentration was multiplied

by dry biomass to calculate P uptake in shoots and roots. Phosphorus use efficiency (PUE) was calculated by dividing plant biomass by total P uptake per tube.

Two grams of air-dried rhizosphere soil was shaken with deionised water (1:2.5 soil to solution ratio) for 1 hour in an end to end shaker and allowed to stand overnight before measuring the rhizosphere pH. Olsen P was assessed according to the procedure of Watanabe and Olsen (1965). The fumigation-extraction method developed by Brookes et al. (1982) was followed for the determination of microbial biomass P. A correction factor Kp of 0.4 was used to calculate microbial biomass P (Brookes et al. 1982). Enzymes assays for acid and alkaline phosphatase activity were performed according to the procedure of Tabatabai (1994). The activity of acid and alkaline phosphatase enzymes was considered as potential (Margenot et al. 2018) and expressed as µmol *p*-nitrophenyl hydrolysed g^{-1} fresh soil h^{-1}.

Phosphorus fractionation was performed according to the original Hedley procedure (Hedley et al. 1982) with the modifications from Condron et al. (1996) and the recommendations proposed by (Condron and Newman 2011) as described elsewhere (Boitt et al. 2018c; Touhami et al. 2020b). In brief, 0.5 grams of air-dried soil was sequentially extracted by 10 mL of 1 M NH_4Cl, followed by 0.5 M $NaHCO_3$ (pH 8.5), 0.1 M NaOH, 1 M HCl, and another 0.1 M NaOH in order to extract readily available P, labile P, moderately labile P, calcium-bound P, and stable P (P in the microaggregates and protected by calcium), respectively. The soil extracts were shaken for 16 hours each time, centrifuged for 15 min at 3500 rpm, filtered and stored in the fridge at 4 °C before P analyses. Olsen and Sommers (1982) method was followed to determine residual P (Pi and Po stabilised by oxyhydroxides of iron and aluminium and organic carbon (Velásquez et al. 2016)) after oven-drying the last soil residue at 50 °C. The determination of inorganic P (Pi) concentration in NH_4Cl-Pi, HCl-Pi, and residual-Pi fractions was performed following the molybdenum blue method (Murphy and Riley 1962). In comparison, the determination of Pi concentration in $NaHCO_3$-Pi, NaOH1-Pi, and NaOH2-Pi fractions was assessed according to Dick and Tabatabai (1977) and the recommendations of He and Honeycutt (2005). Total P concentration (Pt) in the alkali extracts ($NaHCO_3$-P, NaOH1-P, and NaOH2-P) was measured by ICP-OES according to do Nascimento et al. (2015). The difference between Pt and Pi in each fraction represented organic P (Po). The sum of all inorganic and organic P fractions was considered as total P.

The assessment of organic anions by high performance liquid chromatography (HPLC) was performed as described in (Touhami et al. 2020a). Briefly, 5 mL of the rhizosphere soil extract (described above) was filtered through a 0.45 µm Phenex RC syringe (Phenomenex, USA) and one drop of Micropur diluted solution (0.1 mg L^{-1}, Katadyn products, Switzerland) was added to each sample to stop the microbial degradation of organic anions (Cheng et al. 2014; Wang et al. 2016a; Oburger and Jones

2018). The samples were acidified with a drop of orthophosphoric acid and then stored at -20 °C until analysis by HPLC (Pearse et al. 2007; Pang et al. 2015). The organic anion determination was carried out using an HPLC system (Shimadzu Corporation, Kyoto, Japan) as described in Parr et al. (2016). The system had a controller CMM-20A equipped with a pump LC-20 AD, a degas system DGU-20A5, an autosampler SIL-10AF, and a UV detector SPD-20A. The column used to separate and analyse organic anions was a Prevail^{TM} organic acid column (250 x 4.6 mm, 5 µm particle size; Grace Davison Discovery Sciences) coupled with a Guard column (7.5 x 4.6 mm, 5 µm particle size; Grace Davison Discovery Sciences). The mobile phase was 25 m*M* KH_2PO_4 (pH 2.35, adjusted by H_3PO_4), freshly prepared and filtered through a 0.45 µm cellulose acetate membrane. The flow rate was 0.6 mL min^{-1}, while the column temperature was 50 °C. The sample injection volume was 30 µL, and the detector wavelength was 210 nm. Two standard stock solutions were prepared to calibrate the system. The first one contained a mixture of 10 organic acids and was prepared by dissolving all solid organic acids of tartaric acid (Sigma – Aldrich, 99.5 %), formic acid (ANALAR, 98 %), pyruvic acid (Sigma – Aldrich, 98 %), malic acid (Merck, >99 %), malonic acid (Sigma- Aldrich, 99 %), lactic acid (Acros Organics, 85 %), acetic acid (BDH, 100 %), citric acid (Sigma-Aldrich, 99.5 %), shikimic acid (Sigma – Aldrich, 99 %), and succinic acid (Sigma – Aldrich, 99 %) in 0.2 mM CaCl2 matrix solution to have a concentration of 1000 ppm for each organic acid. The second one contained only fumaric acid (Sigma- Aldrich, 99 %) due the presence of traces of fumaric acid in the organic anion mixture and was prepared by dissolving solid fumaric acid in 0.2mM $CaCl_2$ matrix solution to have a concentration of 5 ppm. From these two stock solutions, 6 different working standard curve solutions (from 0.05 to 80 ppm (depending on organic acids)) were prepared and used for calibration. The identification of organic anions was obtained by comparing retention time of standards. Sample quantification was determined by the peak area of chromatograms using the external calibration standard curves. Data were processed in Lab solution software (Version 5.87 SP1). Organic anion concentrations were expressed by unit of root dry matter (Pearse et al. 2007; Wang et al. 2016a).

5.2.4 Statistical analysis

Data were analysed with SPSS version 25.0 (SPSS, Chicago, IL, USA). Plant parameters, rhizosphere properties, and soil P fractions were subjected to a two-way analysis of variance (ANOVA) to test the effects of plant species, CO_2 treatments, and their interactions. A *post-hoc* Tukey test was performed to separate means within plant species at 5 % probability in the presence of a significant effect. Independent samples *t*-test was used to assess the difference in means among CO_2 treatments for each plant species at 5 % probability.

5.3 Results

5.3.1 Plant biomass and plant P uptake

Elevated CO_2 did not affect plant biomass or plant P uptake (Table 5.2). Plant species had a significant impact on plant growth, with blue lupin exhibiting the highest plant biomass (1.98 g tube^{-1}) followed by wheat (1.37 g tube^{-1}) and ryegrass (0.64 g tube^{-1}) (Table 5.3). Moreover, across plant species, blue lupin exhibited the highest shoot biomass (1.38 g tube^{-1}), and wheat the highest root biomass (0.6 g tube^{-1}). Root and shoot P concentrations were higher under ryegrass, followed by wheat and blue lupin, respectively. Total P uptake decreased in the following order: blue lupin ≥ wheat > ryegrass, with blue lupin exhibiting the highest shoot P uptake (0.86 mg tube^{-1}) and wheat the highest root P uptake (0.7 mg tube^{-1}). It is important to mention that direct comparisons between biomass of plant growth are only indicative and not very relevant due to the different number of plants per pot, though comparison between wheat and blue lupin can still be of interest due to similar number of plants per pot. Regardless of CO_2 treatments, the root to shoot ratio was higher under wheat (0.73), while blue lupin had the greatest P use efficiency (1.38) (Table 5.3). We acknowledge that the restricted volume of the PVC tubes used in this experiment may have reduced plant growth (Poorter et al. 2012).

Table 5.2 Summary of the two-way analysis of variance (ANOVA) testing the effect of plant species and CO_2 levels and their interaction on plant parameters, rhizosphere properties, and soil P fractions.

		Plants	CO_2	Plants*CO_2
Plant parameters	Shoot Biomass	***	n.s.	n.s.
	Root Biomass	***	n.s.	n.s.
	Root to shoot ratio	***	n.s.	n.s.
	Shoot P concentration	***	n.s.	n.s.
	Root P concentration	***	n.s.	n.s.
	Total P uptake	***	n.s.	n.s.
	PUE	***	n.s.	n.s.
Rhizosphere properties	Olsen P	***	n.s.	**
	pH	***	n.s.	n.s.
	Microbial biomass P	***	n.s.	n.s.
	Total organic anions	***	n.s.	n.s.
	Acid phosphatase	***	n.s.	n.s.
	Alkaline phosphatase	***	n.s.	n.s.
Soil P fractions	NH_4Cl-Pi	***	n.s.	n.s.
	$NaHCO_3$-Pi	***	n.s.	n.s.
	$NaHCO_3$-Po	***	n.s.	n.s.
	NaOH1-Pi	***	n.s.	n.s.
	NaOH1-Po	***	n.s.	n.s.
	HCl-Pi	***	*	n.s.
	NaOH2-Pi	***	n.s.	n.s.
	NaOH2-Po	n.s.	n.s.	n.s.
	Residual-Pi	n.s.	n.s.	n.s.
	Total P	***	n.s.	n.s.

Pi, Po, PUE mean inorganic P, organic P and P use efficiency, respectively
n.s., *, **, and *** indicate not significant, significant at P <0.05, P <0.01, and P <0.001, respectively.

5.3.2 Phosphorus availability and rhizosphere properties

Elevated CO_2 showed no significant effect on the concentration of Olsen P, microbial biomass P or the concentration and composition of organic anions released in the rhizospheres of the three plant species investigated in this study (Table 5.2, Figure 5.4). Compared to the control, the concentration of Olsen P decreased and exhibited its lowest value under wheat (3.8 mg kg^{-1}) and the highest under blue lupin (5 mg kg^{-1}) (Figure 5.2a). Rhizosphere pH increased under eCO_2 for wheat while it was similar under blue lupin and ryegrass regardless of CO_2 treatment. In comparison with the control soil, rhizosphere pH became more acidic and dropped to an average of 5.3, 5.5, and 5.7 under wheat, blue lupin, and ryegrass, respectively (Figure 5.2b). Microbial biomass P was significantly higher under blue lupin compared to the control soil and other plant species (Figure 5.3a). Acid phosphatase activity increased by an average of 188, 118, and 108 % under blue lupin, wheat, and ryegrass, respectively, compared to the control soil, whereas alkaline phosphatase activity was almost 2-fold

higher under blue lupin compared to the control soil (Fig 5.3b, c). Six organic anions were identified in the rhizospheres of the plant species investigated in this study, including pyruvate, malate, malonate, citrate, shikimate, and fumarate (Figure 5.4). Citrate, malate, and malonate were the major organic anions found in the rhizospheres of the three plant species accounting for 95 % of total organic anions. Citrate contributed to 62 and 55.4 % of total organic anions released in the rhizospheres of ryegrass and wheat, respectively, while malate was the principal organic anion released in the rhizosphere of blue lupin (51 %). The concentration of total organic anions was higher under blue lupin (average: 23.9 µmol g^{-1} root dry matter), followed by ryegrass (17.5 µmol g^{-1} root dry matter) and wheat (11.7 µmol g^{-1} root dry matter) (Figure 5.4).

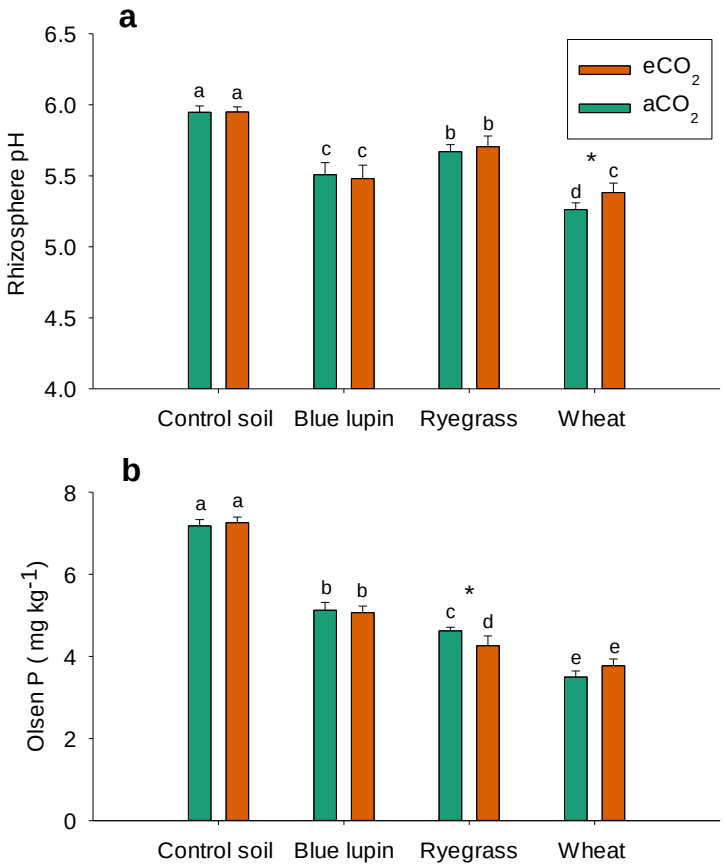


Figure 5.2 Rhizosphere pH (a) and Olsen P (b) in the control soil and the rhizosphere of blue lupin, ryegrass, and wheat grown for six weeks under aCO_2 (390 ppm) or eCO_2 (700 ppm). Values are the mean of 4 replicates ± standard errors. Bars with the same letters are not significantly different (P <0.05). * means significant difference between CO_2 treatments for a given plant.

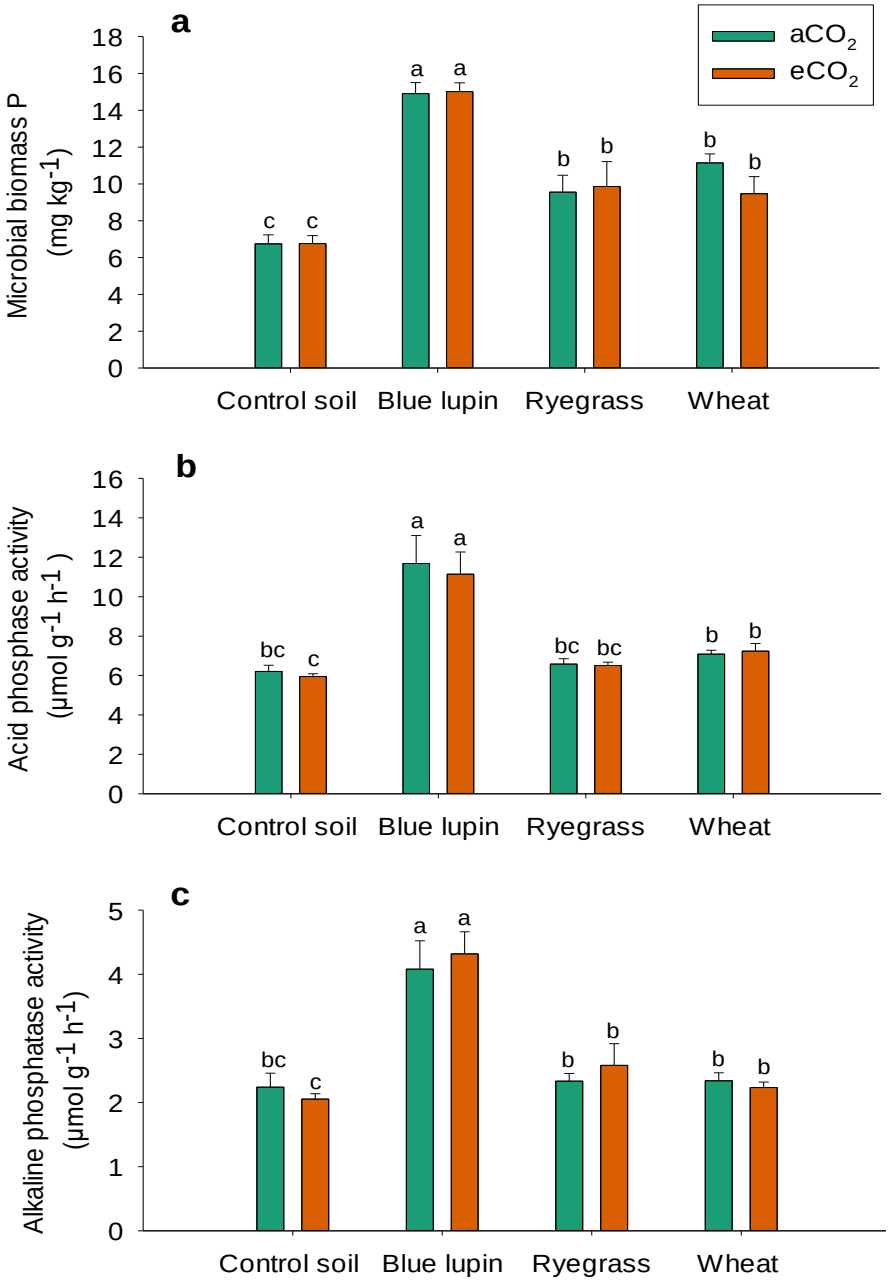


Figure 5.3 Microbial biomass P (a), acid (b), and alkaline phosphatase activity (c) in the control soil and the rhizosphere of blue lupin, ryegrass, and wheat grown for six weeks under aCO_2 (390 ppm) or eCO_2 (700 ppm). Values are the mean of 4 replicates ± standard errors. Bars with the same letters are not significantly different (P <0.05).

Table 5.3 Shoot biomass (SB) (g tube^{-1}), root biomass (RB) (g tube^{-1}), root to shoot ratio (R/S), shoot P concentration (SP) (mg g^{-1}), root P concentration (RP) (mg g^{-1}), total P uptake (TPU) (mg tube^{-1}), and P use efficiency (PUE) (g mg^{-1}) for blue lupin, ryegrass, and wheat under aCO_2 (390 ppm) and eCO_2 (700 ppm). Values are the mean of 4 replicates ± standard errors. Different lowercase letters indicate significant difference (P <0.05) between plant species for a given CO_2 treatment, and different uppercase letters indicate significant difference (P <0.05) between CO_2 treatments for a given plant.

	Blue lupin		Ryegrass		Wheat	
	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2
SB (g tube^{-1})	1.33 ± 0.11 a	1.40 ± 0.12 a	0.40 ± 0.02 cB	0.44 ± 0.02 cA	0.79 ± 0.04 b	0.83 ± 0.03 b
RB (g tube^{-1})	0.56 ± 0.03 a	0.57 ± 0.03 a	0.23 ± 0.03 b	0.25 ± 0.01 b	0.60 ± 0.05 a	0.59 ± 0.08 a
R/S	0.42 ± 0.05 c	0.40 ± 0.03 c	0.59 ± 0.07 b	0.56 ± 0.04 b	0.76 ± 0.03 c	0.70 ± 0.08 c
SP (mg g^{-1})	0.66 ± 0.06 c	0.59 ± 0.03 c	1.15 ± 0.10 a	1.03 ± 0.06 a	0.84 ± 0.03 b	0.80 ± 0.02 b
RP (mg g^{-1})	0.94 ± 0.06 c	0.98 ± 0.04 c	1.35 ± 0.04 a	1.36 ± 0.04 a	1.11 ± 0.08 b	1.24 ± 0.08 b
TPU (mg tube^{-1})	1.39 ± 0.11 a	1.42 ± 0.11 a	0.75 ± 0.01 b	0.79 ± 0.04 b	1.30 ± 0.05 a	1.41 ± 0.12 a
PUE (g mg^{-1})	1.35 ± 0.06 a	1.42 ± 0.07 a	0.82 ± 0.05 a	0.87 ± 0.04 a	1.07 ± 0.05 b	1.00 ± 0.04 b

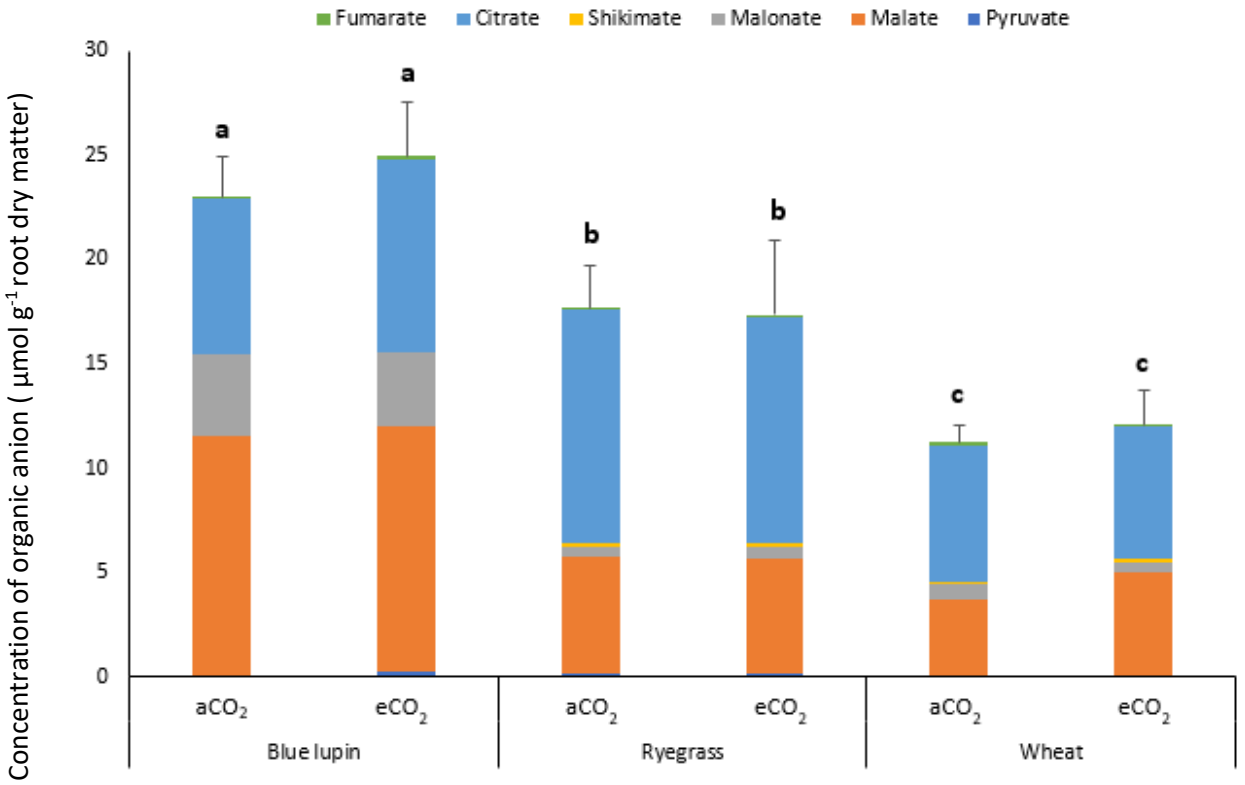


Figure 5.4 Concentration and composition of organic anions in the rhizosphere of blue lupin, ryegrass, and wheat grown for six weeks under aCO_2 (390 ppm) or eCO_2 (700 ppm). Values are the mean of 4 replicates ± standard errors of the mean for total organic anions. Bars with the same letters are not significantly different (P <0.05).

5.3.3 Soil P fractions

Both inorganic and organic P fractions were largely unaffected by eCO_2 concentrations but did show differences across plant species (Table 5.2 and Table 5.4). Phosphorus fractionation data revealed that residual P was the main inorganic P fraction (35.3 %) in our soil, followed by acid extractable inorganic P (HCl-Pi) (26.5 %) and moderately labile inorganic P (NaOH1-Pi) (26.4 %). Moreover, organic P represented 43 % of the total P and was mainly composed of moderately labile organic P (NaOH1-Po) (79 %). In comparison with the control soil, the three plant species depleted all inorganic P fractions regardless of their chemical availability, with a higher depletion observed in moderately labile inorganic P (NaOH1-Pi) and acid extractable inorganic P (HCl-Pi) fractions. Regardless of CO_2 treatments, blue lupin and wheat depleted almost similarly moderately labile inorganic P (NaOH1-Pi) (≈ 19 mg kg^{-1}), whereas wheat depleted the most acid extractable inorganic P (HCl-Pi) (24.2 mg kg^{-1}). Residual P was similar across plant species and CO_2 treatments in this experiment.

Amongst organic P fractions, a significant accumulation (11 mg kg^{-1}) of labile organic P ($NaHCO_3$-Po) was observed in the rhizosphere of blue lupin in comparison with the control soil. On the other hand, blue lupin significantly depleted moderately labile organic P (NaOH1-Po) by an average of 20 mg kg^{-1}, whereas stable organic P (NaOH2-Po) showed no significant changes across plant species and CO_2 treatments. Total P was similar across CO_2 treatments but significantly different under plant species with higher depletion noticed under blue lupin (50 mg kg^{-1}), followed by wheat (47 mg kg^{-1}) and ryegrass (31 mg kg^{-1}).

Table 5.4 Distribution of soil inorganic and organic P fractions (mg kg^{-1}) in the control soil and the rhizosphere of blue lupin, ryegrass, and wheat under aCO_2 (390 ppm) and eCO_2 (700 ppm). Values are the mean of 4 replicates ± standard errors. Within rows, different lowercase letters indicate significant difference (*P* <0.05) between plant species and the control soil for a given P fraction, and within columns, different uppercase letters indicate significant difference (*P* <0.05) between CO_2 treatments for a given P fraction.

	Control	Blue lupin	Ryegrass	Wheat
	aCO_2			
NH_4Cl-Pi	0.3 ± 0.0a	0.3 ± 0.0a	0.2 ± 0.0b	0.2 ± 0.1b
$NaHCO_3$-Pi	16.3 ± 0.7a	12.7 ± 0.5b	12.9 ± 0.5b	12.7 ± 0.6b
$NaHCO_3$-Po	41.9 ± 1.2c	52.4 ± 2.6a	43.8 ± 0.4bc	44.5 ± 0.9b
NaOH1-Pi	114.6 ± 2.5a	96.5 ± 2.7c	99.6 ± 1.6b	91.7 ± 1.9d
NaOH1-Po	254.9 ± 7.8a	236.3 ± 1.9b	250.6 ± 5.2a	248.5 ± 5.8a
HCl-Pi	118.4 ± 4.8aA	96.0 ± 4.9c	109.1 ± 4.2b	89.2 ± 7.9c
NaOH2-Pi	29.2 ± 0.3a	27.9 ± 0.7b	26.5 ± 0.3c	28.6 ± 0.9ab
NaOH2-Po	24.9 ± 1.6b	26.0 ± 1.3ab	26.5 ± 1.2b	25.2 ± 1.6a
Residual Pi	155.8 ± 8.5	154.7 ± 13.6	153.8 ± 9.7	158.7 ± 9.0
Total P	756.4 ± 3.2a	703.0 ± 12.4b	723.2 ± 11.4b	705.5 ± 13.2b
	eCO_2			
NH_4Cl-Pi	0.3 ± 0.0a	0.3 ± 0.0a	0.2 ± 0.0b	0.2 ± 0.0b
$NaHCO_3$-Pi	16.6 ± 0.3a	13.2 ± 0.4b	12.9 ± 0.1b	13.2 ± 0.1b
$NaHCO_3$-Po	42.1 ± 1.6c	53.3 ± 1.5a	43.9 ± 0.6bc	44.5 ± 0.8b
NaOH1-Pi	113.3 ± 2.4a	97.1 ± 1.6c	100.4 ± 2.5b	91.0 ± 1.4d
NaOH1-Po	256.4 ± 5.1a	234.6 ± 1.9b	252.7 ± 5.8a	251.3 ± 2.0a
HCL-Pi	110.2 ± 3.0aB	97.1 ± 2.3c	101.2 ± 4.7b	91.0 ± 3.2d
NaOH2-Pi	29.1 ± 0.4a	27.0 ± 0.9b	26.6 ± 0.4c	28.0 ± 0.7ab
NaOH2-Po	26.5 ± 1.0b	26.7 ± 1.9ab	25.1 ± 1.0b	26.6 ± 1.6a
Residual Pi	157.3 ± 8.3	157.3 ± 13.2	158.6 ± 4.4	157.8 ± 7.4
Total P	752.0 ± 8.2a	704.0 ± 8.16c	721.6 ± 6.4b	705.2 ± 1.6c

Pi and Po mean inorganic and organic P, respectively.

5.4 Discussion

5.4.1 Plant biomass and plant P uptake

Elevated CO_2 is suggested to increase plant biomass due to its impact on net photosynthesis (Ainsworth and Rogers 2007; Pandey et al. 2015b). In contrast to our hypothesis, there was no CO_2 effect on plant biomass for each plant species investigated in this study. Our findings compare well with previous studies when crop plants, including legumes and grasses, were cultivated in low P environment combined with eCO_2 for short-time periods and in different pot sizes (Gentile et al. 2012; Jin et al. 2013; Pandey et al. 2015a; Watts-Williams et al. 2019). The present data is also in accordance with a recent meta-analysis carried out by Jiang et al. (2020) in woody and non-woody species. Phosphorus deficiency and eCO_2 have an opposite effect on plant biomass, and their interactive effects have been described to inhibit plant growth through the negative feedback on photosynthesis; an energy-consuming process highly dependent on P supply; and associated mechanisms (Ainsworth and Long 2005; Singh et al. 2013; Singh and Reddy 2014; Körner 2015; Terrer et al. 2019). Therefore, Pandey et al. (2015b) and Jiang et al. (2020) stressed that plants' responses to eCO_2 depend on soil P status and that P fertilisation could alleviate biochemical limitations on photosynthesis, thereby increasing plant growth responses to eCO_2 and sustaining food security. Provided that the current study was carried out for a short-time period, longer studies (over the whole plant cycle or the critical growth stages) are needed to gain more understanding of the effects of eCO_2 and low P availability on plant growth.

5.4.2 Phosphorus availability

The impact of eCO_2 on soil P availability has shown discrepancies among studies. For instance, Touhami et al. (2020b) found a decrease in P availability in a grazed pasture system subjected to long-term eCO_2 (500 ppm) due to plant P uptake and accumulation of organic P. Other studies highlighted a mobilisation of recalcitrant P pools under forest ecosystems, which contributed to enhancing soil P availability (Khan et al. 2008; Huang et al. 2014; Ochoa-Hueso et al. 2017). Studies looking at the impact of eCO_2 on soil P availability have been mostly carried out under woody species (Lagomarsino et al. 2008; Khan et al. 2008; Bhattacharyya et al. 2014; Huang et al. 2014), and yet few experiments have assessed P availability in the rhizosphere of crop plants (Manoj-Kumar et al. 2011; Bhattacharyya et al. 2014). The present data showed that Olsen P was unchanged under eCO_2 and that plant species were the main factor impacting soil P availability in the rhizosphere. Soil P availability is tightly related to physiological processes taking place in the soil-plant interface, which involves the release of carboxylates and phosphatase enzymes along with proton efflux (Jones 1998; Hinsinger 2001; Lambers 2006; Nannipieri et al. 2011). No changes in P availability across CO_2 treatments suggest that physiological processes involved in P acquisition were unimpacted by the

increased CO_2 levels in our study, maybe because eCO_2 has not influenced plant biomass and biomass partitioning in this low P soil. Nevertheless, this effect of eCO_2 on soil P availability needs to be further investigated under other species or cultivars exhibiting better physiological root adaptations for P acquisition. Moreover, determining the effect of eCO_2 on soil P availability under increasing P applications and different plant species is warranted.

5.4.3 Rhizosphere properties

Soil pH is critical to inorganic P availability in soils (Hinsinger 2001). It has been shown that eCO_2 can decrease rhizosphere pH via several mechanisms, including the release of organic anions, soil and microbial respiration, and efflux of protons H^+ under N_2 fixing plants (legumes) (Hinsinger et al. 2003; Tang and Rengel 2003; Bhattacharyya et al. 2014). We found no change in rhizosphere pH under eCO_2 for blue lupin and ryegrass, while rhizosphere pH increased under wheat. In a study investigating the response of a grazed pasture to eCO_2, Touhami et al. (2020b) found that the pH of sandy soil was not affected by the long-term CO_2 enrichment. In our experiment, wheat decreased rhizosphere pH the most, followed by blue lupin and ryegrass. This pH drop could contribute to the mobilisation of some sparingly available P forms in the rhizosphere (Hinsinger 2001).

Phosphatase activity has been described to be influenced by CO_2 concentrations with contrasting results among studies. For instance, acid phosphatase activity increased in *Arabidopsis thaliana* cultivated in a P deficient hydroponic solution under eCO_2 (Niu et al. 2013a). Whereas, Ochoa-Hueso et al. (2017) found no significant difference in root acid phosphatase activity in a mature Eucalyptus woodland subjected to ambient and eCO_2 for 17 months. Similarly, Jin et al. (2013) described no effect of eCO_2 on acid and alkaline phosphatase enzymes under wheat and chickpea. Further investigations are needed to understand why these contrasting results occur.

The release of organic anions is a physiological response of plants under P stress (Gerke and Meyer 1995; Jones 1998; Raghothama 1999; Jones and Oburger 2011). Organic anions enhance soil P availability for plants via different mechanisms such as soil acidification, chelation, and competition with iron (Fe) and aluminium (Al) oxides (Jones 1998; Hocking 2001; Pearse et al. 2006; Gerke 2015). Among plant species, legumes respond to P deficiency by higher exudation of organic anions (Pearse et al. 2006; Gerke 2015; Pang et al. 2018b). In this study, blue lupin exhibited an average of 1.4 and 2.1-fold higher concentrations of total organic anions compared to ryegrass and wheat, respectively. Elevated CO_2 has been found to increase organic anion release under several crop species in solution cultures (Norby et al. 1987; Watt and Evans 1999; Campbell and Sage 2002; Johansson et al. 2009). However, studies looking at the composition of organic anions in response to eCO_2 in real soils conditions are very scarce. We identified six organic anions in the rhizospheres of the three plant species, with citrate, malate, and malonate representing 51.1, 37.6, and 9.2 % of total organic anions,

respectively. The present data also showed that the composition and concentrations of organic anions were not influenced by CO_2 treatments. Similarly, Watt and Evans (1999) identified five organic anions in the rhizosphere of white lupin and noted that eCO_2 did not influence the rate and composition of organic anions released in comparison with the aCO_2 treatment. Belowground carbon, including carbon derived from roots, was not affected by eCO_2 when ryegrass was grown in a low P soil (Edwards et al. 2005). Therefore, Watt and Evans (1999) concluded that if root biomass increases under eCO_2, it is most likely that root exudation will increase, which was not the case in our study.

5.4.4 Soil P fractions

The response of soil P fractions to eCO_2 has been found to depend on several parameters, including soil P fertility, plant species, soil types, time scale as well as CO_2 enrichment designs (Khan et al. 2008; Jin et al. 2012, 2013, 2017; Huang et al. 2014; Touhami et al. 2020b). Our findings showed that rhizosphere P fractions were significantly impacted by plant species, but not CO_2 treatments. Our findings corroborate the results of Jin et al. (2013), who investigated the transformation of soil P fractions in the rhizospheres of wheat and chickpea cultivated in low P soils and under eCO_2.

Labile inorganic P fractions (NH_4Cl and $NaHCO_3$-Pi), defined as highly available P fractions, were depleted by the three plants species compared to the control soil, most likely due to plant P uptake (Tiessen et al. 1983; Chen et al. 2002; Vu et al. 2008; Rose et al. 2010). Moderately labile inorganic P was the third largest inorganic P pool within our soil and is described as the P adsorbed to the active mineral surfaces of Fe and Al (Hedley et al. 1982; McDowell and Condron 2012). This fraction has been found to be mobilised by plants and can contribute to P uptake under meadow and *Pinus radiata* (Chen et al. 2002; Liu et al. 2019). The addition of organic anions to the soil has been found to mobilise sparingly available inorganic P forms such as NaOH-Pi (Wang et al. 2016b, 2017a; Yang et al. 2019). Although the release of organic anions under blue lupin was significantly higher than wheat, the depletion of moderately labile inorganic P (NaOH-Pi) was similar under the two plant species. Previous studies have highlighted inconsistencies between organic anion exudation and the depletion of sparingly available P pools under plant species (Radersma and Grierson 2004; Wang et al. 2016a; Touhami et al. 2020a). Thus, we suggest that the depletion of NaOH1-Pi in this study was not only related to organic anion release but could be ascribed to other parameters such as rhizosphere pH and root traits or their combination (Pearse et al. 2007; Wang et al. 2016a). Indeed, wheat showed the highest root biomass in our study in comparison with other plant species. Cereals are known to produce fibrous roots, thus having a greater soil exploration ability (Veneklaas et al. 2003; Jin et al. 2013; Sandaña and Pinochet 2016). Moreover, cereals adaptations to low P environments include an increase in root hairs along with the production of longer roots and the

association with mycorrhizal fungi to mobilise more soil P (Singh Gahoonia and Nielsen 2004; Pandey et al. 2015b). In fact, Wang et al. (2010) and Jin et al. (2013) pointed out that longer and finer root system under wheat was the main factor explaining its aptitude in accessing the NaOH-Pi fraction, probably via the greater volume of soil explored by roots and exposed to rhizosphere processes (organic anions and soil acidification).

HCl-Pi or acid extractable inorganic P contributed to more than 26 % of total inorganic, thus being the second largest inorganic P fraction after residual P. This fraction is abundant in calcareous soils and is described as a recalcitrant P fraction representing the P attached to calcium cations (Vu et al. 2008; Richardson et al. 2009; Liao et al. 2020; Tian et al. 2020). To be available to plants, HCl-Pi needs to be dissolved by the action of proton H^+ released in the rhizosphere of plants (Hinsinger 2001; Liao et al. 2020). Our results illustrated higher soil acidification in the rhizospheres of wheat, followed by blue lupin and ryegrass. This pH drop corresponded to a similar trend in HCl-Pi depletion, where wheat showed the greatest depletion of this fraction followed by blue lupin and ryegrass. In a recent study on Karst soils (limestone soils), Tian et al. (2020) pointed out that rhizosphere acidification was probably responsible for the mobilisation of HCl-Pi by soybean. Furthermore, the depletion of HCl-Pi has been observed under several crops (wheat, chickpea, and maize intercropped with *Faba bean*) when grown in calcareous soils (Vu et al. 2008; Liao et al. 2020). In contrast to other P pools, stable inorganic P (NaOH2-Pi) (Pi present in the microaggregates and protected by Ca) and residual P (P stabilised by oxyhydroxides of Fe and Al) were unaffected by plant species and CO_2 treatments, most likely because of their high recalcitrance and the contribution of other P fractions to plant P uptake in this study (Perrott and Mansell 1989; McDowell and Condron 2000; Condron and Newman 2011).

Organic P can represent a significant P source for plants, especially in low P conditions (Nash et al. 2014; George et al. 2018). Organic P in our soil represented ≈ 42 % of total P and was chiefly comprised of NaOH1-Po. Phosphatase enzymes play a critical role in the cleavage of organic P-bounds to liberate orthophosphates for plant uptake (Tabatabai 1994; Nannipieri et al. 2002; Condron and Tiessen 2005). Acid and alkaline phosphatase activity in the rhizosphere of blue lupin was almost 2-fold higher than the control soil. Concomitantly, the depletion of moderately labile organic P (NaOH1-Po) under this plant was greater compared to the other plant species. The release of alkaline phosphatase enzymes has been linked to the activity of soil microbes, mainly bacteria, whereas acid phosphatase enzymes were mostly ascribed to plant roots activity (Wasaki et al. 1997, 2018; Sakurai et al. 2008; Nannipieri et al. 2011; Spohn and Kuzyakov 2013a; Wei et al. 2019a). Our data showed that increased microbial biomass P together with higher phosphatase activity matched a strong depletion of moderately labile organic P (NaOH1-Po) in the rhizosphere of blue lupin. Under P deficient conditions, alkaline phosphatase activity derived from soil microbes has been related to the mobilisation of soil organic P in the rhizospheres of trees (Chen et al. 2002), legumes (Wasaki et

al. 2018), and grasses (Wei et al. 2019a). Acid phosphatase activity was also higher under blue lupin, which implies its potential contribution to organic P mineralisation under this plant. Wasaki et al. (1997) found that lupin roots released acid phosphatase activity when deprived of P. In a rhizobox experiment, acid phosphatase activity was correlated with the depletion of $NaHCO_3$-Po in the rhizosphere of two rice genotypes (Li et al. 2008b). Nevertheless, the relative contribution of acid and alkaline phosphatase activity to organic P mobilisation under blue lupin in this study needs further research.

In contrast to moderately labile organic P (NaOH1-Po), the rhizosphere of blue lupin showed an accumulation of labile organic P ($NaHCO_3$-Po). The rhizosphere of blue lupin also exhibited higher microbial biomass P and organic anion release. Therefore, the accumulation of labile organic P ($NaHCO_3$-Po) in the blue lupin rhizosphere could be associated with microbial P immobilisation triggered by higher rhizodeposition (Jakobsen et al. 2005; Allard et al. 2006; Jin et al. 2012, 2013; Heuck et al. 2015). Soil microbes are carbon deficient (Heuck et al. 2015; Spohn and Widdig 2017; Wei et al. 2019b; Pastore et al. 2020), while the rhizosphere represents a rich environment with carbon sources able to promote microbial growth and P sequestration (Hinsinger et al. 2009; Jones et al. 2009; Achat et al. 2010; Liang et al. 2019; Spohn 2020). Microbial cells are composed of organic P molecules such as phospholipids, DNA, and acid nucleic that can be easily extracted with $NaHCO_3$ (Condron and Tiessen 2005; Condron et al. 2005; Turner et al. 2005; Spohn 2020). Our results compared well with the findings of Chen et al. (2002), who noted that the accumulation of moderately labile organic P ($NaHCO_3$-Po) under ryegrass and *Pinus radiata* was related to increased microbial biomass.

5.5 Conclusions

Crop production is often limited by soil P availability, while eCO_2 can increase plant biomass, thus accelerating soil P cycling. Here we showed that under P limited conditions, plant biomass and rhizosphere properties did not respond to eCO_2 (700 ppm), thus preventing plant species with divergent P acquisition strategies from accessing more recalcitrant and stable P pools. The evidence from this study suggests that P deficiency restricted the response of crop species to eCO_2; therefore, current P-fertiliser recommendations to boost or maintain crop production in low P soils would remain unchanged under future eCO_2. Because the current study was carried out for a short-time period, long-term experiments are needed to affirm this conclusion. Moreover, experiments using bigger pot size are recommended in future studies to avoid the effect of pot size on plant growth and thus the plant response to eCO_2.

Chapter 6

Impacts of Long-Term Atmospheric Carbon Dioxide Enrichment on Soil Phosphorus Dynamics and Availability in Grazed Temperate Pasture

6.1 Introduction

Climate change will impact food production systems globally by a combination of severe weather events, global warming, and increased atmospheric carbon dioxide (CO_2) concentrations (IPCC 2014). While water and heat stresses can limit plant growth, increased CO_2 concentrations may enhance plant growth and thereby result in changes in soil phosphorus (P) concentrations and transformations (Jin et al. 2015; Hou et al. 2018; Bhattacharya 2019; Du et al. 2019). Higher biomass production projected under elevated CO_2 (eCO_2) is likely to increase the demand by plants for P (Khan et al. 2008; Jin et al. 2019). However, most studies looking at eCO_2 have been focused on plant P uptake while neglecting the effect on soil P availability and related changes in different P pools (Bhattacharyya et al. 2014; Deng et al. 2015; Jin et al. 2019; Lal et al. 2019).

The availability of P for plants is driven by the degree of its attachment to iron (Fe), aluminium (Al), and calcium (Ca) cations as well as carbon (C) moieties present in the soil (Condron et al. 2005; Yang and Post 2011). Elevated CO_2 concentrations have been suggested as increasing P availability in low fertility soils due to enhanced rhizodeposition, phosphatase activity, and the mobilisation of recalcitrant organic forms of soil P (Drissner et al. 2007; Bhattacharyya et al. 2014; Jin et al. 2015). Some authors have even stated that eCO_2 combined with nitrogen (N) addition can sustain crop productivity in P deficient conditions (Huang et al. 2014). In another study investigating three soil types (Chromosol, Vertosol, and Calcarosol) in a wheat - pulse rotation system, long-term CO_2 enrichment caused a depletion of $NaHCO_3$-Pi and NaOH-Pi with consequent increases in plant biomass and P uptake (Jin et al. 2017). However, others have found contrasting results. For instance, Jin et al. (2012) and Jin et al. (2014) found an accumulation of $NaHCO_3$-Po and NaOH-Po under legume and wheat plants cultivated in heavy clay soil (Vertosol) and linked that to microbial P immobilisation. Khan et al. (2008) noted an accumulation of HCl and NaOH-Pi fractions in soils under poplar trees grown in the EuroFACE experiment. The discrepancy between these studies could be due to differences in climatic conditions, soil types and P concentrations, plant species, and CO_2

enrichment design. Therefore, a comprehensive understanding of P availability and dynamics under eCO_2 requires further research and investigations.

Elevated CO_2 concentrations not only impact plant biomass but can also influence C fluxes, thus impacting root exudation, phosphatase activity, and microbial biodiversity in the soil (Drissner et al. 2007; Jin et al. 2015; Yu et al. 2016; Mellett et al. 2018). Organic anions are a readily available and rich source of C for soil microorganisms (Jones 1998; Jones and Oburger 2011). Enhanced rhizodeposition expected under eCO_2 is deemed to increase microbial activity, thus accelerating organic matter turnover and mineralising organic P (Drissner et al. 2007; Manoj-Kumar et al. 2011). Elevated CO_2 concentrations can lead to changes in phosphatase activity with conflicting results among studies. For instance, Delucia et al. (1997) reported a decrease in root phosphatase activity of *Ponderosa pine* under eCO_2. In contrast, other researchers described a stimulation effect of CO_2 on phosphatase activity (Moorhead and Linkins 1997; Drissner et al. 2007; Bhattacharyya et al. 2014). Still, interpretation of these results must be done cautiously, taking into consideration plant species, soil organic matter, and CO_2 enrichment design.

Grassland systems are the largest ecosystems in the world, covering about 25 % of the ice-free land area (Phelps and Kaplan 2017) and sustain the livelihood of more than 800 million people (Hynd 2019). Given that projected increases in CO_2 levels may affect P availability (Jin et al. 2015), the assessment of P transformations in response to eCO_2 in pastures is key to sustaining their productivity in the long-term. Different experimental approaches have been used to investigate the effects of eCO_2 on pasture systems ranging from enclosed controlled environment experiments to open-top chambers and Free Air CO_2 Enrichment (FACE) designs (Newton et al. 2006). The only FACE experiment on grazed pasture in the world was established in New Zealand in 1997. Although some detailed work has been done on N and C transformations on this site, only cursory information is available about the impact of eCO_2 on soil and plant P dynamics. The data indicated that soil Olsen P concentrations had decreased in the eCO_2 treatment (Newton et al. 2006; Gentile et al. 2012). Therefore, the objective of this study was to use the FACE experiment to evaluate the impact of long-term eCO_2 on different inorganic and organic P fractions and chemical and biological parameters that may be linked to changes in P forms. Early in the experiment, plant growth showed positive responses to eCO_2 (Ross et al. 2004; Newton et al. 2006); however, in recent years, the responses have been variable with little or no effect of eCO_2 on plant biomass in comparison to the ambient CO_2 (aCO_2) (Ross et al. 2013). We hypothesized that the lower availability of P under eCO_2 is due to an immobilisation of P in organic forms rather than a depletion of P as a result of higher plant uptake.

6.2 Materials and methods

6.2.1 Study site description and design

The New Zealand FACE experiment is located near Bulls, in the Rangitikei district, on the west coast of the North Island (40°14'S; 175°16'E) (Figure 6.1). The climate is temperate, with an average annual rainfall of 892 mm and temperature of 13 °C (min: 8.4 °C, max: 17.6 °C). The soil in the study site is a Pukepuke black sand (USDA: Mollic Psammaquent, World Reference Base for Soil Resources: Kastanozems). The study site was sown 60 years ago with a mixture of grasses and legumes. Carbon dioxide enrichment was initiated in 1997, whereupon the site was fertilised at intervals with the same fertiliser rates applied to both treatments. Over the whole experiment, the amounts applied were P (32.4 g m^{-2}), potassium (K) (105.8 g m^{-2}), and sulphur (S) (105.6 g m^{-2}), while N was supplied by legumes. The fertiliser rate was designed to maintain adequate P, K, and S levels based on annual soil tests and established guidelines for dry stock pasture production (Cornforth and Sinclair 1984). The New Zealand FACE experiment has six rings, three aCO_2, and three eCO_2 with a target enrichment of 475-500 ppm delivered using pulse width modulation during the photoperiod (Newton et al. 2001) (Figure 6.2). Each ring is 12 meters in diameter and grazed by a separate flock of sheep (3-5 animals) between 6 to 10 times a year, according to herbage production (Lieffering et al. 2019) (Figure 6.3). Before each harvest, herbage cuts were taken to estimate the herbage on offer to the animals. When grazing was finished, a post-grazing herbage cut was taken, allowing the amount eaten by the animals to be calculated. A more comprehensive description of the New Zealand FACE and its history can be found in the following references (Edwards et al. 2001; Newton et al. 2001, 2006), while initial soil properties and their evolution are reported in Ross et al. (2004) and Ross et al. (2013).

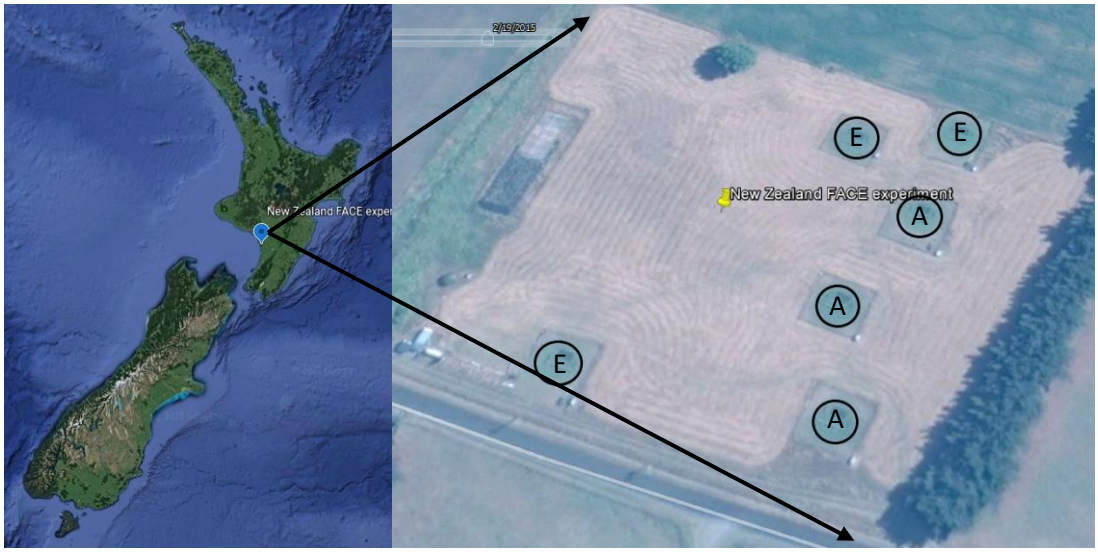


Figure 6.1 Satellite image of the New Zealand FACE experiment showing the localisation of different ambient (A) and elevated (E) CO_2 rings.

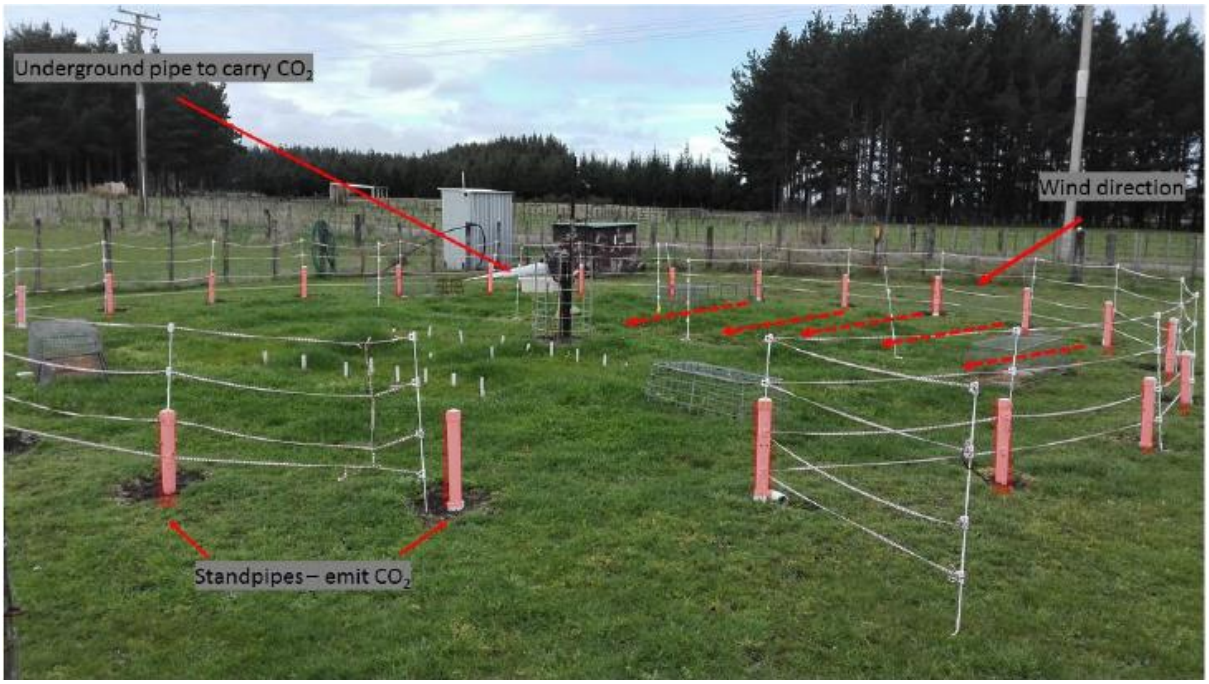


Figure 6.2 General layout and working of a FACE ring. Standpipes are highlighted in light red. The level of CO_2, windspeed and wind direction are monitored in the centre and a computer system controls which standpipes CO_2 is emitted; these are usually those in the upwind direction. Dashed lines show emitted CO_2 moving across the ring with the prevailing wind (Photo courtesy of Mark Lieffering, AgResearch, New Zealand).

Figure 6.3 Sheep grazing a ring in the New Zealand FACE experiment (Photo courtesy of Mark Lieffering, AgResearch, New Zealand).

6.2.2 Soil sampling

In October 2019, soil samples were taken from the top 10 cm of each ring using a 2.5 cm diameter soil corer. At the time of sampling, the mix of plant functional groups was approximately 80 % C3 grasses, 10 % forbs, 8 % legumes, and 2 % C4 grasses (Newton et al. 2014). Due to the small size of the rings and the heterogeneity of dung and urine deposits, two different samples were taken to give a more representative soil sample. The first sample consisted of 12 cores taken from regions with low production (low pasture standing biomass) and the second one from regions with high production (high pasture standing biomass), which represented old patches of urine and dung with high nutrients return, including P and N (Newton et al. 2006). Soil samples were treated separately, and the values reported are the mean of the two soil samples for each replicate ring. The soil samples were sieved to pass through a 2 mm sieve with the consequent removal of plant residues and roots and stored at 4 °C. The samples were then analysed for biological properties during the same week of sampling, while a portion was air-dried (at 30 ^{o}C) and stored for further chemical analyses.

6.2.3 Soil properties measurements and P fractions

Total N and C were measured by an Elementar Vario Max CN analyser. Field moist soil samples were analysed for mineral N (NH_4^+-N and NO_3^--N) (Blakemore et al. 1987), microbial biomass P (MBP) via the fumigation-extraction method (Brookes et al. 1982; Morel et al. 1996) and acid and alkaline phosphomonoesterase activity according to the procedure described by Tabatabai, (1994). Acid and alkaline phosphatase activity were considered as potential enzymes activity (Margenot et al. 2018). Air-dried soil was used to determine soil pH in water at a 1:2.5 soil to solution ratio, Olsen P (Olsen 1954) and P fractionation according to the Hedley et al. (1982) procedure with the modifications from Condron et al. (1996) and Condron and Newman (2011). These modifications were: 0.5 g of air-dried soil was extracted for 16 hours by 10 ml of the following reagents: 1 M NH_4Cl, 0.5 M $NaHCO_3$ (pH 8,5), 0.1 M NaOH, 1 M HCl, and a second 0.1 M NaOH (Condron and Newman 2011). These yielded the following fractions: NH_4Cl-P, $NaHCO_3$-P, NaOH1-P, HCl-P, and NaOH2-P. After each extraction, the soil was washed with 5 ml of NaCl, centrifuged for 5 min and the aliquot was discarded. The last fraction (residual-P) was determined by digesting the residue with concentrated H_2SO_4 and H_2O_2 in a block digestion (Olsen and Sommers, 1982). Alkaline extracts ($NaHCO_3$-Pi, NaOH1-Pi, NaOH2-Pi) were analysed for inorganic P (Pi) following the procedure described by Dick and Tabatabai (1977) and He and Honeycutt (2005) to avoid any mineralisation of organic P in the extracts. The molybdate-ascorbic acid method was used to determine Pi in the acid extracts (NH_4Cl-Pi, HCl-Pi, and Residual-Pi) (Murphy and Riley, 1962). Total P (Pt) was measured using ICP-OES in the alkaline extracts (do Nascimento et al. 2015). Organic P (Po) was calculated by subtracting Pi from Pt in each fraction. The sum of all nine fractions was used to calculate total soil P.

6.2.4 Statistical analysis

An independent samples t-test was performed to differentiate between the two treatments for different biological parameters and soil P fractions. Data were analysed using SPSS version 25.0 (SPSS, Chicago, IL, USA) and plotted with SigmaPlot software version 14.0.

6.3 Results

6.3.1 Soil properties

Our results showed that soil pH was unchanged regardless of CO_2 treatment (Figure 6.4a). In comparison to aCO_2, total C and N increased by 22 and 14 %, respectively, under eCO_2 (Fig.4b, c). However, the concentrations of NO_3^--N and NH_4^+-N decreased by 21 and 38 %, respectively, under eCO_2 compared to aCO_2 (Figure 6.4d, f). Alkaline phosphatase activity increased by 20 % under eCO_2 compared to aCO_2 (Figure 6.5b), whereas acid phosphatase activity was not significantly different between the two CO_2 treatments (Figure 6.5a). Long-term CO_2 enrichment significantly decreased Olsen P by 39 % (Figure 6.5c), while it increased microbial biomass P by 14 % compared to the aCO_2 treatment (Figure 6.5d). The total C/MBP ratio was similar irrespective of CO_2 treatment (Figure 6.6a), whilst the total C/total soil P ratio significantly increased in response to long-term eCO_2 (Figure 6.6b).

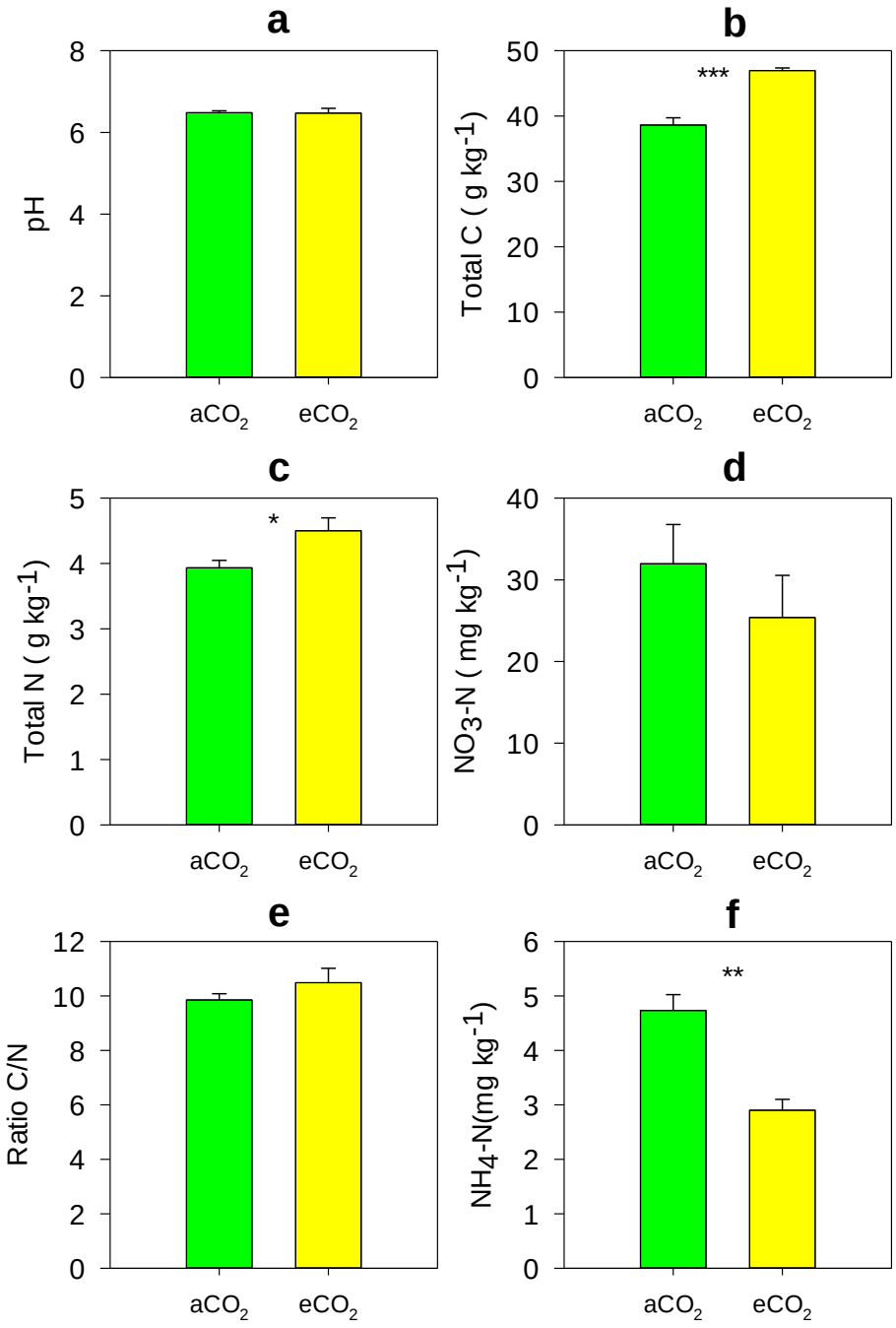


Figure 6.4 Effect of CO_2 on soil pH (a), total C (b), total N (c), NO_3^--N (d), C/N ratio (e), and NH_4^+-N (f) after 22 years of exposure to ambient CO_2 (aCO_2) and elevated CO_2 (eCO_2). Columns represent mean of three replicates and standard errors. *, **, and * indicate significance at the $P < 0.05$, 0.01 and 0.001 level, respectively.**

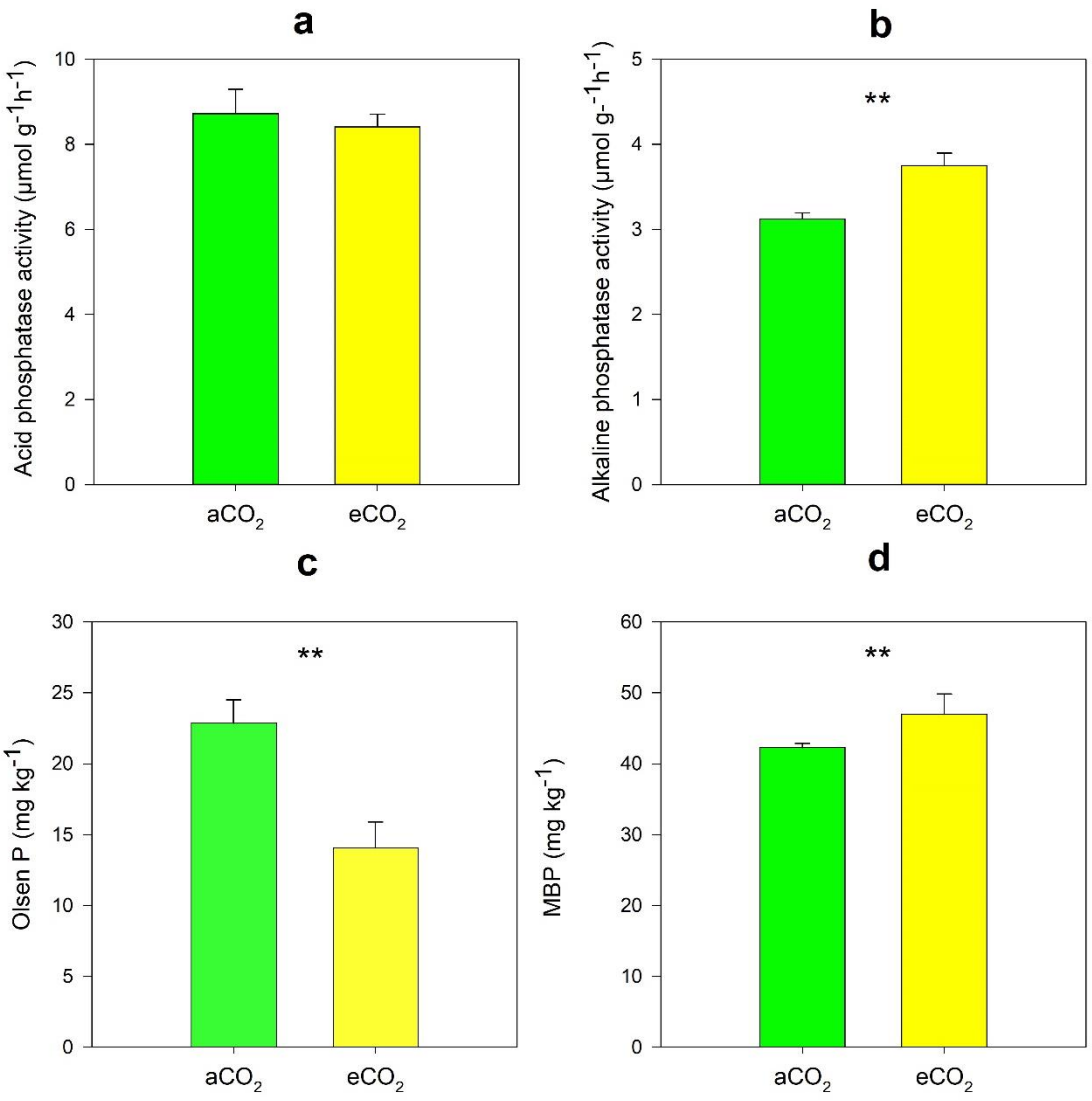


Figure 6.5 Effect of CO_2 on acid (a) and alkaline (b) phosphatase activity, Olsen P (c) and microbial biomass P (d) after 22 years of exposure to ambient CO_2 (aCO_2) and elevated CO_2 (eCO_2). Columns represent mean of three replicates and standard errors. * and ** indicate significance at the $P < 0.05$ and 0.01, respectively.

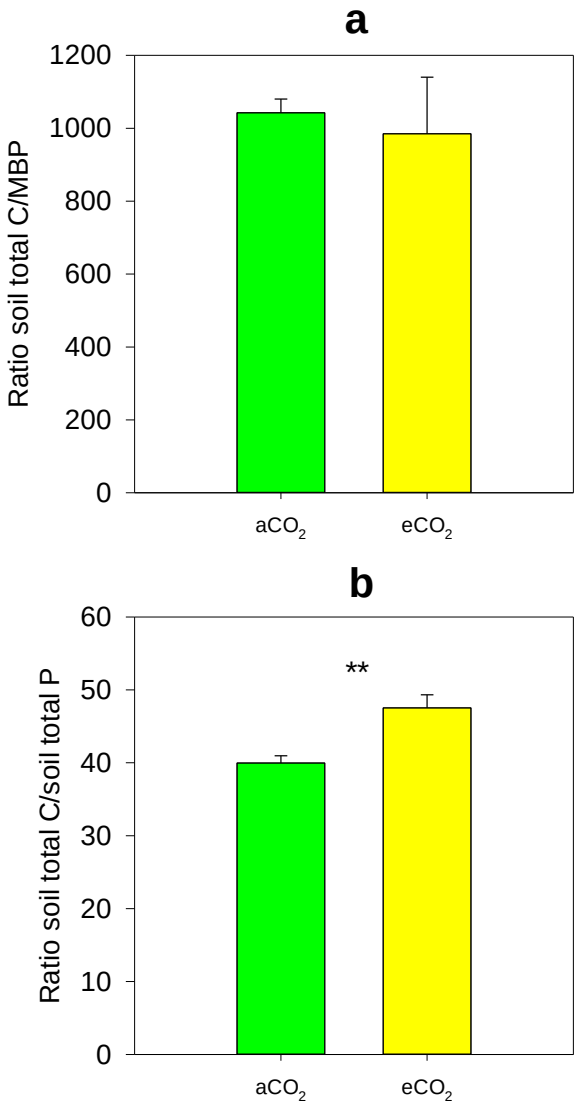


Figure 6.6 Effect of CO_2 on total soil C/MBP (a) and total soil C/total soil P (b) ratios after 22 years of exposure to ambient CO_2 (aCO_2) and elevated CO_2 (eCO_2). Columns represent mean of three replicates and standard errors. ** indicates significant difference ($P < 0.01$).

6.3.2 Soil P fractions

Soil P fractionation data revealed that moderately labile P (NaOH1-P) was the main fraction accounting for 34.4 % of total P, followed by residual P 27 %, calcium P (HCl-P) 24.8 %, labile P ($NaHCO_3$-P) 7.7 %, stable P (NaOH2-P) 6 %, and readily available P (NH_4Cl-P) 0.1 %. Organic P represented 28.2 and 24.4 % of total P under eCO_2 and aCO_2, respectively, with moderately labile Po (NaOH1-Po) being the main organic P fraction (75 %). In comparison to aCO_2, eCO_2 decreased labile Pi ($NaHCO_3$-Pi), moderately labile inorganic P (NaOH1-Pi) and stable inorganic P (NaOH2-Pi) by 38.6, 15.1, and 6.7 %, respectively. Elevated CO_2 concentrations increased residual P by 7.1 % compared to aCO_2, whereas there was no significant difference in total P between the two CO_2 treatments. Our

results showed that NH_4Cl-Pi and HCl-Pi were not different between the two CO_2 treatments (Table 6.1). After 22 years of CO_2 enrichment, a significant accumulation of labile P ($NaHCO_3$-Po), moderately labile organic P (NaOH1-Po), and stable organic P (NaOH2-Po) fractions was observed relative to the aCO2 treatment (Table 6.1). This accumulation was 5, 26, and 17 mg kg^{-1} for $NaHCO_3$-Po, NaOH1-Po, and NaOH2-Po, respectively.

Table 6.1 Concentrations (mg kg^{-1}) of different P fractions in the soil from the aCO_2 and eCO_2 treatments.

	aCO_2	eCO_2	Significance [b]
NH_4Cl-Pi	1.3 ± 0.1	1.4 ± 0.3[a]	ns
$NaHCO_3$-Pi	70.0 ± 2.3	47.4 ± 4.2	**
$NaHCO_3$-Po	25.0 ± 0.6	29.7 ± 2.3	*
NaOH1-Pi	169.5 ± 11.5	145.7 ± 9.1	*
NaOH1-Po	212.0 ± 8.2	238.1 ± 12.5	*
HCl-Pi	276.9 ± 5.0	274.4 ± 17.5	ns
NaOH2-Pi	26.7 ± 0.2	24.9 ± 0.6	*
NaOH2-Po	32.4 ± 1.3	49.0 ± 3.2	*
Residual-P	290.0 ± 4.9	311.3 ± 9.3	*
Total P[c]	1103.8 ± 24.8	1121.8 ± 21.5	ns

[a] Values represent means of 3 replicates ± standard errors.
[b] *, **, *** indicate significance at the $P < 0.05$, 0.01 and 0.001 level, respectively; ns = not significant.
[c] Calculated as the sum of all P fractions.
Pi and Po mean inorganic and organic P, respectively

6.4 Discussion

6.4.1 Soil pH

In our study, soil pH was not affected by eCO_2 and was similar to earlier studies carried out on the same site (Ross et al. 2004; Newton et al. 2006). Soil pH has been reported to decrease through acidification of the soil after exposure to eCO_2 (Andresen et al. 2010), thus potentially increasing available P in cropping systems – usually at neutral or alkaline pH (Jin et al. 2014, 2017). Our soil was already near the optimum pH for P availability; hence soil acidification would likely not increase P availability (Hinsinger, 2001).

6.4.2 Total carbon and nitrogen

Total soil C increased by 22 % under eCO_2 in comparison to aCO_2. Excess C provided by eCO_2 is likely metabolised by plants and transferred to the roots as carboxylases that are released into the rhizosphere or photosynthates accumulated in the root biomass (Wasaki et al. 2005; Drigo et al. 2008; Jin et al. 2014). Previous work on this site showed that long term exposure to eCO_2 caused an increase in rhizodeposition, root turnover, and plant residues (Allard et al. 2005, 2006). Therefore,

increased C inputs under eCO_2 in this grazed pasture system are most likely responsible for the increase in total C observed in our study. It is important to mention that the higher total soil C/total soil P ratio noted under eCO_2 favours P accumulation and immobilisation by microbes (Jin et al. 2014; Wei et al. 2019a).

Like C, our results showed a 14 % increase in total N under eCO_2, but a 21 and 38 % decrease for N-NO_3^- and N-NH_4^+, respectively, indicating a limited N availability compared to the aCO_2. The availability of N under eCO_2 has been linked to increased soil organic matter and microbial activity together with changes in microbial community composition (Newton et al. 2006; Zhong et al. 2018; Yu et al. 2018). Previous studies at the same site also indicated high soil organic matter under eCO_2 due to increased root biomass, principally derived from legumes and C turnover (Allard et al. 2005, 2006). On the other hand, limited N availability can be explained by an increased immobilisation of N in the microbial biomass as well as plant litter (Allard et al. 2006; Newton et al. 2010). Other experiments have described similar patterns of N transformation in different agroecosystems after long-term exposure to eCO_2 (Lagomarsino et al. 2008; Dijkstra et al. 2012).

6.4.3 Phosphatase activities

We found a 20 % increase in alkaline phosphatase activity under eCO_2. Consistent with our findings, several authors have indicated an increase in phosphatase activity in response to eCO_2 (Manoj-Kumar et al. 2011; Bhattacharyya et al. 2014; Mellett et al. 2018). Phosphatase enzymes are released by plants and microorganisms to mineralise organic P for plant uptake (Tarafdar and Jungk 1987; Nannipieri et al. 2011). The activity of these enzymes is affected by climatic conditions, plant species, soil types, and P availability (Lyu et al. 2016; Margalef et al. 2017; Fujita et al. 2017). Alkaline phosphatase activity is largely derived from microorganisms in the soil (Tarafdar and Claassen 1988; Nannipieri et al. 2011) and is believed to be impacted by increased concentrations of CO_2 (Delucia et al. 1997; Drissner et al. 2007). Soil bacteria tend to change more under eCO_2 compared to fungi (Drissner et al. 2007; Jin et al. 2014, 2015). For instance, Drissner et al. (2007) described an increase in microbial biomass after growing white clover for three years under an eCO_2 environment, which was strongly correlated with alkaline phosphatase activity mainly derived from bacteria. Similarly, low P conditions stimulated a decrease in microbial diversity in the rhizosphere of different plant species while enhancing bacterial phyla along with a specific increase in alkaline phosphatase activity (Wasaki et al. 2018; Wei et al. 2019a). Recent findings from the New Zealand FACE experiment have highlighted increases in specific bacterial phyla such as Planctomycetes under eCO_2 (Xia et al. 2017). Planctomycetes have been reported among the dominant phyla integrating *phoD* and *phoX* genes related to alkaline phosphatase activity across a wide range of soil types and environments (Ragot et al. 2015, 2017). Consequently, higher alkaline phosphatase activity measured in the eCO_2 could come

from those specific bacterial phyla enriched under this treatment, although we have no additional data to support this. In contrast to alkaline phosphatase enzymes, acid phosphatases were not affected by eCO_2 in our study. Acid phosphatase activity is considered to be higher in acidic soils (Skujins et al. 1962; Tabatabai 1994). Since soil pH in our study site did not change in response to eCO_2 and its value was close to neutral (pH 6.4), it is likely that acid phosphatase activity and any differences between the eCO_2 and aCO_2 were small.

6.4.4 Microbial biomass P

Microbial biomass P increased by 14 % under eCO_2 compared to aCO_2. As soil microorganisms are C-limited (Drigo et al. 2008; Spohn and Kuzyakov 2013a; Heuck et al. 2015), eCO_2 is believed to increase microbial activity and growth via rhizodeposition (Drissner et al. 2007; Yu et al. 2016). For instance, Jin et al. (2014) found an increase in microbial C and microbial respiration in the rhizosphere of wheat grown at a CO_2 concentration of 800 ppm. Drigo et al. (2009) found an increase in the total sugar concentration in the rhizosphere of sand sedge (*Carex arenaria*) and red fescue (*Festuca rubra*) cultivated under a CO_2 concentration of 700 ppm together with an abundance of *Burkholderia* and *Pseudomonas*. In a recent study investigating root detritus, Wei et al. (2019) reported that a lower C/P ratio in root detritus caused a decrease in P availability via P immobilisation by soil microorganisms. Soil microbes tend to immobilise P when soluble C substrates become available in the soil, inferring that P immobilisation is only a side effect of C nutrition by microorganisms in order to keep their stoichiometric ratios stable (Richardson 1994; Jakobsen et al. 2005; Spohn and Kuzyakov 2013a; Heuck et al. 2015). In our study, a constant total C/MBP ratio under both CO_2 treatments points towards P immobilisation related to higher C inputs under eCO_2 (Jin et al. 2014).

6.4.5 Inorganic P fractions

Phosphorus availability is influenced by the degree of attachment of P to the active mineral surfaces of Fe, Al, and Ca as well as organic molecules present in the soil (Hinsinger 2001; Pierzynski and McDowell 2005; Condron et al. 2005). NH_4Cl-Pi and $NaHCO_3$-Pi fractions represent Pi readily available for immediate plant and microbial uptake (Hedley et al. 1982). We found that eCO_2 decreased labile Pi ($NaHCO_3$-Pi) and Olsen P by 38.6 and 39 %, respectively, compared to aCO_2. Our results concurred with other experiments showing depletion of $NaHCO_3$-Pi, such as Jin et al. (2017), who found a decrease between 17 to 36 % in $NaHCO_3$-Pi under three different cropping soils exposed to 6 years of eCO_2 (500 ppm). NaOH1-Pi is defined as a moderately labile P form associated with Fe and Al oxides (McDowell and Condron 2000). This fraction can contribute to the available P pool after desorption from Al and Fe oxides via organic anions (Jones 1998; Hocking 2001; Oburger et al. 2011; Gerke 2015). Long-term CO_2 enrichment decreased moderately labile Pi (NaOH1-Pi) by 15.1 %. Some studies have reported a similar pattern for this fraction under CO_2 enrichment. For instance, in the

experiment carried out by Jin et al. (2017), NaOH-Pi was depleted under a wheat-pulse rotation by 22 and 77 % in a Chromosol and Vertisol, respectively, whereas no differences were observed in the calcareous soil. HCl-Pi was the third most abundant fraction in our study accounting for 24.8 % of total P and is typical of proportions extracted from sandy soils of New Zealand (Cowie and Hall 1965; Steele 1976). The Ca-P pool is mainly influenced by soil pH and plant species (Vu et al. 2008; Rose et al. 2010; Ye et al. 2018). Since soil pH was the same in the two CO_2 treatments, it was not surprising that no changes were detected in HCl-Pi. Stable Pi (NaOH2-Pi) is considered a more recalcitrant Pi fraction attached to highly structured Fe and Al oxides in micro-aggregates (Condron and Goh 1989; Condron and Newman 2011; McDowell et al. 2016). This fraction needs to be desorbed by organic anions in order to be available in the soil solution (Wang et al. 2016b, 2017a). Our results showed only a minor decrease of this fraction under eCO_2. Described as the occluded inorganic and organic P, residual P is unlikely to contribute to P availability in the short term (Perrott and Mansell 1989; McDowell and Condron 2000, 2012). However, subtropical forest plants were able to access this highly recalcitrant fraction after 5 years of exposure to eCO_2 with N addition (Huang et al. 2014). Moreover, over a long period, intensive cropping systems can mobilise residual P to supply the labile P pool (Jin et al. 2017). In contrast, our study showed that residual P increased after 22 years of eCO_2 enrichment. Khan et al. (2008) also reported an accumulation of this fraction after growing popular trees in a FACE design for 5 consecutive years. Steele (1976) explained that excess Fe could coat inorganic P, thus transforming it into a residual P form. Therefore, we suggest that the build-up of organic matter (Ross et al. 2004; Newton et al. 2006) and its complexation (Steele 1976; Maher and Thorrold 1989; Perrott and Mansell 1989) may be the cause of residual P accumulation in our study. However, without further data, we can only speculate that this was the case.

6.4.6 Organic P fractions

Organic P can account for 20-80 % of total P according to soil types, location, and land use (Nash et al. 2014), being an important source of available P for plants, especially in low P environments. The $NaHCO_3$-Po fraction is readily available for plant uptake after mineralisation by phosphatases. In our study, this fraction accumulated (5 mg kg^{-1}) under eCO_2, while decreases and increases were noted in previous investigations (Manoj-Kumar et al. 2011; Jin et al. 2014). Increased rhizodeposition and higher microbial activity that may occur under eCO_2 could accelerate organic P accumulation. Jin et al. (2014) described a 160 % increase in $NaHCO_3$-Po under wheat plants exposed to eCO_2. During their short experiment (6 weeks), the use of ^{13}C revealed an increased rhizodeposition along with an increase in microbial C and respiration. NaOH-Po is defined as the organic P attached to Al and Fe oxides and is sparingly available for plant uptake (McDowell and Condron 2000; Pierzynski and McDowell 2005) and needs to be mineralised by the combined action of phosphatase enzymes and organic anions (Clarholm et al. 2015; Darch et al. 2016). NaOH extractable P is described as one of

the most important P fractions in grassland soils (Nash et al. 2014). In our study, NaOH-Po accounted for 70 % of organic P, and in line with our hypothesis, NaOH1-Po and NaOH2-Po significantly accumulated after 22 years of CO_2 enrichment. This contrasted the findings by Jin et al. (2017), who observed a decrease of NaOH-Po under a wheat-pulse rotation system subjected to 5 years of CO_2 enrichment. Unlike perennial pastures, intensive cropping systems over a long period of time can accelerate soil organic matter turnover as well as the mineralisation of organic P to meet increased P demand, especially under eCO_2 (Jin et al. 2017).

Accumulations of NaOH-Po have been related to microbial activity and plant rhizodeposition (Jin et al. 2012, 2014). Rhizodeposition can contribute to organic P accumulation via microbial growth. Heterotrophic respiration has been found to be greater under eCO_2 in our study site (Ross et al. 2013), indicating a rapid microbial turnover under these conditions. Microbial cells are primarily composed of nucleic acids, DNA, and phospholipids that could accumulate under eCO_2 and contribute to organic P, which is routinely extracted with $NaHCO_3$ and NaOH (Turner et al. 2003; Cade-Menun and Liu 2014; Nash et al. 2014). Organic P includes P derived from plants and microbial materials such as plant litter, dead roots, and microbial biomass and has been reported to accumulate under grazed pasture systems (Richardson 2001; Condron et al. 2005; McDowell and Condron 2012; Nash et al. 2014). Organic P accumulated under eCO_2 in our study exceeded the difference in microbial P between eCO_2 and aCO_2. This indicated that organic P was also accumulating from other sources. Previous studies in the same site showed that eCO_2 increased root biomass and the turnover of fine roots (Allard et al. 2005, 2006; Newton et al. 2006; Ross et al. 2013). For instance, Allard et al. (2005), using an ingrowth core method, found that root biomass was higher under eCO_2, especially in the summer-autumn period (45 % increase compared to the aCO_2), and root turnover was 2.7 times faster over the same period.

Plant materials are rich with C compounds and represent a potential source of orthophosphate and organic P molecules, including acid nucleic, phospholipids, and phosphoesters (Kuo 1996; McGroddy et al. 2004; Noack et al. 2012; Alamgir and Marschner 2013). In addition to biotic processes, Ca compounds and Fe and Al oxides can adsorb organic P by forming strong bonds, thus preventing its mineralisation by phosphatase enzymes (Turner et al. 2003; Murphy et al. 2009; Menezes-Blackburn et al. 2013). For instance, Celi et al. (2000) and Johnson et al. (2012) found that organic P, mostly inositol-phosphate, is complexed by goethite and calcite due to high amounts of free Fe oxides and calcium cations present in the soil. The data from our P fractionation showed that NaOH-P and HCl-Pi were the first and third main P fractions in this soil, respectively, indicating the presence of high amounts of positively charged minerals, mainly Al, Fe, and Ca. Therefore, we suggest that organic P accumulation observed under eCO_2 may have been exacerbated by the complexation of organic P on the reactive mineral surfaces of Al, Fe, and Ca.

Grazing recycles nutrients from the herbage and may result in losses from the system in animal products. For P, the losses in animal products are small, being less than 10 % of the P ingested (Nguyen and Goh 1992b). The total amount of herbage eaten over the course of the experiment (1997-2019) was 10 % greater under eCO_2, but the difference was not significant (aCO_2: 11809 ± 1134 g m^{-2}; eCO_2: 13000 ± 1500 g m^{-2}; p=0.561). As the herbage P concentrations have previously not been found to differ between CO_2 treatments (Gentile et al. 2012), we conclude that the combination of small removal in animal products, a slight difference in the herbage eaten, and no difference in P concentration in the herbage means it is unlikely that differences between CO_2 treatments in soil P can be ascribed to differences in P removal by animals.

6.5 Conclusions

The findings of this study confirmed that elevated CO_2 (500 ppm) had a major impact on the dynamics and bioavailability of soil P under grazed temperate pasture, which resulted in decreases in soil inorganic P and concomitant increases in organic P. The depletion of inorganic P reflected increased plant growth and P uptake as well as microbial P immobilisation, while accumulation of organic P was attributed to a combination of factors, including enhanced biological activity, plant root turnover, and immobilisation on reactive mineral surfaces. Further studies are required to investigate the biological and biochemical mechanisms responsible for enhanced P immobilisation under elevated CO_2 together with assessing the nature of organic P species to determine if they are labile should CO_2 concentrations continue increasing.

Chapter 7

General Discussion and Future Research

Biotic (plant species) and abiotic (N and P inputs and elevated CO_2) factors can have a significant impact on soil P dynamics by influencing chemical, biological, and biochemical processes. Therefore, an investigation of these factors and the processes linked to P cycling is required to ensure efficient, adaptable, and sustainable agriculture systems. The objective of this thesis was to determine the effects of N and P inputs and elevated CO_2 on soil P dynamics and bioavailability and related processes under different plant species and pasture soils in New Zealand. Soil P fractionation was used to quantify changes in inorganic and organic P fractions while biological and biochemical properties linked to P cycling, including microbial biomass P, organic anions, and phosphatase enzymes were measured.

In a glasshouse experiment, blue lupin (*Lupinus angustifolius*), white clover (*Trifolium repens* L.), perennial ryegrass (*Lolium perenne* L.), and wheat (*Triticum aestivum* L.) were grown in a low P pasture soil (4 mg kg^{-1}) for 8 weeks with and without the single and combined additions of P and N (Chapter 2). Results showed that P addition alone (45 kg P ha^{-1}) or in combination with N (45 kg P ha^{-1} + 200 kg N ha^{-1}) increased plant growth, total P and N uptake, microbial biomass P, and organic anion concentrations, regardless of plant species. In contrast, N addition alone (200 kg N ha^{-1}) had no effect on plant biomass and soil P fractions compared to the control, indicating that N was not a limiting factor for plant growth. This result was attributed to high amounts of available N present in the soil, probably driven from organic matter mineralisation. Organic anion concentrations increased after P addition, especially under legumes. Organic anions are released by plants and soil microbes but can also originate from the degradation of organic matter. The increase in organic anion concentrations after P addition was suggested to be related to a combination of sources, including plant roots, microbial release, and microbial priming effect on soil organic matter. Data from soil P fractionation revealed that moderately labile Pi and stable Po were the most depleted fractions by the four plants species, especially after P addition. This result emphasised that plant species can deplete sparingly available P fractions despite being supplied with soluble inorganic P fertilisers.

In a field trial, the pasture phase (Italian ryegrass (*Lolium multiflorum* Lam.)) after cropping, characterised by low N and C availabilities, was used to study the independent and interactive effects of N and P additions on plant growth and soil P fractions transformations. Seasonal changes in soil biological and biochemical properties were also determined (Chapter 3). Results showed that P addition alone (50 kg P ha^{-1}) increased P availability and shoot N and P uptake but not shoot biomass of Italian ryegrass (*Lolium multiflorum* Lam.), probably because initial soil P (10 to 6 mg kg^{-1}) was not

limiting the growth of this plant species. In contrast, N addition alone (250 kg N ha^{-1}) or in combination with P (50 kg P ha^{-1} + 200 kg N ha^{-1}) increased shoot biomass by an average of 1.6-fold compared to the control treatment. This indicated that plant primary productivity was limited by N availability rather than P. The combined addition of N and P had a significantly greater effect on shoot P uptake than the single nutrient input. Nitrogen addition alone or in combination with P increased microbial biomass P and acid and alkaline phosphatase activities in summer, which was attributed to increased plant growth, alleviation of carbon limitation, and higher temperature in this period. Phosphorus fractionation data revealed that readily available Pi, labile Pi, labile Po, and moderately labile Pi were depleted by Italian ryegrass (*Lolium multiflorum* Lam.); however, the extent of this depletion increased under N treatment by 55, 19, 28, 7 %, respectively, compared to the control. This indicated that N addition to this N-limited pasture soil accelerated the depletion of labile and moderately labile Pi fractions due to higher plant P uptake and removal of plant biomass. In comparison to P addition alone, the combined addition of N and P promoted the mineralisation of labile Po (28%) and the utilisation of adsorbed labile Pi from P fertilisation. Therefore, it was concluded that the combined application of N and P to N-limited pasture soils could provide many advantages over the single input of N or P, including an increase in plant primary productivity, minimising soil P build-up, enhancing organic P mineralisation, and providing better quality feed for animals.

The Winchmore fertiliser trial was investigated to determine short and long-term impacts on soil parameters linked to biological P cycling in response to contrasting P inputs (0, 188, and 376 SSP ha^{-1} yr^{-1}) for more than 67 years under irrigated grazed pastures (Chapter 4). Phosphorus availability increased with increasing P applications, while microbial biomass P was similar under 188 and 376 SSP ha^{-1} yr^{-1}. The latter result was attributed to microbial biomass need to maintain nutrient stoichiometry. Indeed, although P availability was higher under 376P compared to 188P treatment, the similar availabilities of carbon and N, related to plant returns, carbon turnover, and clover content (N source), yielded similar microbial biomass P in both P treatments. Results also indicated that long-term P fertilisation enhanced alkaline phosphatase activity but decreased acid phosphatases under this grazed pasture system. This was attributed to the higher organic P returns (plant detritus and animal dung) under 188P and 376P treatments compared to the control as well as the differentiation in origin and nutrient demand between acid and alkaline phosphatase enzymes. In fact, alkaline phosphatase activity was positively correlated with microbial biomass P and driven by C demand, whereas acid phosphatase activity was inhibited by high P availability and most likely derived from plant roots. Significant seasonal variations in microbial biomass P occurred under 188P and 376P treatments compared to the control. Under these treatments, microbial P immobilisation occurred in summer and winter, while microbial P release was observed in spring and autumn. On

the other hand, similar seasonal patterns were observed for acid and alkaline phosphatase activities across all P treatments. The overall findings from the Winchmore fertiliser trial highlighted yet again the importance of long-term experiments in advancing our understanding of soil P cycling and related processes as affected by management practices.

Results from Chapters 2 and 3 revealed that under P-deficient soil, P addition can increase plant growth and N uptake, while under N-limited soil, N addition increased plant growth and plant P uptake. These results confirmed that the availability of one element can feedback on the dynamics and cycling of the other in soils and plant. Furthermore, the availability of carbon is also an essential factor that needs to be taking into consideration when assessing the effects of anthropogenic inputs on soil nutrient cycling due to the critical role of carbon (energy) in controlling soil microbial biomass and activity.

Seasonal assessment of biological and biochemical processes linked to P cycling showed that temporal changes in microbial biomass P were indirectly affected by nutrient inputs and soil P status, whereas temporal trends of acid and alkaline phosphatase activities were similar regardless of nutrient inputs and soil P status (Chapters 3 and 4). Acid and alkaline phosphatase enzymes exhibited higher activity in summer and lower activity in winter and were driven by environmental conditions (soil moisture and soil temperature), which in turn impacted plant P demand and microbial activity. In contrast to temperate pasture systems in New Zealand, organic P mineralisation was suggested to be maximum in summer due to the presence of adequate soil moisture and higher temperature under the irrigated pasture systems investigated in this work (Chapters 3 and 4). In comparison with phosphatase activities, seasonal changes in microbial biomass P were less affected by environmental conditions and were more likely controlled by complex interactions between several factors, including environmental conditions, plant primary productivity (plant returns, root residues, and rhizodeposits), plant P demand, microbial activity, and potential shifts in microbial community composition.

To gain knowledge on how eCO_2 could affect soil P availability and dynamics in P-poor soils, a six-week pot experiment was carried out where blue lupin (*Lupinus angustifolius*), perennial ryegrass (*Lolium perenne* L.), and wheat (*Triticum aestivum* L.) were grown in a low P soil under aCO_2 (390 ppm) and eCO_2 (700 ppm) (Chapter 5). Results revealed that under P deficiency, plant biomass, P uptake, and rhizosphere properties did not respond to eCO_2 but were significantly affected by plant species. Therefore, soil P availability and changes in P fractions were similar between aCO_2 and eCO_2 treatments but significantly different across plant species. Furthermore, consistent with the results presented in Chapter 2, legumes and grasses were able to mobilise different soil P fractions irrespective of their chemical availability using morphological and/or physiological adaptations.

In Chapter 6, the long-term New Zealand FACE experiment was used to quantify changes in P availability and soil P fractions as well as soil chemical, biological, and biochemical parameters as affected by ambient and elevated CO_2 (500 ppm) concentrations in a grazed pasture system. Long-term CO_2 enrichment increased microbial biomass P and alkaline phosphatase activity by 14 and 20 %, respectively, compared to aCO_2. This indicated that eCO_2 promoted microbial P immobilisation due to increased plant rhizodeposition. Furthermore, long-term CO_2 enrichment decreased labile and moderately labile Pi while promoted moderately labile and stable Po accumulation under grazed pastures. Decrease in soil Pi was attributed to plant P uptake along with immobilisation of Pi in microbial biomass. Accumulation of Po was linked to enhanced biological activity, increased inputs of Po from root detritus, and adsorption onto reactive mineral surfaces (Al, Fe, and Ca).

The findings from Chapters 5 and 6 suggest that the effect of climate change and specifically elevated CO_2 will depend on cropping systems, soil P availability, and the availability of other elements present in the soil such as Al, Fe, and Ca, which could exacerbate soil organic P accumulation. In general, under adequate soil P availability, such as in the FACE experiment investigated in Chapter 5, eCO_2 can increase root biomass, rhizodeposition, and microbial P immobilisation. However, under low P conditions (Chapter 6), an increase in plant biomass is unlikely due to the negative feedback of P deficiency on photosynthesis. This, in turn, prevents plant species from investing in their adaptations strategies to acquire P. Therefore, plants may thrive under P deficiency and eCO_2 conditions if they can capture excess carbon provided by elevated CO_2 to increase their photosynthesis and/or develop better physiological root traits to acquire soil P.

Soil P fractionation data revealed that plant species investigated in this work and their associated rhizosphere microbes were able to mobilise sparingly available and recalcitrant P fractions regardless of their chemical availability, as assessed by sequential P fractionation (Chapters 2 and 5). This result challenges the recalcitrance of some soil P fractions, which is defined by resistance to extraction and clearly not to action by plants or microbes. Plant species used divergent root traits to increase soil P acquisition. Legumes invested more in physiological adaptations (organic anions and phosphatase enzymes) than grasses and accessed more sparingly available P fractions, especially through phosphatase enzymes. In this research, different organic anions were identified from the rhizosphere of grass and legume plant species grown in real soil conditions. Citrate, malate, and malonate were the dominant organic anions found in the rhizosphere of different plant species. Although legume plants exhibited a higher ability in releasing organic anions in their rhizospheres, no correlation was found between the depletion of moderately labile and stable inorganic P fractions and organic anion release. This indicated that organic anions played a minor role in the acquisition of these sparingly available P fractions and that other factors may have controlled inorganic P acquisition, including soil pH, root traits, or their combination.

The ability of microbial biomass to immobilise or release P to plants depends not only on the availability of P but also C and N availabilities. Chapter 2 showed that microbial P increased after P addition to a low P soil, while N addition had no impact on this parameter due to the high availability of N derived from organic matter mineralisation. Chapter 3 showed that microbial biomass P was not affected by N and P inputs in an N-limited but P-sufficient soil. This was possibly attributed to the low availability of C in the investigated soil, which was under an arable cropping system before being cultivated by a grass pasture and used for the study carried out in Chapter 3. Results from Chapter 4 revealed that although inorganic P bioavailability increased with increasing P applications, microbial biomass P was similar under treatments receiving either 188 or 376 kg $ha^{-1}yr^{-1}$ of SSP. This result was ascribed to the similar availability of C and N under these two treatments and the microbial biomass need to maintain nutrient stoichiometry.

Microbial biomass P showed a strong correlation with alkaline phosphatase enzyme activity in most Chapters, emphasising that alkaline phosphatase activity is derived from soil microorganisms. Moreover, there were some indications that an increase in organic anion release concurred with an increase in microbial biomass P, especially under legumes. In fact, organic anions represent a rich and readily available carbon source for soil microorganisms, which can increase microbes' growth and activity but cold also shift their community composition. Results from Chapters 2 and 5 indicated that organic anion release was higher under blue lupin, which coincided with higher alkaline phosphatase activity and microbial biomass P. This indicated that the activity and biomass of soil microbes could be driven by organic anions. The results of chapter 4 also confirmed that alkaline phosphatase enzymes are derived from soil microbes and are dependent on C availability rather than P.

The results of Chapter 3 showed that Italian ryegrass was not limited by P but rather by N. The soil used in Chapter 3 was also used in Chapter 5, However, in Chapter 5 we concluded that the P availability of this soil (7 mg kg^{-1}) was limiting the growth of perennial ryegrass, wheat, and blue lupin and thus their response to eCO_2. At first, these results seem to contradict each other; however, they could be explained by the following. First, while there are some indications in the literature that grass only pastures have lower P requirements compared to legume-grass pastures, the level of optimal Olsen P for Italian ryegrass is not available for New Zealand soils. The control being at 10 mg kg^{-1} Olsen P (or even 6 mg kg^{-1} in the second year) showed no significant difference in terms of plant biomass compared to P addition treatment, suggesting that these levels could be enough for Italian ryegrass growth in sedimentary soils in New Zealand. Secondly, the soil used in Chapters 3 and 5 were sourced from different soil depths and used in different experimental designs. The soil used in Chapter 5 had lower Olsen P (7 mg kg^{-1}), was taken from 0-20 cm soil depth (lower total P) and used in a pot experiment, while soil samples from Chapter 3 were taken from 0-7.5 cm under field

conditions. Phosphorus deficiency and the non-response of plant species to eCO_2 observed in Chapter 5 could be ascribed to the small volume of soil and pot used for the study, which may have induced P limitation. However, in Chapter 3, Italian ryegrass may have been able to take up some P from deeper soil layers, especially knowing that the site was previously under cropping system, where P could be evenly distributed in the soil profile.

The results of this research confirmed our general hypothesis and showed that depletion/accumulation of different inorganic and organic P fractions and the processes related to soil P dynamics and bioavailability were affected by plant species, N and P inputs, and elevated CO_2. However, other factors contributed to the effects observed. These include organic matter content, the initial availability of C, N and P, and probably the abundance of free Fe, Al, and Ca cations in soils. Compared to studies carried out under controlled environments or under field conditions for short-term, long-term field experiments showed that cumulative effects of P fertilisation and CO_2 enrichment on plant primary productivity, plant returns, C inputs and turnover, and plant community composition had a significant influence on P dynamics and soil properties linked to P cycling.

Although the general hypothesis of this work was confirmed, two of our specific hypotheses were not proven. The first hypothesis was that P addition to a P-deficient soil would decrease the release of organic anions due to the supply of available P in fertiliser. However, increased plant growth in response to P addition can increase plant root and, in turn, organic anion release. Results from Chapter 2 showed that organic anion concentrations increased in the rhizosphere of legumes and grasses after P addition. Moreover, organic anion concentrations expressed in a rhizosphere soil or root dry matter basis were similarly higher under P addition compared to the control. This suggested the contribution of soil microbes to organic anion concentrations either directly through microbial exudation or via microbial priming effect on organic matter (organic matter (7.2 %)). However, the analysis of plant biomass increases in legume plants further suggested that organic anion release after P addition was mainly from plant-origin under white clover, but likely from plant and microbial origins under blue lupin. The second hypothesis was that eCO_2 would increase soil P availability by increasing the release of organic anions and enhancing the mobilisation of sparingly available P fractions. Results presented in Chapter 6 showed that total carbon and microbial biomass P significantly increased under eCO_2, which indicated higher rhizodeposition (including organic anions) under eCO_2 compared to aCO_2. However, this high rate of rhizodeposition enhanced microbial P immobilisation, thereby decreasing inorganic P availability and contributing to organic P accumulation. On the other hand, the results presented in Chapter 5 highlighted that under P-deficient soils, an increase of soil organic anions is not expected; thereby, no changes in soil P fractions would occur.

Taking together, the enhanced understanding of soil P dynamics as affected by nutrient inputs and elevated CO_2 can be used to formulate management practices that would increase soil P use efficiency and enhance soil P availability to plants. The main outputs from this study based on our findings are:

- Other techniques need to be combined with chemical P fractionation for a more holistic understanding of soil P dynamics, especially organic P species.

- Legumes have shown higher ability in releasing organic anions and phosphatase enzymes, which concurred with higher mineralisation of organic P. This emphasises the importance of using legumes more often in intercropping systems, rotation systems, and as cover crops to increase P availability and mine P legacy in soils. This builds up on the literature showing that legumes are not only essential to human and animal nutrition but also as soil fertility enhancers.

- Application of organic matter to low P soils can increase microbial biomass and activity. Supplying the soil microbial biomass with carbon (energy) is pivotal to the activation of mechanisms required to increase soil P availability and cycling, such as the release of phosphatase enzymes and organic anions.

- Applications of both N and P in excess of plant requirements can not enhance soil P cycling, but on the contrary, can accelerate soil P build-up and eutrophication of waterways due to N and P losses. Therefore, nutrient inputs to pasture systems need to be tailored according to both plant requirements and soil fertility status.

- Phosphorus deficiency can limit plant ability to grow and acquire P under elevated CO_2 conditions. Therefore, selection for plant cultivars able to cope with P deficiency and elevated CO_2 conditions is of urgent need, especially for regions suffering from P deficiency, high risks of climate change, high fertiliser prices, and low input agroecosystems.

- To maintain the productivity of temperate grazed pasture systems on sandy soils subjected to elevated CO_2 in New Zealand, various management practices can be applied in the short, medium, and long-term. Ongoing applications of P fertilisers will be required to overcome P-limitation, while cultivation may enhance soil aeration and accelerate the mineralisation of Po. Pasture renovation and the introduction of new pasture species may facilitate the mobilisation of recalcitrant P pools, while soil inoculation with beneficial microorganisms could be used to enhance the mobilisation of accumulated Po via the release of organic

anions and phosphatase enzymes. However, the implementation of these management practices needs to take into consideration practicability, cost-efficiency, and unforeseen impacts of each practice.

In light of the findings of this study, further research needs to be undertaken in the following areas:

- The soils used in this work were either P- or N-limited; thus, the response of soil P dynamics and related processes to N and P additions under N and P co-limited soils remains not well understood. Future research on this topic is consequently advised. Moreover, optimum N:P supply ratios need to be further established under pasture systems for better plant, soil, and water quality.

- Arbuscular mycorrhizal fungi (AMF) play a critical role in soil P acquisition, especially under P deficient conditions; however, AMF can compete with the plant host for nitrogen. Therefore, the effects of single and combined additions of N and P on AMF colonisation, soil P fractions, and the release of phosphatase enzymes and organic anions need to be elucidated in future studies.

- Long-term P fertilisation had a significant effect on acid and alkaline soil phosphatase activities at the Winchmore fertiliser trial, while previous studies in the same site have revealed significant changes in microbial taxa linked to soil P cycling. Thus, investigations using metagenomics and phosphatase genes in this trial could unravel the contribution of different microbial taxa to acid and alkaline phosphatase activities.

- Due to the challenges associated with sampling and measuring phosphatase enzymes and organic anions in soils and rhizospheres (adsorption, root breakage), the use of soil zymography and DGT-like gels (diffusive gradients in thin films) is warranted. These methods can advance our understanding of the role of phosphatase enzymes and organic anions in biogeochemical P cycling with minimal soil disturbance and with the ability to map spatial variations of these processes in the bulk soil and rhizospheres. Developing efficient methods to sample organic anions under field conditions is also required.

- Legumes and grasses have shown no response to elevated CO_2 in terms of P availability and rhizosphere processes when grown in P-deficient soil. Nevertheless, the interactive impacts of elevated CO_2 and soil P deficiency need to be further investigated under other plant species exhibiting better physiological root adaptations for P acquisition, such as white lupin (*Lupinus albus* L.). The impact of intercropping legumes and grasses under elevated CO_2 and P deficiency is still unclear.

- An assessment of the effect of elevated CO_2 on soil P availability and dynamics under dominant soil types used for pastures in New Zealand is required. This will allow a better estimate of the effect of elevated CO2 on a national scale to target specific measures to be implemented in the future.

- Besides the concentrations of atmospheric CO_2, climate change is believed to increase temperature and drought events. Therefore, future studies looking at the combined effects of CO_2, temperature, and water stress are recommended to give realistic insights on soil P availability and dynamics in response to future climate change scenarios. Studying the response of AMF under similar conditions is also necessary.

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