Sub-Antarctic plant-soil interactions in a changing world: plant N acquisition and growth under warming on Marion Island

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Abstract

It is widely accepted that in cold ecosystems, strong abiotic (e.g., temperature) controls over soil decomposition processes result in N-limited plant productivity and soils replete in organic N (oN, e.g., amino acids) but not inorganic N (iN, i.e., NH_4^+ and NO_3^-). Recent advances in our understanding of plant N use have shown that cold ecosystem plants meet the bulk of their N requirements through direct oN acquisition. Climate warming in cold ecosystems under global change is expected to alleviate the temperature limitations to soil decomposition, effectively increasing N-availability through iN release. This has resulted in predictions for stronger indirect than direct effects on plant productivity. Additionally, increasing iN fractions may affect cold-ecosystem plant nutrition by altering the N-form predominantly acquired from oN to iN. Investigations into plant oN acquisition and the effects of soil warming on plant productivity are largely restricted to northern high latitudes, leaving southern cold ecosystems such as the sub-Antarctic underrepresented. Sub-Antarctic soils are typically replete in oN but not iN, however, plant oN uptake has not been accounted for in terrestrial N-budgets. Furthermore, the islands are experiencing high rates of climate change including increasing temperatures. This thesis examines sub-Antarctic plant-soil interactions, investigating whether sub-Antarctic grasses acquire oN and how soil warming affects plant growth and nutrition. Potted and field experiments were run with four common grasses (*Polypogon magellanicus*, *Poa cookii*, *Agrostis stolonifera*, and *Poa annua*) from sub-Antarctic Marion Island (MI, -46.9, 37.8). I hypothesised that sub-Antarctic grasses acquire oN which affects plant growth relative to iN and that soil warming influences plant growth directly and indirectly through stimulating microbial iN mineralisation and plant nutrition by altering bioavailable oN and iN soil fractions. Grasses supplied with either ^{15}N-enriched oN (glycine) or iN (NO_3^-) provided evidence for oN acquisition in hydroponics. Experimental oN and iN provision (in hydroponics) resulted in higher relative growth rates (RGR) on iN compared to oN, but species-specific differences in biomass allocation under the different N-forms and [N]. Grasses supplied with ^{15}N-glycine *in situ* resulted in significant ^{15}N enrichment, although intact oN acquisition *in situ* cannot be determined with the use of only ^{15}N-labelled glycine. Considering the high [oN] in MI soils, this evidence suggests that oN represents an important N-resource for sub-Antarctic vegetation. A five-month warming experiment (MI ambient summer temperatures +3°C) resulted in limited biomass increases, which was only significant ($p < 0.05$) for *P. annua* (by 42%), and soil NH_4^+ increased by 12%. A fertilisation (NPK) treatment resulted in substantially higher plant biomass increases than warming (449% relative to 24%, respectively), suggesting that warming-induced N-release should not be assumed to strongly stimulate plant biomass. Soil warming did not influence plant acquisition of oN or iN. A soil incubation experiment (42 d; 5°C control, +3°C and +6°C warming) showed no effect of warming on soil iN, oN, or PO_4^{3-}, although iN increased and PO_4^{3-} decreased with increasing total organic C (TOC). Microbial biomass (C) increased with soil TOC but was not affected by warming. While microbial P increased with TOC and the +6°C warming treatment, microbial N was unaffected by warming and did not change with TOC. These results highlight the importance of investigating prevailing assumptions on soil-plant processes in cold ecosystems, including outdated assumptions that sub-Antarctic grasses only acquire iN, and that warming-induced N-release strongly stimulates plant productivity. The evidence presented here suggests that early work on MI underestimated total plant-available N, thus the sub-Antarctic terrestrial N-budget requires re-evaluation. Plant biomass and microbial mineralisation only showed limited or non-significant responses to soil warming, challenging the predictions for large, widespread effects of soil warming on N-release in cold ecosystems. The large differences in plant biomass under NPK relative to warming suggest that plant responses to increased N are limited if other nutrients, e.g., P, do not increase concomitantly. Despite strong temperature controls on cold-ecosystem soil decomposition rates, the stimulatory effects of short-term warming may be curtailed by a combination of interactive plant-soil processes.

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Chapter 1

General Introduction

Cold ecosystems typically have low rates of plant productivity due to climate limitations such as temperature (Billings and Mooney 1968). It is however argued that the main limitations to productivity in these systems are due to indirect rather than direct climate limitations (Chapin 1983). Nitrogen is one of the most limiting nutrients in terrestrial ecosystems, and its relative availability for plant uptake is a key determinant of terrestrial productivity (Aerts and Chapin 1999). Soil decomposition processes, which largely control N availability, are limited by abiotic drivers such as low temperatures. Low N availability is thus widely accepted as a strong indirect temperature limitation to cold ecosystem productivity (Chapin 1983). These processes include extracellular enzyme activity which releases bioavailable organic N (oN) through soil organic matter (SOM) depolymerisation (Schimel and Bennett 2004), microbial mineralisation of bioavailable oN compounds which releases inorganic N (iN), i.e., NH_4^+ which may then be nitrified to NO_3^- (Clarholm 1985), and soil invertebrate grazing activity on SOM which releases iN (Aerts 2006).

The temperature limitations to soil decomposition also control the bioavailability of oN and iN forms, which may affect plant N nutrition. In cold ecosystems, temperature-limited nutrient release by extracellular enzymes limits microbial activity, resulting in soils that typically contain higher fractions of bioavailable oN than iN (Kielland 1995). Plants have the physiological capacity to acquire a variety of oN compounds in addition to iN, including monomers such as the amino acids glycine or glutamine, but also polymers such as the peptide tri-alanine (Kielland 2001; Lipson and Näsholm 2001; Kuster et al. 2016). The relative availability of these N-forms can affect plant growth on an individual level due to the energetics associated with their respective uptake (Franklin et al. 2017) but also on a community level, as it may influence resource partitioning or competition (Miller et al. 2007a; Kuster et al. 2016). Under current rates of global change (IPCC 2022), increasing temperatures are predicted to stimulate decomposition rates (Kirschbaum 1995, 2004a). These increasing temperatures could alleviate N-limitations to cold ecosystem productivity by stimulating decomposition, but also change the relative fractions of oN and iN available for plant acquisition (Kuster et al. 2016).

Most research investigating cold ecosystem plant N uptake and plant-soil responses to climate warming is based on Northern Hemisphere systems, while cold terrestrial systems in the Southern Hemisphere have received less attention (Pendlebury and Barnes-Keoghan 2007; Yergeau et al. 2012). This is unsurprising, considering that vegetated cold ecosystems are predominantly found in northern high latitudes in addition to the concerns for potential feedbacks to warming within these systems, such as the fate of historical C-sinks in permafrost or surface albedo effects (Kirschbaum 2000; Winton 2006; Huang et al. 2017). Sub-Antarctic terrestrial systems, which comprise several small islands near the Antarctic Convergence, may however represent a useful area for investigating plant N use and plant-soil interactions under climate warming. The geographic isolation of oceanic islands results in a simple ecological system with depauperate flora and fauna (Harter et al. 2015). For example, sub-Antarctic Marion Island (MI; -46.90, 37.75) has no trees and low herbaceous and graminoid diversity, with only 21 native vascular plants (Chau et al. 2020). Indeed, the paucity of species and simple biotic interactions (compared to continental systems) have rendered oceanic islands pivotal for the development of seminal theories towards ecology, evolution, and biogeography, leaving their neglect in current climate change investigations surprising (Harter et al. 2015). Furthermore, sub-Antarctic islands experience a hyperoceanic climate with low seasonality and high sensitivity to climate perturbations, with evidence for significant climate change trends including temperature increases and changes in precipitation (Pendlebury and Barnes-Keoghan 2007; le Roux and McGeoch 2008a; Adams 2009).

1.1. Is oN an important N source for cold ecosystem and sub-Antarctic flora?

Recent decades have witnessed important paradigm shifts in our understanding of plant-soil-N interactions, with the ecological relevance of direct oN acquisition at the forefront (Aerts and Chapin 1999; Lipson and Näsholm 2001; Schimel and Bennett 2004). Although the physiological capacity for direct oN acquisition has been acknowledged for more than a century (e.g., Hutchinson and Miller 1912), only iN sources were assumed ecologically relevant for plant N nutrition until the late 20th century (Schimel and Bennett 2004). However, oN is now understood to be an ecologically relevant N source utilised by plants across a wide range of ecosystems from tundra to tropics (Kielland 1994; Andersen and Turner 2013; Moe 2013). Furthermore, the membrane transporters associated with oN acquisition have been identified (Miller and Cramer

2004; Tegeder and Rentsch 2010), and all plant species investigated have the physiological capacity for oN acquisition (Näsholm et al. 2009).

Despite the evidence for oN uptake, quantifying the extent to which oN is available and directly used by plants *in situ* is still relatively new and challenging (Jones et al. 2005a; Warren 2012, 2014). Direct (i.e., intact) oN acquisition is commonly measured with dual-labelled $^{13}C^{15}N$ amino acids, where intact uptake is determined by a positive regression between ^{13}C and ^{15}N in the plant tissue (Persson and Näsholm 2001a; Jones et al. 2005a). This method has the advantage of potentially allowing for the distinction between intact uptake and uptake following microbial mineralisation, which may occur if only ^{15}N oN isotopes are used. However, this is not without complications, with evidence that both the ^{13}C and ^{15}N can be acquired separately following mineralisation, and that ^{13}C is also respired rapidly following intact uptake; both cases resulting in false interpretation of the data (Warren 2012). Furthermore, although oN makes up a substantial component of soil N, only soluble oN is available to plants and our knowledge of the constituents, flux, and transformations within this N pool is limited (Schimel and Bennett 2004; Jones and Kielland 2012; Warren 2014). For example, free amino acids account for <5% of the total soluble oN pool, whereas proteins and peptides represent a substantially larger fraction (*ca*. 50%) in boreal forest soils (Jones and Kielland 2012). However, these measurements are complex, and results are known to vary with the techniques used. The methodological limitations to quantifying oN uptake and accurately describing soil oN constituents represent an important gap in our understanding of plant nutrition and ultimately terrestrial N cycling.

Plant oN acquisition is prevalent in soils with high oN and low iN concentrations (Jones et al. 2005b; Moe 2013). This is substantiated by evidence from gradients of fertility (i.e., iN availability) that show high oN uptake in otherwise infertile soils (Streeter et al. 2000; Weigelt et al. 2003; Kahmen et al. 2009). In cold systems, temperature-limited microbial mineralisation is often too low for plants to rely on iN, and there is evidence that direct oN acquisition accounts for a substantial proportion of plant N requirements (Kielland 1994, 2001; Lipson and Näsholm 2001). However, because evidence for the ecological relevance of oN to plant nutrition was largely unaccounted for in the 20^{th} century (Schimel and Bennett 2004), some literature from oN-replete ecosystems still assumes that plants only use iN. For example, despite soil oN contributing to >90% of the total N on MI (Smith 1978), none of the soil N cycling literature investigates plant oN acquisition (e.g., Smith 1977, 2008). Plant oN acquisition thus represents a relatively new but potentially important component to our understanding of plant-soil-N fluxes.

1.2. The ecological significance of oN acquisition under global change

Organic N acquisition is energetically beneficial to plants and may affect plant growth and biomass partitioning (Näsholm et al. 2009; Cambui et al. 2011). The uptake of an already reduced N compound and the simultaneous uptake of C with N confers an energetic benefit compared to iN acquisition, which has been linked to increased relative growth rates (RGR) (Franklin et al. 2017). Furthermore, oN relative to iN provision influences plant biomass allocation, although responses in plant root:shoot ratio may differ (e.g., Miller et al. 2008; Cambui et al. 2011). However, investigations into the effects of N-form on plant growth are largely based on agricultural plants in laboratory settings and may therefore have important but under-studied ecological implications for wild plants.

In addition to forming an important N-resource, there is evidence that different forms of iN and oN differentiate ecological niches *in situ*. For example, niche-partitioning by N-form has been shown between NO_3^- and NH_4^+ (Kahmen et al. 2006) and between iN and oN sources, including amino acids such as glycine, glutamine, lysine, and alanine (Weigelt et al. 2005; Miller et al. 2007a; Tian et al. 2020) and peptides such as tri-alanine (Kuster et al. 2016). However, the affects this may have on individual plants and indeed species coexistence, given the energetic differences between oN and iN acquisition (Franklin et al. 2017), remains largely unknown. Moreover, climate warming is hypothesised to influence soil decomposition rates, which may affect the relative concentrations of bioavailable oN and iN. For example, increased microbial mineralisation and concomitant iN increases have been linked to alterations in N-form uptake by grasses grown in competition, thus affecting ecological niche partitioning by N-form (Kuster et al. 2016). This may represent an important indirect effect of climate warming on plant growth, should different N-forms impact plant growth and species interactions. Therefore, determining the ecological effects of different N-form uptake at a community level, such as plant competition and co-existence, requires better knowledge of how oN relative to iN supply affects plant growth on an individual level.

1.3. Consequences of climate warming for plant growth in cold ecosystems

Plant productivity is intrinsically linked to soil processes, as plants meet the bulk of their nutritional requirements through root uptake from the soil. Under anthropogenic climate change, mean global temperatures have increased by > 1°C over the last century (IPCC 2022), and warming is predicted to elicit strong responses in cold ecosystem plant-soil interactions (Kirschbaum 1995). Soil decomposition processes involving microbes have higher temperature sensitivities to warming than plant productivity, and this sensitivity is higher in cold ecosystems (Kirschbaum 1995). Should the process of climate warming continue accelerating, soil decomposition processes at high latitudes may be increased by global change. Indeed, concerns that increasing temperatures in northern high latitudes, which hold significant C-stocks, will create positive feedback to climate warming through elevated soil respiration has attracted a wealth of scientific interest (e.g., Kirschbaum 2000, 2004b; Christensen et al. 2004; Hilasvuori et al. 2013; Pold and DeAngelis 2013; Tiemeyer et al. 2016; Lei et al. 2021). Terrestrial C-cycles are however intrinsically linked with N-cycles through the interplay of plant and soil processes, highlighting the importance of understanding the effects of warming on plant-soil interactions and not soil processes alone (Mack et al. 2004; Beier et al. 2008; Classen et al. 2015; Zou et al. 2022).

The high temperature sensitivity of soil decomposition processes has resulted in predictions for increased microbial activity and thus mineralisation rates, increasing soil [iN] (Kirschbaum 1995). Microbial activity is expected to increase due to direct alleviated temperature limitations as well as increased nutrient availability through warming-induced increases in extracellular enzyme activity (Wallenstein et al. 2009; Brzostek et al. 2012; Sistla and Schimel 2013; Classen et al. 2015). Considering the evidence for strong N-limitations to cold system primary productivity and higher temperature sensitivities of decomposition than productivity, there are predictions that the indirect stimulatory effects of warming, i.e., N-release, will outweigh the direct effects (Kirschbaum 1995). Indeed, there is evidence for increased iN release under warming, which has been linked to increases in plant productivity and N uptake (Rustad et al. 2001; Bai et al. 2013; Sistla et al. 2013; Salazar et al. 2020). Furthermore, recent long-term evidence shows larger increases in soil respiration at high latitude, cold ecosystems relative to temperate or tropical systems (Lei et al. 2021), as evidence for the early predictions.

In addition to the indirect effects of warming on plant productivity, warming directly influences plant growth and vegetation processes. There is evidence for alterations in plant phenology, trending towards earlier leaf emergence and flowering with warming at high latitudes (Bjorkman et al. 2020). Furthermore, vegetation composition changes with warming, although this likely interacts with below-ground processes (Rinnan et al. 2007; Elmendorf et al. 2012; Bjorkman et al. 2020). Soil warming directly influences plant root processes, such as significantly increasing root respiration which may influence N requirements, although this effect is largely transitory (Atkin et al. 2000). Experimental warming increases N uptake, which may arise from a combination of plant morphological and physiological responses, as well as soil responses such as increased N release (BassiriRad 2000). For instance, fine root biomass and length increase under warming, which may assist plant N capture (Yin et al. 2013). Furthermore, warming increases root capacity for nutrient uptake by influencing ion movement across the root and may alter root transport properties and thus plant preference for various N sources (BassiriRad 2000). These mechanistic responses are related to and affected by whole plant responses to warming, such as increased productivity and thus nutrient demand, plant N status, and root allocation (BassiriRad 2000; Yin et al. 2013). The direct warming responses interact with indirect responses such as increased soil bioavailable N and thus may amplify or curtail plant below-ground responses to warming. However, untangling the extent to which temperature directly or indirectly affects plant growth remains challenging.

Across the literature investigating cold ecosystem plant-soil interactions under warming, the wide diversity of systems explored provides an equally diverse record of plant and soil responses. While general trends across different latitudes show an increase in N availability with warming, this is not ubiquitous and some sites have negative or non-significant responses (e.g., sites in Rustad et al. 2001; Bai et al. 2013). Furthermore, study duration may have an important influence on the results reported. For example, increased microbial activity may be a transitory response that is only significant in the short term (Romero-Olivares et al. 2017). On the other hand, microbial biomass and community responses may only be significant in the long term, with evidence that they follow vegetation change; a slow process that takes years or decades (Rinnan et al. 2007). This wide diversity of soil decomposition responses leaves the potential indirect effects of warming on plant productivity unclear. However, there is little doubt that high latitude systems are sensitive to increasing temperatures, with evidence for northern high latitude vegetation change (e.g., Jia et al. 2003; Elmendorf et al. 2012), suggesting that while cold ecosystems respond to warming, the mechanisms driving this change remain unknown.

An important source of variation in the literature that arises from the strong representation of northern hemisphere systems is the effect of seasonality on experiment results. Northern high latitude sites typically experience extreme seasonality, with both extreme temperature ranges and photoperiod. This may be impacted by seasonal instead of year-round experimental manipulation in Arctic and sub-Arctic studies (Poppeliers et al. 2022). For example, although several long-term studies have imposed summer warming treatments (e.g., Campioli et al. 2012; Deslippe et al. 2012; Sistla et al. 2013), there is evidence for an increased frequency of extreme winter warming events with detrimental effects on the vegetation productivity and reproduction (Bokhorst et al. 2008, 2009; Bjorkman and Gallois 2020). For example, with 20 years of summer warming treatments, Sistla et al. (2013) documented increased woody biomass and thus increased winter snow trap, increasing winter soil temperatures. However, Bokhorst et al. (2008, 2009) show that extreme winter warming events cause snowmelt, exposing soils to freezing temperatures and ultimately leading to reductions in shrub growth. The prevalence of methodological variables that curtail potential stimulatory warming responses, such as seasonal experimental manipulation, emphasises the need for year-round experimental approaches. It may also indicate the value of the sub-Antarctic as a site for long-term climate experiments, where the thermo-stable climate removes confounding effects of extreme temperature ranges. The simple ecological system and biotic interactions, along with low seasonality, may allow for investigations into the mechanisms of plant and soil processes that drive ecosystem change.

1.4. Sub-Antarctic Marion Island

The current study was based on sub-Antarctic MI, the larger of the two Prince Edward Islands in the Southern Ocean. Marion Island experiences a low mean annual temperature of 6°C with a *ca*. 4°C seasonal range, 2 000 mm precipitation per annum, and gale force winds every one in three days (le Roux 2008). The soils are typically rich in organic matter, and the lowlands support a highly productive vegetation community compared to other tundra systems due to the extended growing season (Huntley 1972; Smith 1988; Smith and Steenkamp 1992a). Soils near the MI coastline have high salt content due to salt spray, and localised vertebrate colonies around the island have a large influence on the nutrient status (Smith 1978). The marine vertebrates include *Aptenodytes patagonicus* (King Penguin), *Eudyptes chrysolophus* (Macaroni Penguin), and *Eudyptes chrysocome* (Southern Rockhopper Penguin), and other seabirds such as *Diomedea exulans* (Wandering Albatross) and *Macronectes* sp. (Northern and Southern Giant Petrels), as well as pinnipeds *Arctocephalus gazella* (Antarctic Fur Seal), *Arctocephalus tropicalis* (Sub-Antarctic Fur Seal), and *Mirounga leonina* (Southern Elephant Seal). They contribute to allochthonous N (and other nutrient) input, which has large but localised effects on the nutrient status and vitality of the surrounding vegetation, with most of the nutrients lost to the sea in runoff (Smith 1978).

The vegetation of MI is relatively depauperate in vascular plant species, with only 21 native vascular plants (Chau et al. 2020) and 18 alien species, six of which are currently considered invasive (Greve et al. 2017). The four species investigated in the current study were *Polypogon magellanicus* (Lam.) Finot (previously known as *Agrostis magellanica*), *Poa cookii* (Hook.f.) Hook.f., *Agrostis stolonifera* L. and *Poa annua* L. Both *P. magellanicus* and *P. cookii* are native, while *A. stolonifera* and *P. annua* were anthropogenically introduced and are invasive (Gremmen and Smith 2004; Greve et al. 2017). *Poa cookii* is a tussock-forming, highly nitrophilous species that commonly occurs in areas influenced by vertebrates on the island, including the coastline and further inland proximal to Procellariidae (petrel or prion) burrows (Smith 1976; Gremmen and Smith 2004). *Polypogon magellanicus* is a tussock-forming, generalist species more widespread across the island. It is a common coastal and mire species but also found further inland on fellfield to the edge of the polar desert where it grows epiphytically on *Azorella selago* Hook.f. cushions (Gremmen and Smith 2004). It is distributed across most sub-Antarctic islands but also occurs in New Zealand, Chile, and Peru (Govaerts et al. 2021). *Agrostis stolonifera* is a stoloniferous, invasive species that was first recorded in 1965 and has since had major impact on the island, having spread along the coastline and where there has been human activity (le Roux et al. 2013; Greve et al. 2017). It is native to parts of Europe, Asia, and north Africa, but invasive on all other continents except for Antarctica (Govaerts et al. 2021). *Poa annua*, another stoloniferous and invasive species, was first recorded on MI in 1948 but was likely introduced by sealers in the 1800s and has also spread along the coastline (le Roux et al. 2013; Greve et al. 2017). It is native to parts of Africa, Europe, and Asia, but is invasive on all other continents including Antarctica (Molina-

Montenegro et al. 2019; Govaerts et al. 2021). All four grasses co-occur along sections of the MI coastline.

Marion Island and other sub-Antarctic islands represent a gap in the literature regarding plant N uptake and growth under global change. Descriptions of sub-Antarctic terrestrial N-cycling are based on early work that assumes plants do not access oN (see Smith 1977, 2008). Should sub-Antarctic MI flora have the capacity for oN uptake, sub-Antarctic soil N-cycling literature may have underestimated the amount of plant-available N. Moreover, MI has experienced substantial climate change, including decreased rainfall by *ca*. 27% over 50 years (1950-2000) and increased temperatures, with an increased frequency of hourly temperatures rising over 10°C but decreased frequency falling below 0° (le Roux and McGeoch 2008a; Ripley et al. 2020). While the effects of warming on sub-Antarctic plant-soil interactions have not yet been assessed, there is some evidence for increased microbial responses to warming (Yergeau et al. 2012), and for higher photosynthetic responses by invasive than native grasses to increasing temperature (Ripley et al. 2020). Furthermore, there is evidence for upward vegetation range expansion on the island by 3.4 m yr^{-1} with 1.2°C warming (le Roux and McGeoch 2008b), indicating that sub-Antarctic vegetation communities have been affected by the documented increases in island temperature.

1.5. Hypothesis and thesis outline

This thesis investigated the forms of N that are acquired by sub-Antarctic grasses, and how plant growth and soil N are affected by warming. I hypothesised that sub-Antarctic grasses acquire oN which affects plant growth relative to iN, and that soil warming influences plant growth directly and indirectly through stimulating microbial iN mineralisation and alters plant-available oN and iN. To test this hypothesis, the thesis was divided into two data chapters.

Chapter 2 investigated the sub-hypotheses that sub-Antarctic grasses acquire oN under hydroponics conditions and that oN relative to equimolar iN supply affected plant growth. Furthermore, I hypothesised that the grasses acquire oN *in situ*, but that oN acquisition would be higher at sites with low soil [iN].

Chapter 3 investigated the sub-hypotheses that soil warming affects plant growth directly and indirectly due to increased iN-release with microbial activity and that increased soil iN would affect the N-form acquired by sub-Antarctic grasses. I also hypothesised that soil warming would increase soil iN and PO_4^{3-} and microbial biomass and nutrient immobilisation, due to stimulatory effects on microbial activity.

Chapter 2

Evidence for organic N acquisition by sub-Antarctic grasses

2.1. Abstract

Organic N (oN, e.g., amino acids) represents an important resource for plants in soils replete with oN but not inorganic N (iN: NH_4^+ and NO_3^-). However, literature from the oN-replete sub-Antarctic assumes that plants only acquire iN, potentially underestimating plant-available N. I hypothesised that sub-Antarctic Marion Island (-46.90, 37.75) grasses (*Polypogon magellanicus*, *Poa cookii*, *Agrostis stolonifera* and *Poa annua*) acquire oN and that N-form affects plant growth. To determine oN acquisition, grasses were supplied with ^{15}N-glycine or $^{15}N\text{-}NO_3^-$ in hydroponics. Two hydroponics experiments measured plant growth on oN and iN, one with 6 mM-N treatments (NO_3^-, NH_4NO_3, and three glycine:NO_3^- ratios) in nutrient-poor mire water, and another with 4 mM-N or 0.4 mM-N as glycine or NO_3^- in nutrient-rich Long Ashton solution. Furthermore, ^{15}N-glycine, $^{15}N\text{-}NO_3^-$, or $^{15}N\text{-}NH_4^+$ were supplied to grasses at three field sites along an increasing [oN] gradient. Sub-Antarctic grasses acquired oN, with higher uptake rates (nmol g^{-1} s^{-1}) and root $\delta^{15}N$ enrichment with glycine- than NO_3^--supplied plants. The Long Ashton experiment had lower relative growth rates (RGR) on glycine than NO_3^-, but N-form or [N] resulted in species-specific root:shoot ratios. The mire experiment had consistently low RGR and root:shoot ratio on oN and NH_4NO_3, suggesting that [nutrient] influences growth responses under high [N]. Field glycine-supplied plants at high [oN] sites had higher $\delta^{15}N$ enrichment than those at high [iN] sites. This study demonstrates the importance of investigating plant oN acquisition in regions it is unaccounted for and shows that sub-Antarctic soil N-cycling requires re-evaluation.

2.2. *Introduction*

Plant productivity is a key component to the terrestrial N-cycle, thus identifying plant-available N sources influences our understanding of it. Until the late 20th century, the soil N cycle was understood to be regulated by microbial mineralisation of soil organic matter (SOM) (Schimel and Bennett 2004). Most literature assumed inorganic N (iN; i.e., NH_4^+ and NO_3^-) was the only N source ecologically relevant for plant acquisition (e.g., van Cleve and Alexander 1981; Smith and Steenkamp 1992a), despite the capacity for direct organic N (oN, e.g., free amino acids) acquisition being long established (e.g., Hutchinson and Miller 1912; Virtanen and Linkola 1946; Wright 1962). However, instead of microbial mineralisation, the soil N-cycle is largely driven by the depolymerisation of SOM by extracellular enzymes, releasing oN monomers (such as free amino acids) for acquisition by both plants and microbes (Schimel and Bennett 2004). This challenges two important assumptions in N-cycling literature. First, that microbes are superior competitors for oN compared to plants due to rapid growth rates, higher substrate affinity, and large surface-area:volume ratios (Hodge et al. 2000). There is evidence that plants compete successfully with microbes for oN due to the spatiotemporal heterogeneity of soil N (Lipson and Monson 1998; Hodge et al. 2000; Schmidt et al. 2002; Bardgett et al. 2003). Secondly, and the focus of this study, that plants rely on iN pools to meet N requirements. The potential for oN acquisition is particularly relevant in cold ecosystems, where temperature-limited iN mineralisation results in higher fractions of bioavailable oN than iN (Kielland 1994; Atkin 1996). Therefore, investigations into cold systems that only considered plant iN uptake may have greatly underestimated plant-available N, with important implications for describing and quantifying plant-soil N fluxes.

There is widespread evidence for plant oN acquisition across a range of ecosystems. For example, oN acquisition has been documented in Arctic tundra (Kielland 1994, 2001; Nordin et al. 2004), temperate grasslands (Streeter et al. 2000; Weigelt et al. 2003), temperate forests (Finzi and Berthrong 2005), boreal forests (Nordin et al. 2001), and tropical montane forests (Andersen and Turner 2013). Furthermore, plant oN acquisition is most prevalent in soils replete in oN, with evidence for intact acquisition of amino acids, such as glycine, as well as larger oN molecules such as peptides (Jones et al. 2005b; Kahmen et al. 2009; Moe 2013; Wilkinson et al. 2015; Tian et al. 2020). In cold ecosystems, temperature-limited microbial iN release through mineralisation results in soils that are typically replete in oN but not iN (Kielland 1995). Indeed, cold ecosystem flora, such as that in the Arctic and sub-Arctic, meet the bulk of their N requirements through oN acquisition (Atkin 1996; Kielland 2001; Persson and Näsholm 2001b; Näsholm et al. 2009).

Organic N acquisition has energetic advantages over iN for plant nutrition, and there is evidence that N-form provision affects plant growth. Despite the energetic cost of pH regulation with the uptake of amino acid zwitterions (molecules with both positively and negatively charged groups), the simultaneous uptake of C with N provides a net assimilation advantage to oN acquisition: the so-called "C-bonus" (Zerihun et al. 1998; Franklin et al. 2017). Additionally, oN sources exist in an already reduced state, eliminating the energetic costs of a reduction step as is necessary for NO_3^- (Miller et al. 2007b). Moreover, oN relative to iN provision may influence plant relative growth rate (RGR) and biomass allocation. For example, oN (6 mM glutamine) relative to equimolar iN (NO_3^- and NH_4NO_3) increases *Arabidopsis thaliana* (L.) Heynh. RGR and root fractions due to increased N use efficiency (NUE) and the low diffusibility of oN compounds in soil, respectively (Cambui et al. 2011; Franklin et al. 2017). However, amino acid application may decrease root growth, with suggestions that high external oN signals sufficient N availability, eliminating the need for root proliferation (Walch-Liu et al. 2006; Miller et al. 2007b; Hassan et al. 2020). Furthermore, [oN] may influence these responses, with evidence for low [oN] increasing *A. thaliana* root development but high [oN] inhibiting it (Lonhienne et al. 2014). The type (e.g., amino acids, NH_4^+ or NO_3^-) and concentration of N and their effects on plant growth and allocation have been widely studied. Most of the focus has, however, been on agricultural species (e.g., Padgett and Leonard 1996; Näsholm et al. 2001; Tian et al. 2008; Hassan et al. 2020) or the model plant *A. thaliana* (e.g., Cambui et al. 2011; Lonhienne et al. 2014; Kiba and Krapp 2016). With less literature investigating how N-forms or [N] affect wild plant growth, little is known of the ecological significance of oN or iN uptake such as how it affects plant growth traits, competition, or co-existence *in situ*.

Despite the wealth of literature on plant oN uptake and its importance in northern high latitudes, oN acquisition by sub-Antarctic vegetation has been largely overlooked, with one exception from Macquarie Island (see Schmidt and Stewart 1999). This represents a substantial gap in the literature for southern cold ecosystems. Sub-Antarctic soils are highly organic, with low [iN] unless proximal to large vertebrate colonies (e.g., seabirds or pinnipeds; Smith 1978; Erskine et al. 1998). For example, soil oN on sub-Antarctic MI make up *ca*. 99.9% of total soil N (20 mg g^{-1} oN and <

0.1 mg g^{-1} iN, Table S1) if uninfluenced by vertebrates (Smith 1978). Such high oN fractions are similar to Arctic system soils (Nadelhoffer et al. 1992) but larger than other terrestrial systems, e.g., oN makes up 4.8% of total N in a temperate grassland (Oelmann et al. 2007). Moreover, Smith and Steenkamp (1992a) found that plant N requirements of a sub-Antarctic mire-grassland community during the growing season (158 mg N m^{-2} d^{-1}; Smith 1988) would not be met by the low iN mineralisation rates (net mineralisation 48 mg N m^{-2} d^{-1}). At the time, under the assumption that only iN was relevant for plant nutrition, iN excretion by soil macrofauna was assumed to account for this discrepancy (Smith and Steenkamp 1992b, c, 1993; Smith 2008). However, increased predation pressure by the invasive *Mus musculus* (house mouse) has diminished large invertebrate populations, presumably reducing their roles in nutrient cycling (Crafford 1990; Bergstrom and Chown 1999; Smith 2008; McClelland et al. 2018). MI thus represents an ecosystem with high oN and low microbial activity, and while invertebrates may contribute to gross N release, the role of oN in plant nutrition remains unaccounted for.

This study investigated oN uptake and the effects of oN vs iN provision on plant growth in four MI graminoid species, *Polypogon magellanicus* (Lam.) Finot, *Poa cookii* (Hook.f.) Hook.f., *Agrostis stolonifera* L. and *Poa annua* L. I hypothesised that MI grasses have the capacity for oN uptake, that oN influences RGR and biomass partitioning, and that the grasses acquire oN *in situ*, predicting that oN acquisition would be highest in soils with high oN and low iN. The capacity for potted and *in situ* oN and iN uptake was determined by providing plants with ^{15}N labelled oN (glycine) and iN (NO_3^- and NH_4^+). The effects of oN and iN on plant growth and biomass partitioning were explored in two hydroponics experiments.

2.3. Methods and Materials

2.3.1. Study site and species

The four graminoid species investigated in this study, *P. magellanicus*, *P. cookii*, *A. stolonifera*, and *P. annua*, are commonly found on sub-Antarctic MI and co-occur along the coastline. For all hydroponics experiments, mature grass clumps were collected within 100 m of each other on the east coast of the island (-46.88, 37.86) and tillers were separated and planted from these clumps. Three of the four experiments presented in this study took place on MI, two hydroponics and one field based. The fourth experiment took place in a controlled environment growth chamber at the University of Cape Town, South Africa.

For the MI hydroponics experiments, the grasses were planted in 1 L mire water that had been collected from a large mire on the east coast of the island (-46.876, 37.857), which had *P. magellanicus* and other vascular plants growing in it. Mires cover *ca*. 50% of the island below 300 m above sea level and thus contribute to a substantial fraction of the island's annual productivity (Smith 1988; Smith et al. 2001). These experiments were conducted outdoors under natural light and temperature but sheltered from high winds near the main base over the growing season of 2019-2020. The hydroponics experiment conducted in South Africa used grasses collected from the same site as the MI experiments, and grasses were grown in Long Ashton (LA) nutrient solution (Hewitt, 1966).

The field experiment investigated *in situ* uptake of glycine, NO_3^-, and NH_4^+ at three sites on the east coast of MI. The sites had varying degrees of vertebrate influence (thus allochthonous iN input) and were characterised according to the descriptions in Smith et al. (2001) as 'Biotic' for the site with high animal influence near the beach, 'Slope' for the site along a fern slope-complex, and 'Fellfield' for the site on a grey-lava ridge (Fig. 1; Table S2). All four species were present within 30 m of each other at the 'Biotic' site, which had high vertebrate influence by seabirds such as *Macronectes giganteus* (Southern Giant Petrel); *Diomedea exulans* (Wandering Albatross); *Aptenodytes patagonicus* (King Penguins), and *Eudyptes chrysocome* (Southern Rock Hopper Penguins) and pinnipeds *Mirounga leonina* (Southern Elephant Seals) and *Arctocephalus gazella* (Antarctic Fur Seals). This site was mostly covered by the four grasses, bryophytes, and the herb *Leptinella plumosa* Hook.f. The 'Slope' site had only *P. cookii* present, and there were inactive Procellariidae (petrels and prions) burrows, thus it represented an intermediately vertebrate-influenced site (Smith 1976). The slope was dominated by the fern, *Austroblechnum penna-marina* (Poir.) Gasper & V.A.O.Dittrich, with some *P. cookii* tussocks. The 'Fellfield' site had *P. magellanicus* and *P. annua* present and had no visible vertebrate influence, although it may have been frequented by mice. This site was sparsely vegetated, with some *Azorella selago* Hook.f. cushions, cushion bryophytes, *P. magellanicus* and *P. annua* individuals (Table S2).

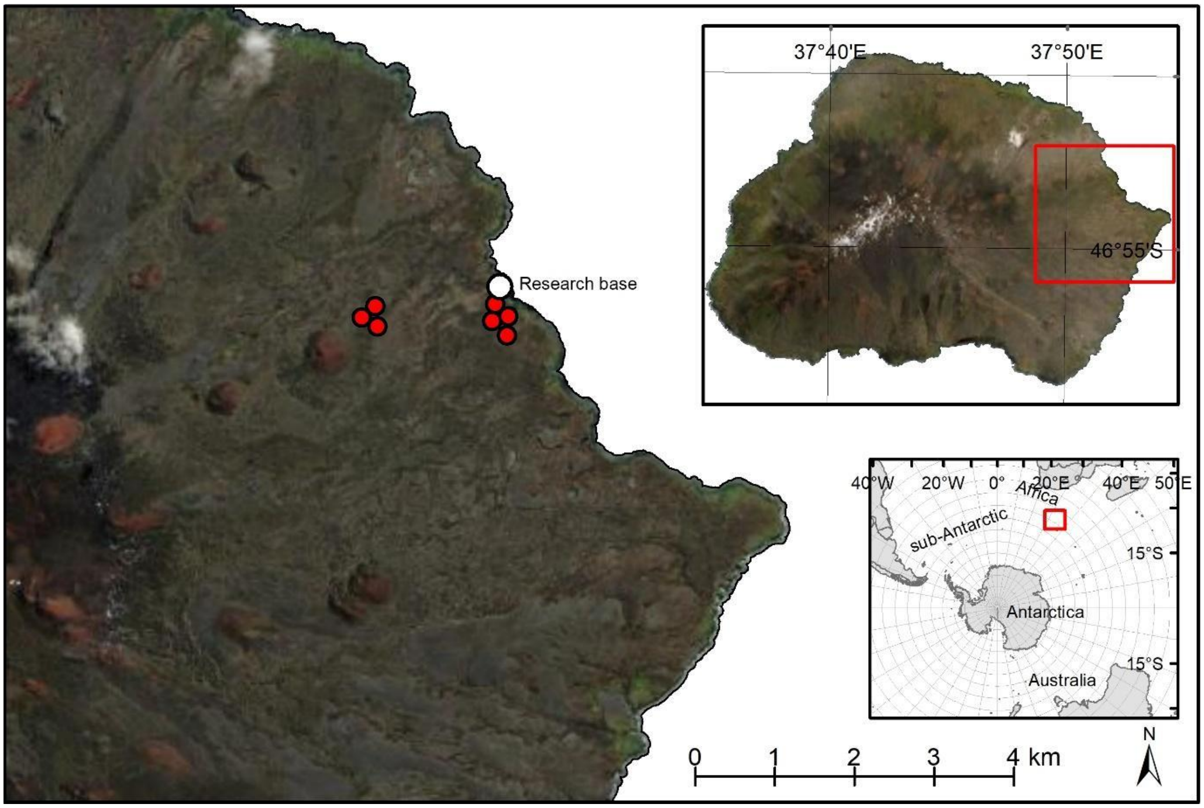

Fig. 1: Map of sub-Antarctic Marion Island. The white point shows the research base on the east coast, and the red points show the locations where ^{15}N enriched oN and iN were provided to the four grasses. The points near the research base are for *P. magellanicus*, *P. cookii*, *P. annua* and *A. stolonifera* and the points further inland near the base of a scoria cone (Junior's Kop) are for *P. magellanicus* and *P. annua* on a grey-lava outcrop and *P. cookii* on a slope *ca*. 150 m from the grey lava.

2.3.2. Hydroponics experiments: uptake of and plant growth on oN and iN

To determine whether sub-Antarctic grasses have the capacity for oN uptake, one mature, non-flowering tiller of each species was grown in 1 L mire water with 6 mM $NaNO_3$ (n = 12 per species). Grasses were grown in this treatment for five weeks at the end of the growing season (March - April 2020), with water change and nutrient replenishments every two weeks. Following five weeks of growth, plants (n = 6 per species) were supplied with either 6 mM ^{15}N-glycine (98% ^{15}N, Cambridge Isotope Laboratories, Andover, MA, USA) or ^{15}N-$NaNO_3$ (98% ^{15}N, Cambridge Isotope Laboratories). The ^{15}N solutions consisted of 20 mL 10.5 µM glycine and 20 mL 10.5 µM

NO_3^-, one of which was isotopically distinct (^{15}N). Unenriched (natural abundance) $\delta^{15}N$ values were obtained from plants grown in 6 mM $NaNO_3$ in the mire growth experiment, to determine whether 'enriched' treatments significantly increased $\delta^{15}N$ following ^{15}N provision. One leaf was harvested at 12 h, and the whole plant was harvested at 24 h following ^{15}N supply.

To investigate growth parameters on different N sources in mire water, plants (one tiller per pot of 1 L mire water, n = 5 replicates per species X treatment) were grown in five 6 mM-N treatments with water changes and nutrient replenishment every two weeks. The treatments were 1) 3 mM NH_4NO_3, 2) 6 mM $NaNO_3$, 3) 4 mM $NaNO_3$ + 2 mM glycine, 4) 3 mM $NaNO_3$ + 3 mM glycine, and 5) 2 mM $NaNO_3$ + 4 mM glycine, following Cambui et al. (2011). At every water change, fresh plant biomass was recorded to determine RGR, which was calculated as the difference between the mean natural logarithm-transformed plant weights at the start and end of the experiment divided by the number of days the experiment ran (Hoffmann and Poorter 2002). The plants were harvested at 14 weeks, with roots and shoots separated. One leaf was harvested from each plant for leaf [N] analyses. During this experiment, 12 replicates died, resulting in low replication for some treatments (see Fig. S1). Furthermore, low RGR resulted in a few replicates without a leaf suitable for N analysis, further decreasing replication (Fig. S1). The mire used to supply the growth solution for the two MI hydroponics experiments was sampled on six occasions during the experiments, and frozen at -20°C until submission to the Elsenberg Laboratory (Western Cape Department of Agriculture, Stellenbosch, South Africa) for water chemistry analyses, including elemental concentrations (iCAP 7000 ICP OES, Thermo Fisher, UK), NH_4^+ and NO_3^- concentrations (RQflex10 reflectometer, Merck, Germany), and pH.

The LA hydroponics experiment investigated plant growth, biomass partitioning, and N uptake rates on different [N] (4 mM or 0.4 mM) and N-forms (glycine or NO_3^-). Plants (n = 6 per species) were grown in 1 L LA solution (4 mM $CaCl_2$, 2 mM K_2SO_4, 1.1 mM $MgSO_4$, 0.14 mM H_3BO_3, 90 µM Fe-EDTA, 20.8 µM $MnSO_4$, 3.3 µM $CuSO_4$, 2.3 µM $ZnSO_4$, 0.25 µM Na_2MoO_4, and a phosphate buffer (pH 5.5) of 1.87 mM NaH_2PO_4 and 0.13 mM Na_2HPO_4), modified from Hewitt (1966). This experiment was run in a controlled growth chamber with a 12/12 light-dark cycle, and PAR ca. 250 µmol m^{-2} s^{-1}, at 10°C. A pilot experiment showed that 4 mM glycine in LA solution formed a precipitate within 12 h due to changes in solution pH. To avoid this, an asynchronous design was adopted, where plants were transferred between 1 L LA solution and 1

L 2 mM $CaCl_2$ + 4/0.4 mM-N every 24 h, maximising time for nutrient acquisition but minimising movement and thus potential root damage. Every eight days, nutrients were replenished and plants were weighed for RGR determination. At 40 d, plants were left in the respective N treatments under constant light and 1 mL solution was sampled every 12 h for 48 h, to determine N uptake rates. To account for water loss through evapotranspiration, initial and final solution volumes were incorporated into uptake rate calculations. Plants were then harvested.

All leaves harvested for $\delta^{15}N$, or N analyses were the second fully expanded leaf from the apical meristem. Harvested plants were dried for 48 h at 70°C, and the dry weights used to calculate root:shoot ratios.

2.3.3. oN and iN uptake in situ

To determine whether plants access oN *in situ* and whether this was influenced by soil [oN] and [iN], plants were provided with ^{15}N-glycine, ^{15}N-NO_3^-, or ^{15}N-NH_4^+ ($^{15}NH_4Cl$, 98 % ^{15}N, Cambridge Isotope Laboratories, Andover, Ma, USA). One leaf (n = 10 per species per site) was sampled 24 h before enrichment to determine unenriched $\delta^{15}N$ (i.e., natural $\delta^{15}N$ abundances). ^{15}N treatments were added to plants (n = 10) at each site between 8 and 10 am, and one leaf harvested 24 h later. Each labelling solution (60 mL) comprised of 20 mL 10 µM glycine, 20 mL 10 µM NO_3^- and 20 mL 10 µM NH_4^+, one of which was isotopically distinct (i.e., contained ^{15}N). All leaves harvested for $\delta^{15}N$ analyses were the second fully expanded leaf, were dried for 48 h at 70°C, and stored at room temperature until $\delta^{15}N$ analyses. Enrichment by the respective ^{15}N source was determined if leaf $\delta^{15}N$ following ^{15}N provision was significantly higher than the unenriched $\delta^{15}N$ from the same species at each respective site.

2.3.4. Colorimetric assays of glycine and NO_3^-

Colorimetric assays were used to determine [glycine] and [NO_3^-] in the LA growth experiment nutrient solution, to calculate N uptake rates. NO_3^- was measured using the method described by Doane and Horwáth (2003), where VCl_3 reduces NO_3^- to NO_2^-, which is captured by Griess reagents (N-1-naphthylethylenediamine dihydrochloride and sulphanilamide). Glycine was measured according to Moore and Stein (1954), where NH_2-containing groups are reacted with a ninhydrin reagent. In samples without glycine addition, there were negligible amounts of NH_2-containing groups, and therefore no correction was carried out for the ninhydrin protocol. Absorbance of colorimetric reactions was measured with a ThermoSpectronic spectrophotometer (Helios Epsilon model, Thermo Scientific, USA) at 540 and 570 nm for NO_3^- and glycine, respectively.

2.3.5. *Leaf $\delta^{15}N$ and leaf [N] analyses*

For $\delta^{15}N$ analyses, plant leaves were ground to a fine powder using a ball mill (MM200, Retsch, Germany), and 2 mg of each sample weighed into tin capsules. The tin capsules were combusted in a Flash 2000 organic elemental analyser (Thermo Scientific, Germany), and the gasses passed into a DELTA V Plus IRMS (isotope ratio mass spectrometer) via a ConFlo IV gas control unit. Results were calibrated according to in-house standards. Leaf [N] for the LA experiment was also measured using this method. Leaf [N] for the mire experiment was measured with this method or with the Flash EA 1112 Series (Thermo Scientific, Germany), which uses a similar method of combustion and gas chromatography. Here, 5 mg of each sample was weighed into tin capsules, combusted in a high-temperature reactor, the gasses were separated by retention rates in a chromatograph column, and results calibrated according to in-house standards.

2.3.6. *Statistical analyses*

All statistical analyses were performed using R statistical software, version R.4.1.1 (R Core Team, 2021). Data were analysed with an Analysis of Variance (ANOVA), testing for interacting effects between species and the respective fixed effects in each experiment (i.e., plant tissue or site and N-form in the ^{15}N enrichment experiments; N-form and [N] for the mire and LA growth experiments). Where analyses showed evidence of significant effects ($p < 0.05$), post-hoc tests were performed in the ‘emmeans’ package (Lenth 2023), calculating Tukey Honest Significant Differences. For the LA and field experiments, evidence for interactions and subsequent post-hoc tests on the three-way ANOVAs resulted in complicated outputs for graphical interpretation. Because the interaction indicated species-specific responses to the treatments, an ANOVA was run for each species separately to aid interpretation. To determine the specific uptake rates over 48 h in the LA growth experiment, linear models were fitted to the solution [N] (glycine or NO_3^-) over time. Uptake rates were considered detectable if there was a significant relationship ($p < 0.05$) between time and [N], and if significant, the slopes from these models were used to calculate uptake rates per gram of plant biomass (nmol g^{-1} s^{-1}). These were then analysed with an ANOVA. Where further investigations into *a-priori* hypotheses were relevant (i.e., in the mire growth experiment, to examine overall differences between iN and oN treatments, and for iN and oN uptake rates across all species in the LA experiment), contrasts were performed using the 'emmeans' package. All ANOVAs were performed in the 'car' package (Fox and Weisberg, 2019). All residuals were checked to conform to model assumptions, and where they did not, the data were log-transformed to meet normality and homoscedasticity assumptions.

2.4. Results

2.4.1. Hydroponics experiments: uptake of and plant growth on oN and iN

Plants took up both ^{15}N-glycine and ^{15}N-NO_3^- in hydroponics, and there were species-specific differences in the $\delta^{15}N$ enrichment of these N-forms (Fig. 2a, b). *Agrostis stolonifera* and *P. annua* had significantly higher $\delta^{15}N$ in the glycine- and NO_3^--supplied plants relative to plants unenriched with ^{15}N and had significantly higher $\delta^{15}N$ enrichment with glycine- relative to NO_3^--supply (Fig. 2a). However, *P. magellanicus* and *P. cookii* showed no evidence for significant increases in $\delta^{15}N$ in either glycine- or NO_3^--supplied plants. There was evidence for a significant statistical three-way interaction between species, treatment, and plant tissue ($p < 0.001$) for root and shoot $\delta^{15}N$, signifying species-specific uptake (Fig 2b). All species had high $\delta^{15}N$ in both roots and shoots, suggesting that all species acquired the ^{15}N sources over 24 h, and that there was transport (i.e., from root to shoot) of the two ^{15}N treatments (Fig 2b). Compared to the unenriched leaf $\delta^{15}N$, with a mean of 9 ± 0.4‰ (mean ± SE), root and shoot $\delta^{15}N$ was high for NO_3^--provided plants with means of 173 ± 44‰ and 220 ± 51‰ (respectively), and higher in glycine-provided plants with means of 1543 ± 153‰ and 394 ± 66‰ (respectively). Root $\delta^{15}N$ in glycine-provided plants was significantly higher than NO_3^--provided plants for all species except *P. cookii* (Fig. 2b).

In the mire growth experiment, there was no evidence for an interaction between species and N-treatment for RGR, root:shoot ratio, or leaf [N]. There was however evidence for a significant effect of species ($p < 0.05$, Fig. S1) and of N-treatment ($p < 0.05$, Fig 3) for these three variables. Both RGR and root:shoot ratios were significantly higher in the iN relative to the oN treatments (contrasts: $t = 6.67$, $p < 0.001$ for RGR; $t = 8.64$, $p < 0.001$ for root:shoot ratio; Fig. 3a, b). Furthermore, there was a strong difference in root growth between the oN and the NO_3^- treatments, where NO_3^- resulted in the growth of new roots, whereas most replicates in the oN treatments had little to no new root growth (Fig. 3d). Leaf [N] was significantly lower in the NO_3^- treatment compared to the highest oN treatment (1:2 NO_3^-:glycine; Fig. 3c). When compared to the other four treatments, leaf [N] was significantly lower in the NO_3^- treatment (contrast: $t = -3.23$, $p = 0.002$); the treatment that had the highest RGR and root:shoot ratios.

In the LA growth experiment, there was no significant interaction between treatments and species for plant RGR. However, there was evidence for an effect of N-form ($p < 0.05$), where the NO_3^- treatment resulted in higher RGR than glycine, and an effect of species ($p < 0.001$), where *P. annua* had significantly higher RGR than the other three species (Fig 4a). Throughout the N-uptake experiment, a decrease in [N] in the 4 mM treatment solutions was only detected for *P. annua* ($p < 0.05$; Fig. S2), whereas the other three species showed no detectable N loss over time. Uptake rates were thus only calculated and analysed for the 0.4 mM treatments. There was a significant interaction between species and N-treatment in specific N uptake rates ($p < 0.05$; Fig. 4b), with evidence for a significantly higher uptake rate for glycine than NO_3^- by *A. stolonifera*. When uptake rates between the glycine and NO_3^- treatments were contrasted across all species, there was evidence for an overall higher uptake of glycine (contrast: $t = 3.28$, $p = 0.002$; Fig. 4b).

The LA growth experiment resulted in a significant interaction between treatments and species for root:shoot ratio and leaf [N] (Fig. S3). To aid visual interpretation, species were analysed with separate ANOVAs for these two variables. Two replicates of *P. cookii* had the main, large tiller die during the experiment in the 4 mM NO_3^- treatment, which caused excessive variation in root:shoot ratio due to the low mass of the emerging tillers (Fig. S4), and thus the two replicates were excluded from the analysis. N-form affected *P. magellanicus* root:shoot ratio, which was significantly higher on NO_3^- relative to glycine ($p < 0.05$), whereas leaf [N] was influenced by an interacting effect of N-form and [N] ($p < 0.05$), with high leaf [N] in the 4 mM NO_3^- treatment.

Poa cookii showed evidence for an interacting effect of [N] with N-form in both root:shoot ratio and leaf [N], ($p < 0.05$) with the highest values for these variables in the 4 mM NO_3^- treatment (Fig. 5a). Contrastingly, *P. annua* showed evidence for strong effects of [N] on both root:shoot ratio and leaf [N] ($p < 0.001$), which were significantly higher in the 4 mM N treatment, irrespective of N-form. While there was no significant difference in *A. stolonifera* root:shoot ratios, there was evidence for an interacting effect of [N] and N-form on leaf [N] ($p < 0.05$), with higher leaf [N] in the 4 mM relative to the 0.4 mM treatments (Fig. 5a, b).

The composition of the mire water was compared to that of the LA solution. Mire water had low concentrations of macro- and micro-nutrients (typically < 0.1 mM; Table S3) except for Cl^- and Na^+, possibly from sea spray. Natural [NH_4^+] and [NO_3^-] were only 0.02 and 0.05 mM, respectively. The nutrient concentrations in the LA solution were much greater than those in mire water (Table S3).

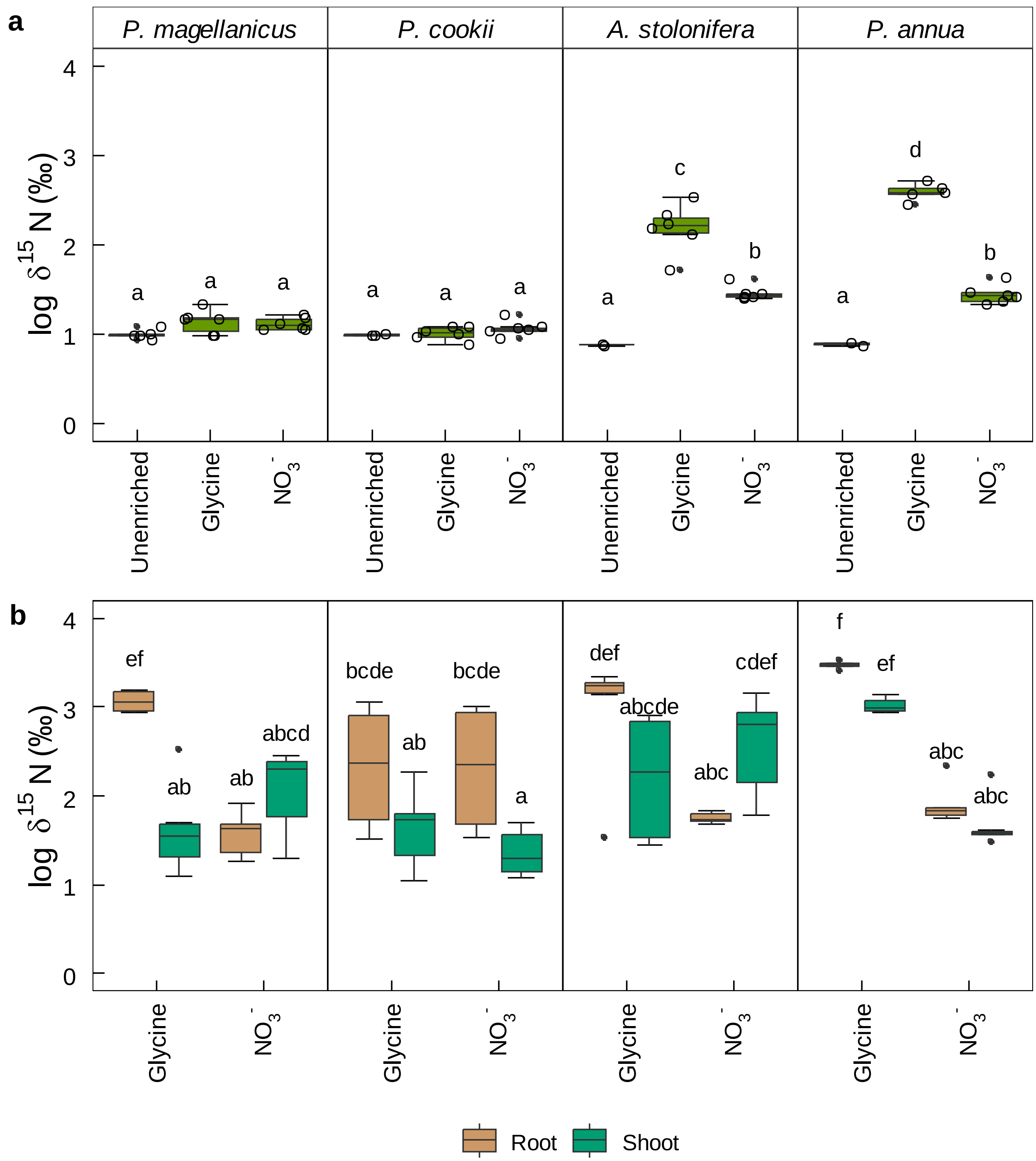

Fig. 2: $\delta^{15}N$ of the four study species supplied with ^{15}N-glycine or ^{15}N-NO_3^-. **a**) $\delta^{15}N$ 12 h after ^{15}N enrichment in either unenriched leaves, or leaves supplied with ^{15}N-glycine or ^{15}N-NO_3^-. There was a significant interaction between species and N treatment ($F_{6, 47} = 53.04$, $p < 0.001$). Points are included to indicate the replication. **b**) $\delta^{15}N$ 24 h after ^{15}N enrichment with either ^{15}N-glycine or ^{15}N-NO_3^-. There was a significant interaction between species, plant tissue, and N-form ($F_{3, 79}$

= 7.83, $p < 0.001$). Data were plotted on a log scale due to large variation, to aid visual interpretation. Letters indicate significant differences between treatment and species from an emmeans post-hoc test at the $\alpha = 0.05$ significance level. Green boxplots show the shoot and leaf $\delta^{15}N$, and brown boxplots the root $\delta^{15}N$.

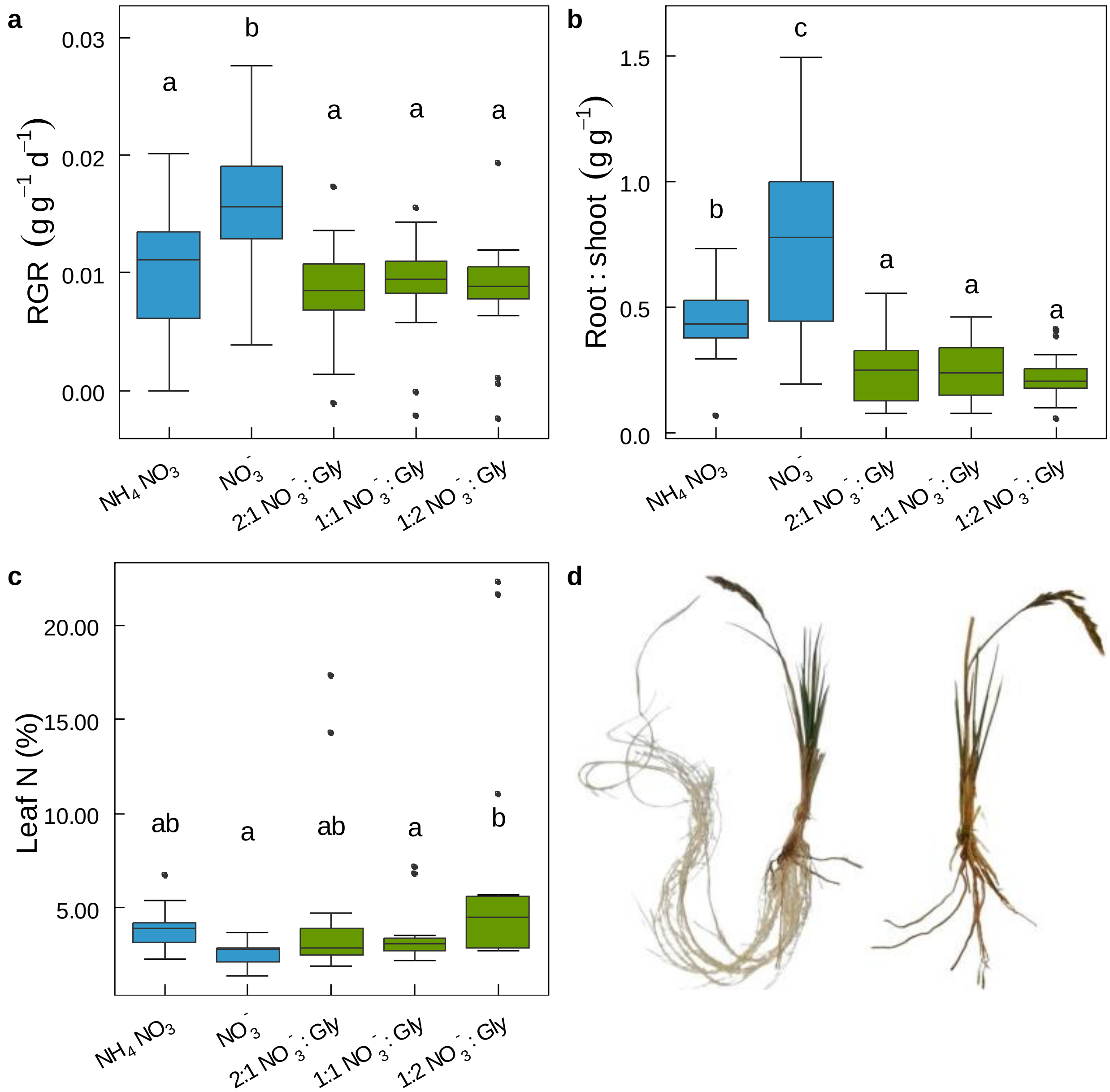

Fig. 3: Growth variables for all study species grown in five N treatments in the mire growth hydroponics experiment. **a** Relative growth rate (RGR), **b** root:shoot ratio, **c** leaf [N] concentration, and **d** a comparison of root growth in two replicates of *P. magellanicus* at NO_3^- (left) and 1:2 NO_3^- :glycine (right). There was a significant difference in RGR, root:shoot ratios, and leaf [N] between the five N-treatments ($F_{4,\,80} = 14.85$, $p < 0.001$; $F_{4,\,80} = 20.46$, $p < 0.001$; $F_{4,\,70} = 4.87$, $p = 0.002$, respectively). Letters indicate significant differences between N-treatments from an emmeans post-hoc test at the $\alpha = 0.05$ significance level. Blue boxplots show the iN treatments, and green boxplots the oN treatments.

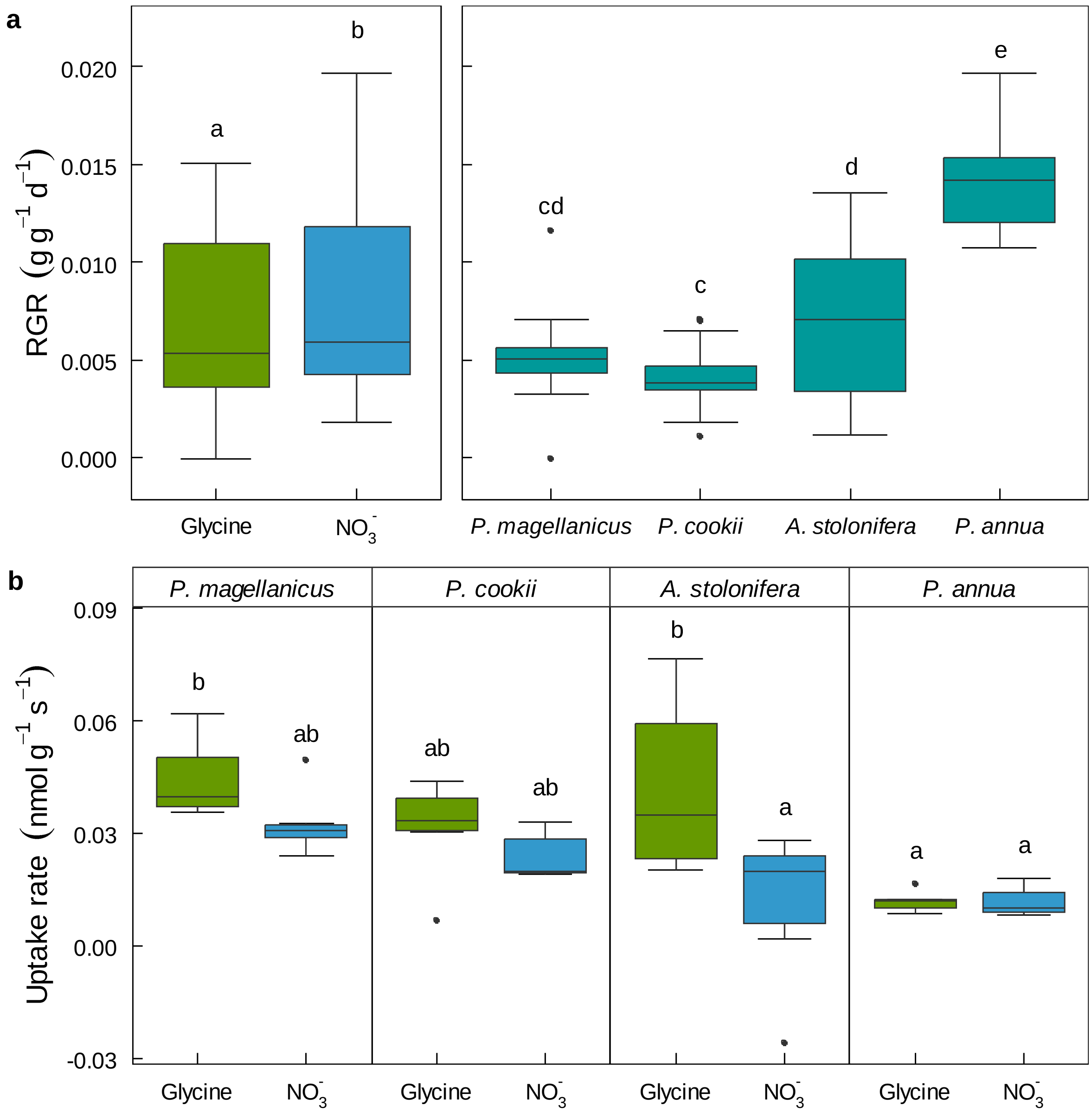

Fig. 4: Growth in the four N treatments and uptake rates in the two 0.4 mM treatments for all study species in the LA experiment. **a** Relative growth rate (RGR), where there was a significant difference in RGR between the N-form ($F_{1,\,90}$ = 5.87, p = 0.017; left panel) and between the four species ($F_{3,\,90}$ = 80.58, p < 0.001; right panel). **b** N uptake rate (nmol g^{-1} s^{-1}), where there was a significant interaction between N-form (NO_3^- or glycine) and species ($F_{3,\,40}$ = 2.92, p = 0.046). Letters indicate significant differences between treatments and species, from an emmeans post-hoc test at the α = 0.05 significance level.

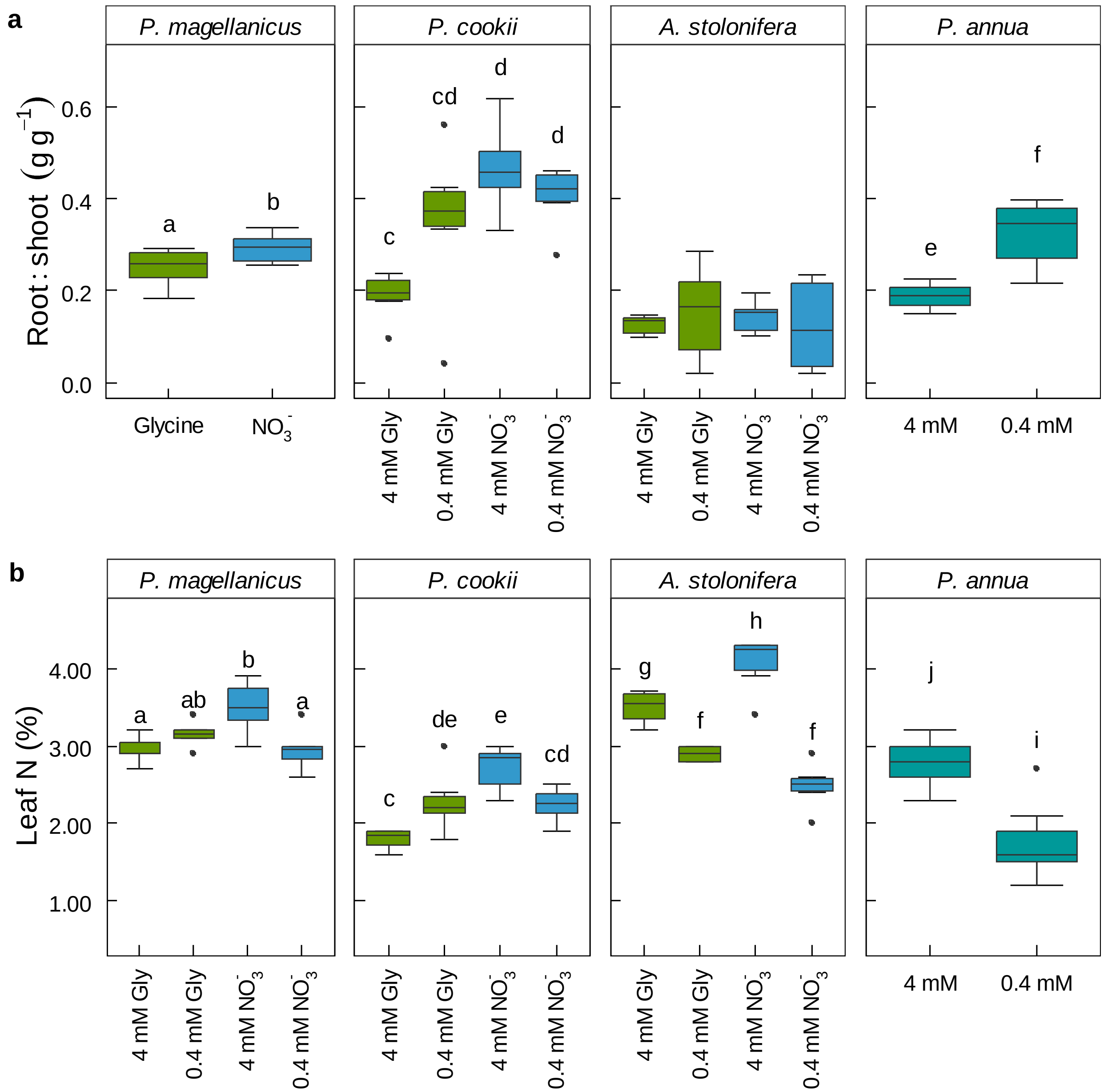

Fig. 5: Growth variables for the study species in the four N treatments in the LA growth experiment. For root:shoot ratio and leaf [N], there was a significant interaction between species and N treatments ($F_{3, 78} = 4.25$, $p < 0.001$; $F_{9, 80} = 8.90$, $p < 0.001$), thus separate models were run for each species. **a** root:shoot ratio, with a significant effect of N-form for *P. magellanicus* ($F_{1, 21} = 9.72$, $p = 0.005$) but no effect of [N]; a significant effect of [N] for *P. annua* ($F_{1, 21} = 46.60$, $p < 0.001$) but no effect of N-form; and a significant interaction between [N] and N-form for *P. cookii* ($F_{1, 18} = 5.38$, $p = 0.032$). **b** leaf [N], where there was a significant interaction between [N] and N-form for *P. magellanicus* ($F_{1, 20} = 14.00$, $p = 0.001$), *A. stolonifera* ($F_{1, 19} = 19.56$, $p < 0.001$) and

P. cookii ($F_{1, 20}$ = 18.09, $p < 0.001$); and a significant effect of [N] for *P. annua* ($F_{1, 21}$ = 67.29, $p < 0.001$) but no effect of N-form. Letters indicate significant differences from an emmeans post-hoc test at the $\alpha = 0.05$ significance level.

2.4.2. oN and iN uptake in situ

At both the 'Biotic' and 'Fellfield' field sites, there was significant $\delta^{15}N$ enrichment of both glycine- and iN- (NO_3^- and NH_4^+) supplied plants, whereas plants at the 'Slope' site only showed significant $\delta^{15}N$ enrichment with NO_3^- and NH_4^+ (Fig. 6). The uptake of these N-forms differed between both site and species, as shown by the significant interaction between these factors ($p < 0.05$). When analysed for each species independently, site had a strong effect on $\delta^{15}N$ enrichment with the various N-forms. While there was only evidence for $\delta^{15}N$ enrichment in iN-provided *P. magellanicus* and *P. annua* at the 'Biotic' site, both species showed significant $\delta^{15}N$ enrichment in glycine- and iN-provided plants at the 'Fellfield' site (Fig. 6). *Agrostis stolonifera*, which was only present at the biotic site, had significant enrichment with all ^{15}N treatments. *Poa cookii*, on the other hand, only showed $\delta^{15}N$ enrichment with iN at both the 'Biotic' site (NO_3^-) and 'Slope' site (both iN forms) (Fig. 6).

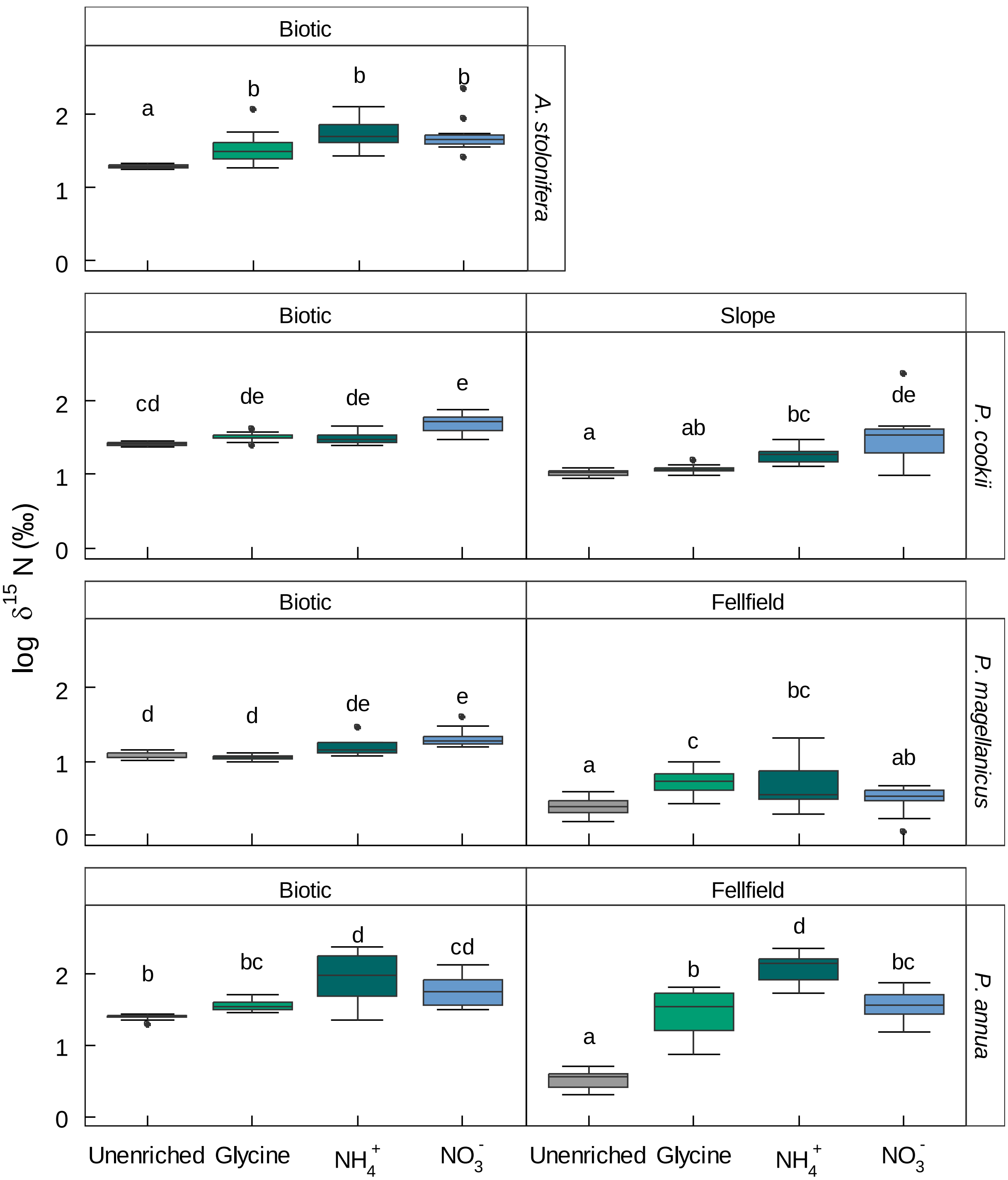

Fig. 6: Leaf δ^{15}N in the grasses at the three sites *in situ*, for unenriched plants and plants supplied with ^{15}N-glycine, ^{15}N-NH_4^+, or ^{15}N-NO_3^-. There was evidence for a significant interaction between species, site, and N-form ($F_{3,\,251}$ = 11.32, p < 0.001), thus separate models were run for each species. There was a significant effect of N-form on *A. stolonifera* δ^{15}N ($F_{3,\,36}$ = 9.80, p < 0.001),

and a significant interaction between site and N-form for *P. cookii* ($F_{3, 72}$ = 3.31, p = 0.025), *P. magellanicus* ($F_{3, 71}$ = 7.99, p < 0.001) and *P. annua* ($F_{3, 72}$ = 19.14, p < 0.001) $\delta^{15}N$. Data were plotted on a log scale due to large variation, to aid visual interpretation. Letters show significant differences between N-forms and sites for each species from an emmeans post-hoc test at the α = 0.05 significance level.

2.5. Discussion

Consistent with the literature showing all plant species have the capacity for oN uptake (Näsholm et al. 2009; Paungfoo-Lonhienne et al. 2012), the sub-Antarctic grasses investigated all acquired oN in the pot experiments under controlled conditions. The high root $\delta^{15}N$ values provide evidence for glycine uptake by all the grasses investigated, and indeed, most ^{15}N-glycine-provided plants had significantly higher $\delta^{15}N$ enrichment compared to those provided with ^{15}N-NO_3^-. Furthermore, the LA uptake experiment resulted in overall higher glycine uptake rates than NO_3^-, demonstrating the capacity for sub-Antarctic grasses to readily access oN sources when they are provided in high concentrations. Considering the higher soil [oN] on MI and indeed most sub-Antarctic soils (Smith 1978; Smith and Steenkamp 1992a), this suggests that direct oN acquisition may play an important role in plant N nutrition in the sub-Antarctic, and that previous literature that only accounted for iN acquisition (Smith 1977, 2008) underestimated total plant-available N.

Direct oN uptake by plants has energetic benefits due to the simultaneous acquisition of C with N (Franklin et al. 2017) and can affect plant growth. In support of the hypothesis, oN supply influenced RGR and biomass allocation compared to iN supply. However, contrary to other literature such as Cambui et al. (2011) and Franklin et al. (2017) who showed increased RGR with oN, both growth experiments resulted in lower RGR with glycine supply. Furthermore, while other literature (Cambui et al. 2011; Franklin et al. 2017) shows increased root fractions with oN provision, the currently study found consistently low root growth under high oN (2 mM, 3 mM, and 4 mM glycine). Low root growth with amino acid or protein supply has been documented in other studies, however, this is concentration- and compound- specific. For example, low [protein] (1.67 µM bovine serum albumen) stimulated *A. thaliana* root growth, whereas higher concentrations (45 µM) inhibited it (Lonhienne et al. 2014). Root inhibition with low L-glutamate supply (0.05 – 0.5 mM) has also been documented for *A. thaliana*, however, does not occur with similar concentrations of related amino acids, and varies between the ecotypes of *A. thaliana* tested (Walch-Liu et al. 2006). This study recorded different responses to [N], where *P. cookii* leaf [N] increased with root fraction but *P. annua* leaf [N] decreased with root fraction. The other species showed significant responses to the treatments, where *A. stolonifera* increased leaf [N] with high [N], and *P. magellanicus* showed higher root growth with NO_3^- relative to glycine. This suggests a greater complexity to growth responses under different N-forms than that presented by Franklin et al. (2017), varying between species, concentrations, and N compounds.

Evidence for different growth responses to N-form and [N] is however largely limited to laboratory experiments and may have different results in natural settings. The evidence for species-specific growth responses to [N] and N-form may influence ecological interactions between co-existing plants on MI. For instance, the native tussock species (*P. magellanicus* and *P. cookii*) showed lower root growth on high [oN] relative to high [iN], whereas the invasive *P. annua* showed large responses to [N] irrespective of N-form. While many potted experiments interpret low root growth under high [N] as root regulation based on external [N] or plant N status (Miller and Cramer 2004; Walch-Liu et al. 2006; Miller et al. 2007b), field evidence shows root proliferation under high [N] can represent a competitive adaption (Robinson et al. 1999; Hodge 2009). Increased root investment by *P. annua* under low N may indicate a 'foraging' strategy (Miller and Cramer 2004), and indeed, *P. annua* outcompetes native Antarctic species due to high N-acquiring strategies (Cavieres et al. 2018). Root proliferation under high iN may however confer a competitive advantage to plants growing in an area with highly localised iN addition, such as sub-Antarctic grasses that grow along the coastline with localised iN input by faunal activity. Although the low sample size precludes sufficient evidence to fully support this interpretation, the evidence for different growth responses indicates the importance of investigating plant growth under different N-forms in light of their different growth and N-acquisition traits. Furthermore, such experiments in a field environment may aid our understanding of how N-form acquisition relates to species interactions, competition, and co-existence.

The results from the current study suggest that sub-Antarctic grasses acquire oN in the field, particularly in sites with low iN. Due to the use of only singly labelled isotopes (^{15}N), intact acquisition of oN cannot be confirmed in the present study (Persson and Näsholm 2001a; Jones et al. 2005a; Warren 2012). To experimentally confirm intact oN acquisition in field environments, the use of dual-labelled $^{13}C^{15}N$ oN is recommended, as isotope analysis of both ^{13}C and ^{15}N allows for distinction between oN acquired intact or following microbial mineralisation (Persson and Näsholm 2001a). Intact oN utilisation at sites replete with oN is however common (Nordin et al. 2004; Schimel and Bennett 2004; Jones et al. 2005b). Indeed, bacterial activity was likely highest at sites with high faunal influence (e.g., Grobler et al. 1987) such as the 'Biotic' and 'Slope' sites. These sites showed little evidence for $\delta^{15}N$ enrichment in ^{15}N-glycine-provided grasses. Bacterial activity at the site 'Fellfield' with significant $\delta^{15}N$ enrichment in the ^{15}N-glycine-provided grasses was likely lower than at sites with faunal influence. Therefore, as a working hypothesis, this suggests that sub-Antarctic plants use oN at sites with high oN and low iN. The hydroponics experiments had a lower risk of microbial mineralisation prior to glycine uptake, and therefore glycine uptake or $\delta^{15}N$ enrichment following ^{15}N-glycine supply provides evidence for intact oN acquisition. There are accounts for relatively low bacterial presence in MI mires (French and Smith 1986; Grobler et al. 1987). The LA nutrient solution had a low risk of contamination because it was made with deionised water in laboratory settings. However, further investigations into the extent to which oN meets sub-Antarctic plant N requirements in field conditions with varying oN and iN fractions are necessary.

Plant growth in response to oN and iN forms was affected by the [N] relative to other nutrients in hydroponic solution. Growth variables (RGR, root:shoot ratio, and leaf [N]) differed substantially between the mire and LA experiments, which provided N in similar concentrations (i.e., 6 mM and 4 mM as maximum [N] in mire and LA experiments, respectively). However, the solutions were either highly depauperate (mire water) or more concentrated (LA solution) with nutrients. The low growth, increased mortality, and low root:shoot ratio in the oN and NH_4NO_3 treatments in the mire experiment are symptoms attributed to N-toxicity, which has been described on high NH_4^+ (Britto and Kronzucker 2002) and high glycine (Mohammadipour and Souri 2019). This suggests that the large discrepancy between N and other nutrient concentrations in the mire experiment induced N-toxicity symptoms, however, when provided in LA solution with higher [nutrient], there were no toxicity symptoms, and the plants grew well on glycine. Interestingly, the mire experiment NO_3^- treatment resulted in increased RGR and root:shoot ratio, i.e., no symptoms of N-toxicity. Furthermore, leaf [N] in this treatment was significantly lower than in the other four treatments. Although largely speculative, I suggest that the lack of toxicity symptoms in the NO_3^- treatment is related to its decreased uptake rate documented in the LA experiment and in other studies compared to NH_4^+ and oN (Näsholm et al. 2009). Low NO_3^- uptake rates may be due to the reduction step necessary before assimilation which is not necessary for NH_4^+ or amino acids (Miller and Cramer 2004). The low uptake rates of NO_3^- thus allowed plants to meet other nutritional requirements (e.g., P, Mg, Ca) in this treatment, whereas the higher uptake rates for glycine and NH_4^+ relative to low nutrient acquisition resulted in N-toxicity. This evidence, although unrelated to the hypothesis investigating plant oN use, indicates the importance of other macronutrients in addition to N to support plant growth.

Although previously unaccounted for, oN acquisition may be important to support MI vegetation productivity and needs to be accounted for in future investigations into sub-Antarctic N budgets. This is particularly important given the evidence for current warming in the region (le Roux and McGeoch 2008a), as warming may alter the relative availability of oN and iN and thus the N-form predominantly acquired by plants (Kuster et al. 2016). This may have important implications for vegetation N and C demands due to the differences in plant NUE between these N-forms (Franklin et al. 2017) and the different growth responses shown here. Alterations in N-form availability may also result in species changes, should certain species compete better for different N-forms (Pang et al. 2019). In a MI context, the evidence for high N acquisition and biomass allocation as a 'foraging' strategy under low N by *P. annua* may result in competition with native flora. Indeed, *P. annua* outcompetes native Antarctic flora due to high N-acquisition capacity (Cavieres et al. 2018), and MI invasive flora have shown recent range expansions under climate warming (le Roux et al. 2013). However, in light of the evidence that topographic and edaphic features limit range expansions (Cramer et al. 2022), the role of [N] and N-forms in future range expansions by MI flora warrants investigation.

2.5.1. Conclusion

This study shows the importance of investigating the capacity for plant oN uptake in regions where it has remained unaccounted for. The direct acquisition of oN by plants forms an important part of a paradigm shift in our understanding of soil N cycling. However, as a relatively recent paradigm shift from the late 20th century, some literature still assumes that only iN is ecologically relevant to plants. This study contributes to the evidence for plant oN uptake, showing that sub-Antarctic grasses acquire oN. Given the substantial oN pool of MI soils, compared to the paucity of iN, oN may form an important N resource that supports plant growth in the sub-Antarctic. Furthermore, the evidence for species-specific growth and allocation on oN and iN treatments suggests an interesting angle for future field experiments to determine whether N-form partitioning *in situ* affects plant growth.

Chapter 3

Limited effects of short-term sub-Antarctic soil warming on microbial mineralisation and plant growth

3.1. Abstract

Cold ecosystem plant productivity is N-limited due to temperature-limited decomposition, resulting in predictions for higher indirect than direct warming effects on productivity through microbially-mediated N-release. Plant growth under soil warming was investigated on sub-Antarctic Marion Island (MI, -46.9, 37.8). I hypothesised that 1) warming increases plant growth indirectly through stimulating microbial mineralisation, 2) warming-induced increases in soil iN influences the N-form acquired by plants, and 3) warming influences on microbial activity affect soil inorganic N (iN; NH_4^+ and NO_3^-), PO_4^{3-}, and microbial C, N and P. Four grasses (*Polypogon magellanicus*, *Agrostis stolonifera*, *Poa cookii*, and *Poa annua*) were subjected to soil warming (MI ambient +3°C) and NPK fertilisation (positive control) in pots for five months. Additionally, soils with varying organic content were incubated at 5°C (control), +3°C and +6°C for 42 d. While leaf [N] increased in all species, only *P. annua* increased biomass (by 42%) with warming. By contrast, fertilisation increased biomass by 449% across all species. Soil [NH_4^+] increased by 12% with warming in the pot experiment but was uninfluenced by temperature in the incubation experiment; PO_4^{3-} remained unchanged overall. Warming did not affect plant oN or iN uptake, and incubation at different temperatures did not affect soil [oN] or microbial C, N or P. These results suggest that indirect plant growth stimulation to warming is not ubiquitous and warming-induced "fertilisation" should not be assumed. These muted responses to short-term temperature manipulations question the direct and indirect responsiveness of plant and soils on MI to climate warming.

3.2. Introduction

Plant productivity is intrinsically linked to soil processes, as plants predominantly meet their nutritional requirements from soil nutrient pools. In cold ecosystems, while plant growth and productivity are limited directly by low temperatures, the indirect effects of temperature are considered stronger determinants of productivity, such as low N-availability due to temperature-limited decomposition rates (Chapin 1983). Under current trends of anthropogenic climate change (IPCC 2022), temperature increases are hypothesised to alleviate these limitations to cold ecosystem plant productivity. Evidence for a higher temperature sensitivity of soil decomposition processes than plant productivity has led to predictions that the indirect effects of warming, through stimulated decomposition and thus N-release, will outweigh the direct effects of warming to plant productivity (Chapin 1983; Kirschbaum 1995, 2004). Considering the ecological consequences increased plant productivity may have on cold ecosystem functioning, it is necessary to test the predictions for warming-induced nutrient release and how this may affect plant growth and nutrition.

In support of these predictions, there is evidence that soil warming affects microbial activity and decomposition processes. Experimental soil warming has shown evidence for N-release in cold ecosystems, through stimulating extracellular enzyme activity and soil organic matter (SOM) proteolysis (Brzostek et al. 2012; Jiang et al. 2018), detritivore activity (Aerts 2006), and microbial mineralisation of inorganic N (iN) and other nutrients such as PO_4^{3-} (Nadelhoffer et al. 1991; Hobbie 1996; Rustad et al. 2001; Bai et al. 2013; Salazar et al. 2020). This has been linked to evidence for increased plant productivity and plant N-uptake (Rustad et al. 2001; Bai et al. 2013; Sistla et al. 2013). However, the effects of warming on microbial nutrient cycling do not always result in nutrient release. Stimulating microbial populations and activity may increase microbial nutrient status, known as nutrient immobilisation, as it renders these nutrients unavailable for plant uptake (Rustad et al. 2001).

Soil warming therefore has the capacity to indirectly influence plant growth through its direct effects on microbial activity and thus nutrient availability. Additionally, soil warming directly affects plant growth and nutrient uptake (BassiriRad 2000), which may implicate or act in concert with warming-induced N-release to stimulate plant growth. For example, soil warming stimulates root biomass and alters root morphology, which is linked to increased N uptake (Yin et al. 2013;

Qiao et al. 2016). Considering the evidence for increased N-release with warming, the direct effects of root warming may effectively increase the plant’s capacity to utilise the increased bioavailable N.

Across the wealth of literature investigating cold ecosystem warming, evidence for site-specific responses and only transitory stimulations in microbial activity questions the extent to which N-release has affected and will affect changes in high latitude vegetation. For example, although the meta-analyses by Rustad et al. (2001) and Bai et al. (2013) show a general trend of iN increases with warming, some sites (including cold, high latitude systems) have non-significant or decreased soil iN. The large variations in site responses to warming are often linked to confounding effects such as soil water content (SWC) (Allison and Treseder 2008; Beier et al. 2008), and litter C:N ratios or vegetation cover, which exert stronger controls over decomposition than warming (Nadelhoffer et al. 1991; Hobbie 1996; Steinauer et al. 2015; Ward et al. 2015). Furthermore, while short-term warming may stimulate microbial activity, these effects may be transitory and diminish over time (e.g., > 10 y shown in Romero-Olivares et al. 2017) to a steady-state similar to the controls. This is presumably due to a combination of substrate depletion and microbial acclimation (Callaghan et al. 2004; Romero-Olivares et al. 2017) and indicates that long-term N-release under warming should not be assumed. Despite this, multiple studies have used fertilisation treatments to approximate the long-term effects of warming (e.g., Jonasson et al. 1999; Graglia et al. 2001; Mack et al. 2004; Rinnan et al. 2007; Lamb et al. 2011; Campioli et al. 2012). When compared to long-term warming treatments, however, biomass increases under fertilisation are often substantially stronger than warming (e.g., Graglia et al. 2001; Rinnan et al. 2007). The lack of evidence for a ubiquitous N-release under warming and the transitory nature of this response indicates that the extent to which plant productivity may be indirectly stimulated by warming remains unclear.

If soil warming increases microbial mineralisation, this may influence the relative concentrations of bioavailable organic N (oN) and iN in cold ecosystem soils and affect the N-form predominantly available for plant acquisition (Kuster et al. 2016). Cold ecosystems are typically replete with oN but not iN, and plants in these marginal environments meet their N requirements largely through oN acquisition (Kielland 1994; Lipson and Näsholm 2001). Increased microbial mineralisation under (short-term) soil warming and concomitant increases in soil iN may however lead to higher iN availability and uptake. Organic N acquisition relative to iN has an energetic benefit to plants due to the simultaneous uptake of C with N (Franklin et al. 2017), thus an alteration in N-form use may implicate plant nutrient use efficiency (NUE). Indeed, there is evidence for a change in N-form use under warming, especially when plants are grown in competition. For example, Kuster et al. (2016) link increases in iN availability to higher plant iN acquisition, and Jiang et al. (2018) show that long-term warming and grazing increased soil oN (through increased extracellular enzyme activity and denitrification of soil iN) and therewith oN acquisition.

While the effects of warming on plant-soil interactions have been widely investigated in northern high latitudes, sub-Antarctic terrestrial systems have received less attention despite similarly high rates of climate change (Smith 2002). Sub-Antarctic islands may however represent a useful study site for such investigations, with relatively simple ecological systems and a thermo-stable climate (Smith 2002; le Roux 2008). On sub-Antarctic MI, a lack of vertebrate grazers results in a simple soil nutrient cycle, with low invertebrate populations due to high predation by the invasive *Mus musculus* (house mouse) (Crafford 1990; Bergstrom and Chown 1999; Smith et al. 2002). The soils are highly organic, but coastal N deposition by seabirds or pinnipeds leads to varying amounts of bioavailable oN and iN (Smith 1978). This study explored how short-term warming on MI affects plant productivity through its effects on soil nutrient availability. I hypothesised that soil warming would stimulate plant growth directly but also indirectly through increased soil [iN], due to stimulated microbial mineralisation rates. I also hypothesised that the increases in soil [iN] would affect the N-form acquired by sub-Antarctic grasses, predicting a change from oN to iN with warming. These hypotheses were investigated in a five-month potted experiment on MI, with half of the pots warmed to +3°C above ambient and a fertilisation treatment included as a positive control. Additionally, I hypothesised that the effects of soil warming on microbial activity is influenced by both the degree of warming, with a positive relationship between temperature increases and warming responses, and soil organic content. Soils with high organic content have higher microbial biomass and substrate availability, and thus may have stronger responses to warming compared to soils with low organic content. These hypotheses were investigated in an incubation experiment under three temperature treatments and with soils collected from sites with varying total organic C (TOC) content. Microbial responses to warming were assessed by analysing soil nutrients, microbial biomass, and microbial nutrient immobilisation.

3.3. Methods and Materials

3.3.1. Study site and species

The grasses in the planted warming experiment, *Polypogon magellanicus* (Lam.) Finot, *Poa cookii* (Hook.f.) Hook.f., *Agrostis stolonifera* L., and *Poa annua* L., were collected on sub-Antarctic MI where they co-occur within 100 m of each other near the main base on the east coast (-46.88, 37.86). Soil cores (20 cm long, 8 cm diameter) were collected in this vicinity with a soil corer, and 5-7 grass tillers were planted in each core. For the duration of the experiment, pots were kept outdoors under natural light and rainfall conditions.

Soil cores for the incubation experiment were excavated from five sites on the east coast of the island, with varying degrees of vertebrate influence and vegetation cover (Table S4), thus varying TOC. Animal influence from vertebrate colonies was categorised based on proximity to and extent of vertebrate activity. Soil macroinvertebrates were present at three of the sites (only Oligochaeta, earthworms), and their presence/absence at sites was recorded. Their presence and the presence of smaller invertebrates (such as Collembola) were assumed to represent natural abundances, so were not removed from the study. Soil cores were collected in May 2020 and stored in polyethylene zip-lock bags at 5°C until the start of the experiment in November 2020. The site with low vertebrate activity ('Low', Table S4) was the same soil site that cores were sampled from for the planted warming experiment.

3.3.2. Plant growth under soil warming and fertilisation

To investigate the effects of warming on grass biomass and N-form acquisition, the four grass species were planted in pots with two treatments (soil warming and NPK fertilisation) applied in a full factorial design (n = 12 per species per treatment). Soil warming was achieved with a heating cable (25 W m^{-2}, Thermon BSX 8-2 Heating Cable, Durham, UK) at 15 cm soil depth (Fig. S5a). To monitor soil temperature, five temperature sensors (107 Temperature Probe, Campbell Scientific, USA) were placed in warmed pots and one temperature sensor in a pot at ambient temperature, at 5 cm soil depth. Half of the pots were warmed to 3°C above ambient soil temperatures, with the heating cable output regulated by a measurement and control data logger

(CR1000X, Campbell Scientific, USA). Soil temperatures in ambient and warmed pots were logged for the duration of the experiment (Fig. S5a). Half of the pots were fertilised as a positive control for soil warming, with 0.75 g (28 g N m^{-2}, 13 g P m^{-2}, 15 g K m^{-2}) of slow-release fertiliser (Osmocote Pro 5-6, NPK 19-9-10 + 2 MgO + TE) added to the soil surface. Fertiliser addition in this study was higher than that used in other studies (28 g N m^{-2} compared to 10 g N m^{-2} of e.g., Rinnan et al. 2007), but deemed appropriate given the high oN content of MI soil (e.g., 26 mg N g^{-1}; Smith 1978), indicating high substrate concentrations for potential mineralisation. The four experimental treatments were thus control ("C", no warming, no fertiliser), heated ("H", +3°C, no fertiliser), fertilised ("C+NPK", no warming, fertiliser) and heated and fertilised ("H+NPK", +3°C warming with fertiliser). To account for the effects of warming on soil inorganic [N] and [P] without plant nutrient uptake, six pots without plants were maintained in the C and H treatments.

The experiment took place over five months in austral summer (November 2019 – April 2020) on MI. Pots were kept well-watered (untreated rainwater was added if there were >2 consecutive days without rain) to mimic natural conditions, as most habitats on MI where the grasses occur have naturally waterlogged soils. Non-destructive biomass assessments took place roughly every four weeks to determine relative growth rates (RGR), calculated according to Hoffmann and Poorter (2002). In these assessments, tillers, developing and fully expanded leaves (expanded leaves identified by a visible ligule), and inflorescences were counted, and approximate biomass was determined according to a proxy determined by Ibrahim (2007) (Fig. S6). At the end of the experiment, a soil core (*ca*. 20 g) was collected from each pot, sealed in a polyethylene bag, and frozen at -20°C. For soil analyses, samples were thawed, extracted with 0.5 M K_2SO_4 in a 1:5 *w*:*v* (soil wet weight:solution volume) ratio through thorough stirring and then 1 h shaking on an oscillating shaker, and filtered through a 0.45 µm Whatman filter membrane before analyses for soil inorganic N and P (details below). The remaining soil was dried to constant weight at 70°C.

3.3.3. oN and iN uptake under soil warming and fertilisation

Plant iN and oN uptake was assessed at the end of the planted warming experiment with the addition of either ^{15}N-glycine glycine (98% ^{15}N, Cambridge Isotope Laboratories, Andover, MA, USA), ^{15}N-NH_4^+ ($^{15}NH_4Cl$, 98% ^{15}N, Cambridge Isotope Laboratories), or ^{15}N-NO_3^- ($Na^{15}NO_3$, 98% ^{15}N, Cambridge Isotope Laboratories) to the potted plants. ^{15}N-forms were provided by pouring 60 mL of ^{15}N-containing solution onto the soil at the base of the plant (n = 4 per species, growth treatment, and ^{15}N treatment). Each solution contained 20 mL 10.5 µM glycine, 20 mL 10.5 µM NH_4Cl, and 20 mL 10.5 µM $NaNO_3$, one of which was isotopically distinct (i.e., containing ^{15}N). One leaf was harvested from each replicate the day before ^{15}N enrichment, to determine unenriched (i.e., natural $\delta^{15}N$ abundance) $\delta^{15}N$ values and the leaf [N]. Plants received ^{15}N enrichment shortly before sunrise on a day without rainfall (to ensure no dilution of the ^{15}N solution), and one leaf was harvested 24 h following that. A 24 h chase period was chosen based on separate experiments which showed slow N uptake rates and higher $\delta^{15}N$ enrichment at 24 h relative to 12 h harvests. All leaves harvested were the second fully expanded leaf and were immediately dried for 48 h at 70°C, and then stored at room temperature until mass spectrometry analyses. Evidence for ^{15}N acquisition was assessed from the difference in $\delta^{15}N$ between unenriched and enriched leaves (see statistical analyses). Some replicates did not have two fully expanded leaves due to low growth rates, and therefore could not be used to determine $\delta^{15}N$ enrichment. Plant aboveground material was harvested after the uptake experiment, dried at 70°C for 48 h, and analysed for total N content.

3.3.4. Soil N and P and microbial C, N, and P under warming and increasing TOC

The incubation experiment tested whether warming influenced the relationship between soil TOC and soil N, P, and microbial C, N, and P. The experiment had a paired design, with each core halved and half kept as control (5°C) and the other warmed by either +3°C or +6°C relative to the controls (Fig. S5b) using the same heating cable, data logger, and regulator setup as the planted experiment (n = 10 control-heated pairs per heating treatment per site). After a 42 d incubation, each replicate was separated into sections, one for gravimetric SWC (difference between wet and dry soil cores), and the other for determining inorganic N and P and organic C and N. Soil moisture levels were maintained by watering with deionised water every two to three days. Soil nutrient and microbial parameters were regressed against SWC to determine whether SWC influenced the results, as there is evidence that excessive soil water confounds microbial mineralisation (e.g., Beier et al. 2008). Replicates were weighed at the beginning and end of the experiment to determine whether soil water content increased during the experiment.

3.3.5. *Chloroform fumigation extraction for microbial C, N, and P*

To determine how incubation at three temperatures (control = 5°C, +3°C and +6°C) influenced soil microbes, microbial C, N, and P were measured with the chloroform ($CHCl_3$) fumigation-extraction technique. In this method, $CHCl_3$ lyses microbial cells, releasing microbial C (a proxy for biomass), N, and P (Vance et al. 1987). Microbial C, N, and P was calculated as the difference in TOC, total dissolved N (TDN; the sum of iN and oN) and PO_4^{3-} (respectively) between fumigated and unfumigated soil samples. The extractability of microbial C, N, and P was not accounted for, and therefore the data represented an index of the variables that are comparable between treatments. Fresh soil was fumigated with ethanol-free $CHCl_3$ in a vacuum desiccator by boiling and venting the desiccator twice before a final boiling, after which the desiccator was left for 24 h before samples were removed (Vance et al. 1987). Soils were then immediately extracted with 0.5 M K_2SO_4 at a 1:5 *w:v* ratio (Beck et al. 1997), through thorough stirring and then shaking on an oscillating shaker for 1 h. A separate sub-sample of each replicate was extracted without fumigation. Soil extracts were then centrifuged (Hermle, Germany) at 671 *g* for 15 minutes, the supernatant separated, filtered through a 0.45 µm Whatmann filter membrane, and stored at -20°C until analyses for TOC, TDN, and PO_4^{3-}. A white precipitate formed in the extracts after freezing, but this does not compromise the respective C, N, or P measurements, and solutions were centrifuged for 5 minutes at 671 *g* to ensure no interference by the crystals.

3.3.6. *Soil inorganic N and P and organic N and C measurements*

The effects of warming on soil iN and PO_4^{3-} from both the planted and incubation experiments were determined using colorimetric assays on the 0.5 M K_2SO_4 extracts. Nitrate was measured based on the method described by Schnetger and Lehners (2014), where VCl_3 reduces NO_3^- to NO_2^-, which is then captured by Griess reagents (N-1-naphthlyethylenediamine dihydrochloride and sulphanilamide). No correction for NO_2^- was made due to measured negligible concentrations. NH_4^+ was measured using the indophenol blue method (Dorich and Nelson 1983), using Berthelot reagents (phenol, nitroprusside, sodium citrate, and sodium hypochlorite). PO_4^{3-} was measured based on the ammonium molybdate method described by Strickland and Parsons (1972), where the reagents ammonium molybdate, ascorbic acid, sulphuric acid, and potassium antimonyltartrate forms a phosphomolybdate complex. Colorimetric absorbance measurements were made with a spectrophotometer (ThermoSpectonic, Helios Epsilon model, Thermo Scientific, USA) (planted experiment) or using a multiplate reader spectrophotometer (incubation) (Multiskan Spectrum, Thermo Electron Corporation, Finland), at 540 (NO_3^-), 630 (NH_4^+), and 885 (PO_4^{3-}) nm. Soil extracts from the incubation experiment were analysed for oN (oN = TDN – iN) and TOC. Soil TOC was measured using a TOC Torch Combustion Analyser (Teledyne Tekmar, USA) where, after an initial sparging with phosphoric acid to eliminate inorganic C, the oxidation of carbon material produced CO_2 which was measured using non-dispersive infrared detection. Samples were measured against a 100 ppm potassium phthalate (KHP; $C_8H_5KO_4$) standard made in 0.5 M K_2SO_4. Soil TDN was measured using a protocol based on Yu et al. (1994) and Hagedorn and Schleppi (2000), where samples are treated with a persulfate and NaOH reagent, autoclaved for 1 h, resulting in persulfate oxidation of all TDN compounds to NO_3^-, which were then measured colorimetrically according to Schnetger and Lehners (2014). The use of 1 mM urea standards resulted in urea oxidation to NO_3^- with 98.7% accuracy, and this discrepancy was accounted for. All analyses used standards prepared in 0.5 M K_2SO_4. All soil C, N, and P data are presented per g (dry weight). Dry weights were recorded for every core in the incubation experiment, and for all cores without plants in the MI planted experiment. SWC in the MI planted experiment was measured on combined control and warmed treatments after no significant difference between control and warmed SWC was established. I assumed negligible effects of transpiration on SWC due to the high SWC (> 500%) and relatively low growth rates of the grasses.

3.3.7. *Leaf δ^{15}N and N analyses*

To determine how warming and fertilisation influenced leaf N and the type of N acquired, leaves from the planted experiment were analysed. For leaf δ^{15}N and [N], samples were ground to a fine powder using a ball mill (MM200, Retsch, Germany), and weighed (2 mg) into tin capsules. These were combusted in a Flash 2000 organic elemental analyser (Thermo Scientific, Germany), gases passed into an isotope ratio mass spectrometer (DELTA V Plus IRMS) via a ConFlo IV gas control unit and calibrated according to in-house standards.

Total N content (g) was determined on total aboveground biomass. Aboveground [N] was determined using the Flash EA 1112 Series (Thermo Scientific, Germany), where 5 mg of finely ground plant biomass was combusted in a high-temperature reactor. The resultant gases were measured by chromatography after separation by retention rates in a chromatograph column, and results calibrated according to in-house standards.

3.3.8. Statistical analyses

All statistical analyses were performed using R statistical software, version 4.1.1 (R Core Team, 2021). Data from the potted experiment (plant RGR, total biomass, leaf [N], total N (g); soil iN and P) were subjected to analysis of variance (ANOVA), testing for interacting effects between the two treatments (warming and fertiliser) and species. To determine whether there was sufficient evidence for enrichment with the ^{15}N form (i.e., enrichment > 0), one-sample t-tests were run on each species x treatment x ^{15}N-form combination. The Benjamini Hochberg correction was used to account for an increased Type 1 error rate (Benjamini and Hochberg 1995), using the 'rstatix' package (Kassambara 2023). To determine whether warming, fertilisation, and N-form affected $\delta^{15}N$ enrichment, a three-way ANOVA was run for each species.

For soil incubation, TOC data were analysed with a linear mixed effects model (LMEM), with core replicate as a random effect to account for the paired experimental design, and site and warming treatment as fixed effects. Thereafter, the soil nutrient and microbial data were analysed with a LMEM, testing for a relationship between the response variables (soil iN, PO_4^{3-}, oN, and microbial C, N, and P) and interacting effects of TOC (i.e., site) and warming treatments, with core replicate as a random effect. Significant differences (p-values) and F-values from the LMEM were determined with an ANOVA. Models for oN, PO_4^{3-}, and microbial C, N, and P resulted in a singular fit, where the random effect (core replicate) had a variance of 0. For these models, the random effect was removed, and a linear model was used. To determine whether SWC had changed throughout the experiment, differences in core mass between the start and end of the incubation were analysed with an ANOVA. Thereafter, core SWC was analysed for an effect of incubation temperature, to determine whether warming had influenced SWC.

All ANOVAs were performed in the 'car' package (Fox and Weisberg, 2019), and LMEMs with the 'lme4' package (Bates et al. 2015). Where there was evidence for a significant effect ($p < 0.05$), post-hoc pairwise comparisons were calculated in the 'emmeans' package (Lenth 2023), using

Tukey Honest Significant Differences. All residuals were checked, and where they did not conform, were log-transformed to meet normality and homoscedasticity assumptions.

3.4. Results

3.4.1. Plant growth under soil warming and fertilisation

The heating cable warmed the soil cores in the MI pot experiment to *ca*. 3°C above ambient soil temperature, with mean control and warmed temperatures of 9.0 ± 4.5°C (mean ± SE) and 11.70 ± 1.8°C, respectively (Fig. S5a). As a field comparison, *in situ* temperatures from five sites across MI (Table S5; temperature measured using iButtons at 5-10 cm soil depth from 08-11-2019 to 10-04-2020) had a combined mean of 7.6 ± 1.2°C and range 2.5 - 25.0°C (Schoombie and le Roux, *pers. comm*).

Over five months of growth under soil warming and fertilisation, *A. stolonifera* and *P. annua* showed positive RGR in all treatments while *P. magellanicus* and *P. cookii* decreased in biomass over time in the unfertilised treatments (Fig. S6). At the end of the experiment, there was evidence for species-specific aboveground biomass responses to the two treatments, with a significant interaction between species and warming ($p < 0.05$) and species and fertilisation ($p < 0.001$) (Fig. 1a). The post-hoc test revealed that only *P. annua* showed a significant increase in biomass with warming in the unfertilised treatments (Fig. 1a), whereas all other species' biomass did not respond to soil warming. By contrast, there was a strong biomass response to fertilisation with an overall (significant for all species) increase in plant aboveground biomass of 449% compared to the smaller (and only significant for *P. annua*) 24% overall increase with warming (Fig. 1a). There was no evidence for an effect of warming within the fertilisation treatments. Aboveground N content followed a similar trend, with species- and treatment-specific responses ($p < 0.05$) and only *P. annua* showing evidence for increased N content under warming (in unfertilised treatments) (Fig. 1c). All species showed significant increases in total N content when fertilised (Fig. 1c). There was a significant difference between species leaf [N] ($p < 0.001$), where *P. magellanicus* was significantly higher than the other three species (Fig. 1d). Furthermore, there was a significant interaction between warming and fertilisation treatments on leaf [N] ($p < 0.05$), where warming increased leaf [N] in the unfertilised treatments but showed no response in the fertilised treatments (Fig. 1b). Leaf [N] under fertilisation was significantly higher than under warming (Fig. 1b).

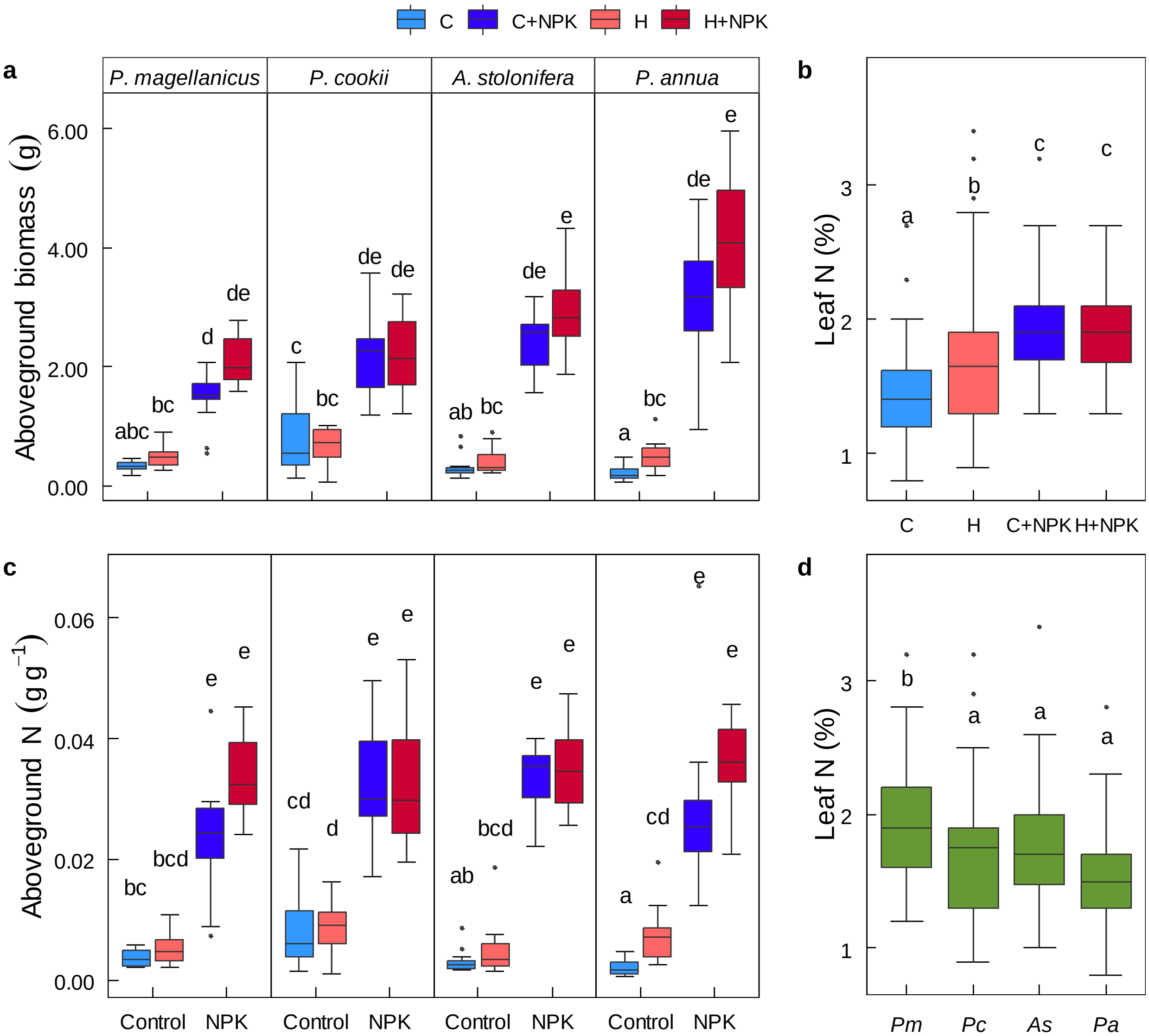

Fig 1: Plant biomass and N content following five months of growth in the warming and fertilisation treatments, for the four species, where "*Pm*" is *P. magellanicus*, "*Pc*" is *P. cookii*, "*As*" is *A. stolonifera*, and "*Pa*" is *P. annua*. **a** Dry biomass of the aboveground plant material. There was evidence for significant interactions between warming and species ($F_{3,176} = 4.67$, $p = 0.0037$), and between fertilisation and species ($F_{3,176} = 13.91$, $p < 0.001$). **b** Leaf [N] between the four treatments, where there was evidence for a significant interaction between warming and fertilisation treatments ($F_{1,185} = 6.68$, $p = 0.011$). **c** Aboveground N content (g g^{-1}), where there was evidence for a significant interaction between warming, fertilisation, and species ($F_{3,176} = 3.58$, $p = 0.015$). **d** Leaf [N] where there was evidence for a significant difference between the

species ($F_{3, 185} = 9.77$, $p < 0.001$). Letters indicate significant differences between treatments and/or species from an emmeans post-hoc test at the $\alpha = 0.05$ significance level.

3.4.2. Soil inorganic N and P after five months of warming

There was evidence for an increase in soil iN with warming ($p < 0.05$; Fig. 2a) due to an increase in [NH_4^+], but not [NO_3^-] (Fig. S7). There were species-specific differences in soil [iN] following five months of growth, where only pots with *A. stolonifera* showed evidence for a significant increase in soil [iN] with fertilisation (Fig. 2a). Soil [PO_4^{3-}] increased significantly with fertilisation ($p < 0.001$), but there was no evidence for significant differences with soil warming or between the four species (Fig. 2b). When soil inorganic nutrient concentrations were compared between pots with and without plants, [NH_4^+] was significantly higher in unplanted pots, but neither [NO_3^-] nor [PO_4^{3-}] showed any difference (Fig. S8).

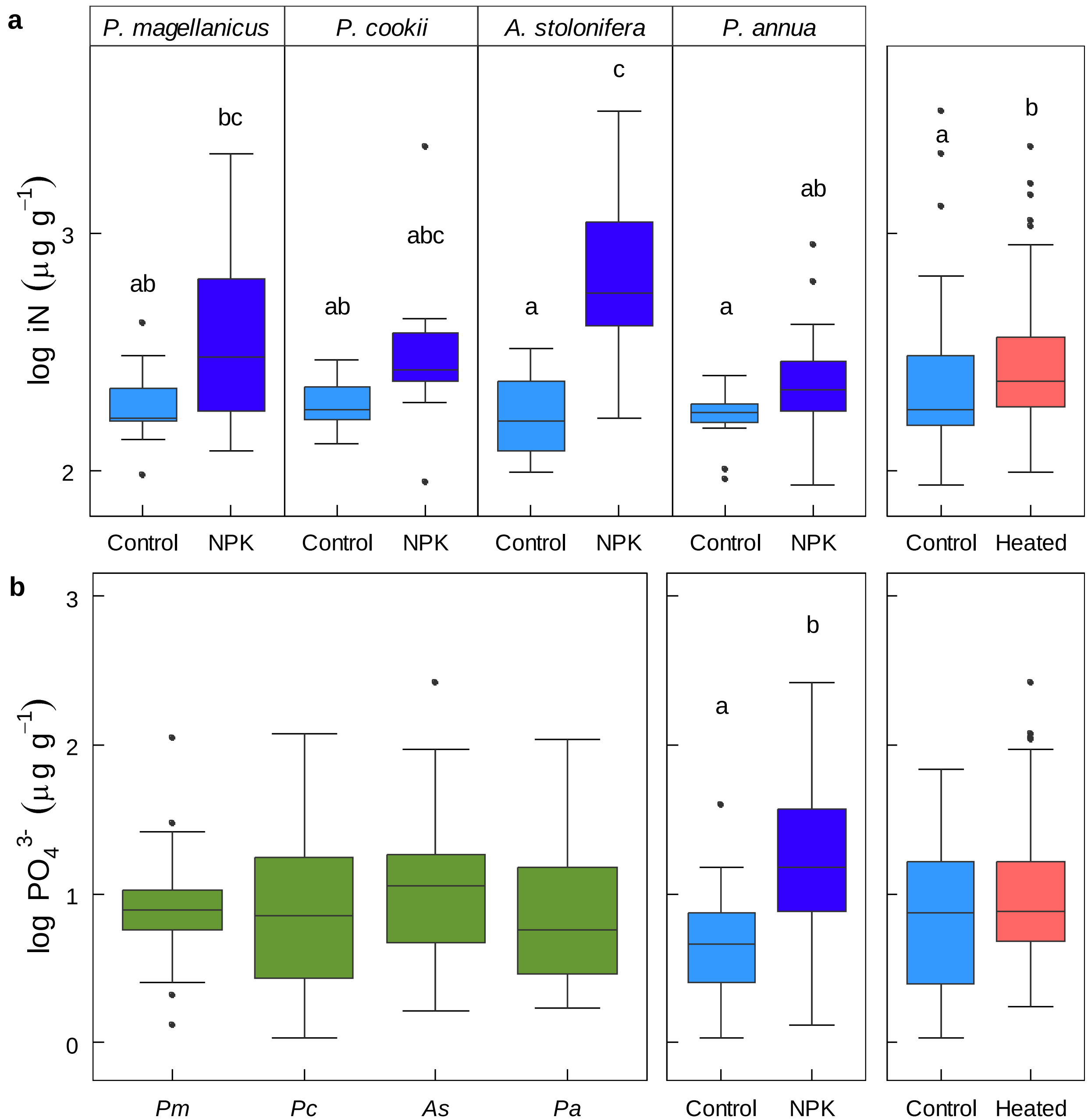

Fig 2: Soil [iN] (sum of NO_3^- and NH_4^+) and [PO_4^{3-}] following five months of warming and fertilisation treatments. **a** Soil [iN], where there was evidence for a significant interaction between fertilisation treatments and species ($F_{3,\,87}$ = 3.21, p = 0.027) and a significant effect of warming ($F_{1,\,87}$ = 4.10, p = 0.046). **b** Soil [PO_4^{3-}], where there was evidence for a significant effect of fertilisation treatment ($F_{1,\,90}$ = 39.7, p < 0.001), but no evidence for significant differences between species or warming treatments. Data are logged to improve the visual display, due to large variation

in the data. Letters indicate significant differences between treatments and/or species from an emmeans post-hoc test at the $\alpha = 0.05$ significance level.

3.4.3. oN and iN uptake under soil warming and fertilisation

The one-sample t-tests showed very few species X treatment X ^{15}N-form combinations had resulted in significant enrichment (leaf $\delta^{15}N$ enrichment > 0; Fig. 3, Table S6). While the other three species showed a low number of treatment combinations that were $\delta^{15}N$-enriched, *P. annua* had higher enrichment (Fig. 3, Table S6). Significant enrichment in ^{15}N-glycine-provided plants was not higher in the control treatments, and there was no increased significant enrichment in ^{15}N-iN-provided plants in the warmed treatments.

Soil warming did not influence the N-form acquired for any species, as indicated by no significant interaction between N-form and warming on leaf $\delta^{15}N$ (Fig. 4). The models run on each species did however indicate evidence for species-specific leaf $\delta^{15}N$ enrichment under the different treatments. Only *P. annua* showed an effect of soil warming on $\delta^{15}N$, with lower $\delta^{15}N$ enrichment in the warmed than control treatments ($p < 0.05$) (Fig. 4). The ^{15}N-form (glycine, NH_4^+, or NO_3^-) supplied to the grasses significantly affected $\delta^{15}N$ enrichment ($p < 0.05$), with significantly higher $\delta^{15}N$ in NO_3^- (*P. cookii*), NH_4^+ (*P. annua*) or both iN forms (*A. stolonifera*) compared to glycine (Fig. 4). Fertilisation treatments influenced both *A. stolonifera* and *P. annua* $\delta^{15}N$, which was significantly higher in unfertilised treatments ($p < 0.05$; Fig. 4). There was a significant interaction between ^{15}N-form and fertilisation treatment for *P. magellanicus* ($p < 0.05$), where unfertilised NO_3^--supplied plants had significantly higher $\delta^{15}N$ (Fig. 4).

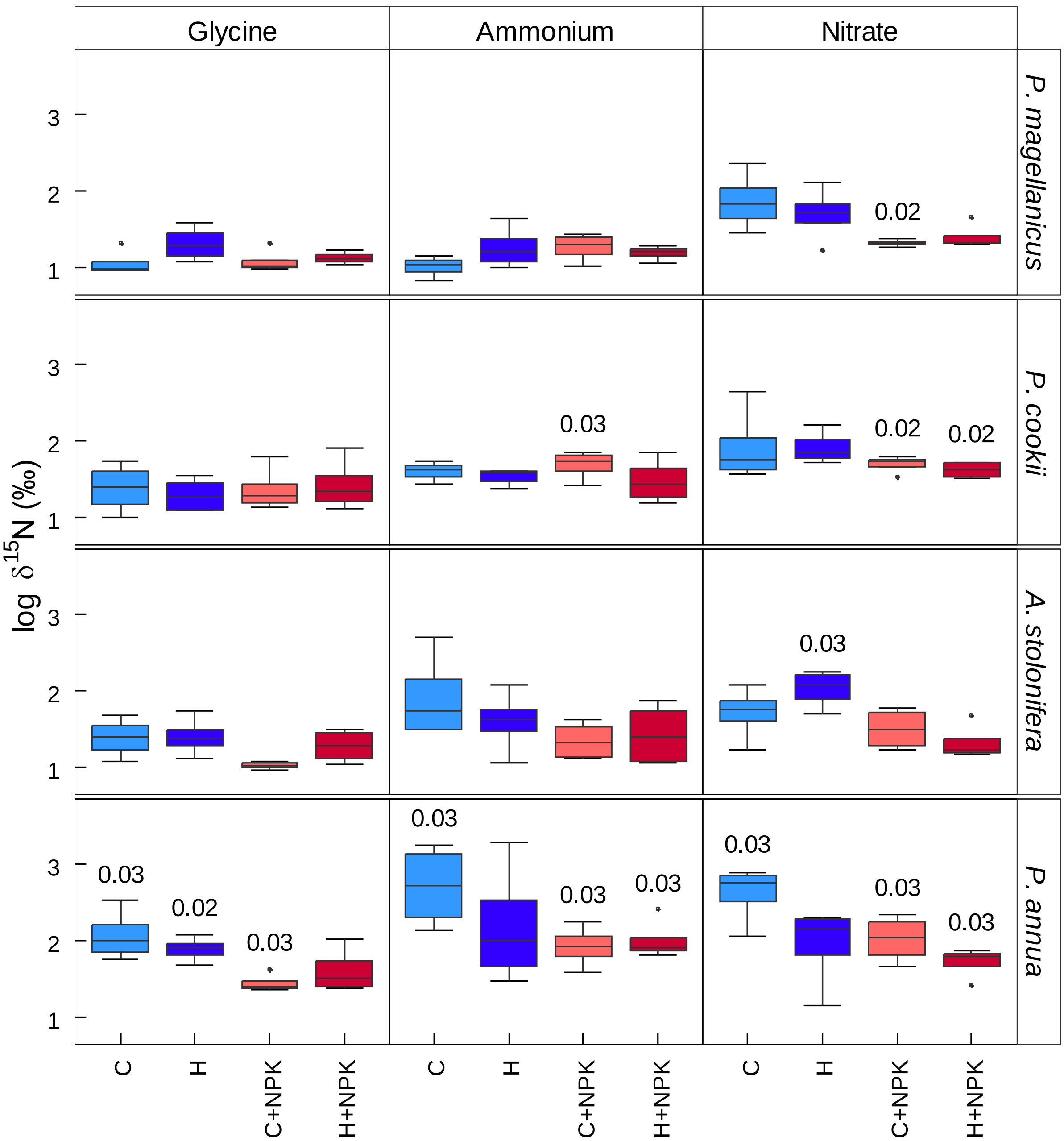

Fig. 3: Leaf $\delta^{15}N$ enrichment ($\delta^{15}N_{enrichment} = \delta^{15}N_{enriched} - \delta^{15}N_{unenriched}$) for each species on the warming, fertilisation, and ^{15}N-form treatments. Boxes with *p-values* are the treatments with significant enrichment ($\delta^{15}N > 0$) according to the one-sample t-test. The *t*-values, *p*- values, and adjusted *p*-values for all species and treatments are in Table S6. Data are logged (due to negative values, $\delta^{15}N + 10$ were logged) to improve visual display, due to large variation.

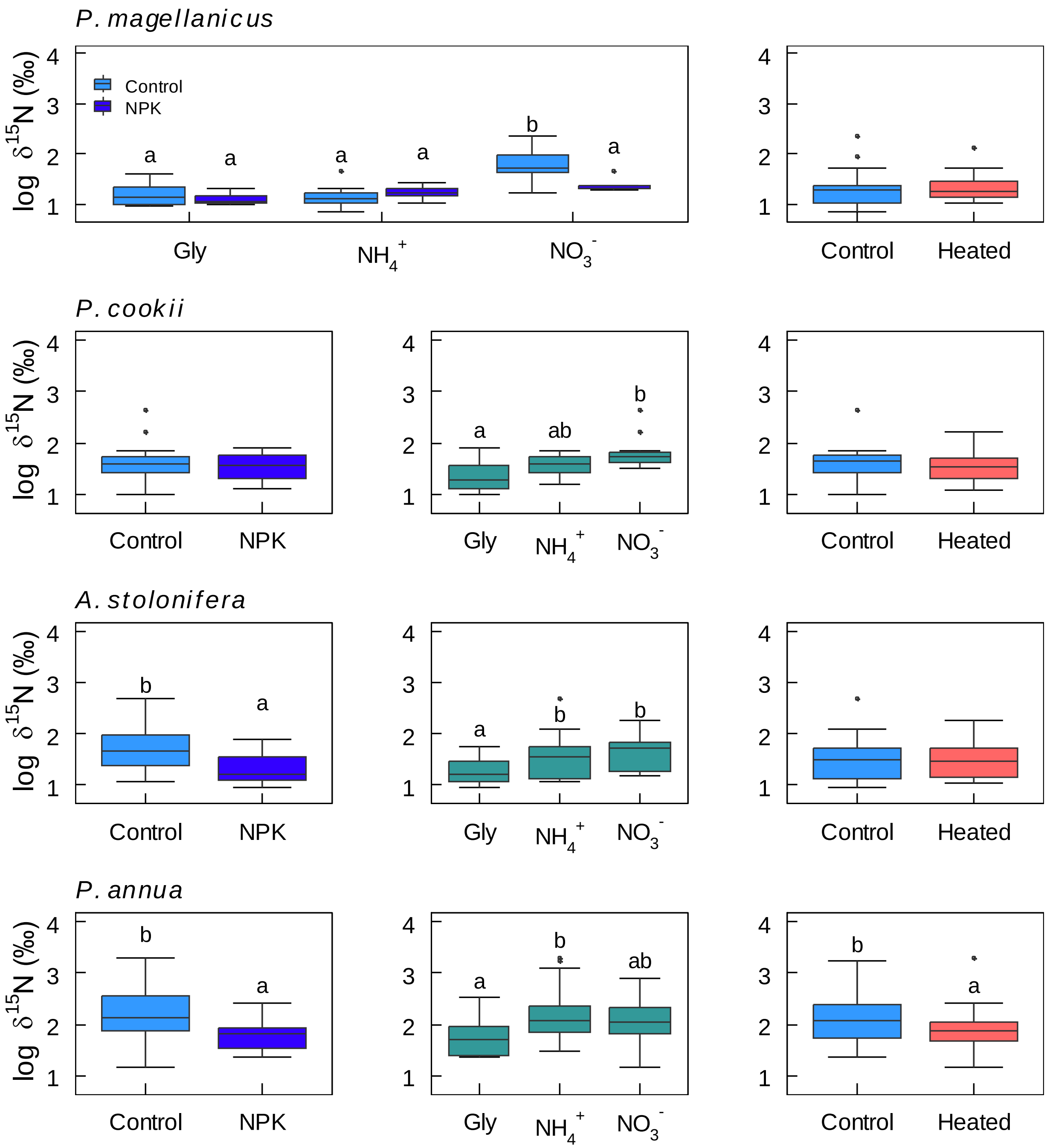

Fig. 4: Leaf δ^{15}N enrichment ($\delta^{15}N_{enrichment} = \delta^{15}N_{enriched} - \delta^{15}N_{unenriched}$) for each species on the warming, fertilisation, and ^{15}N-form treatments. There was evidence for a significant interaction between fertilisation and ^{15}N-form for *P. magellanicus* ($F_{2,\,40} = 5.06$, $p = 0.011$). Furthermore, there was a significant effect of ^{15}N-form on δ^{15}N for *A. stolonifera* ($F_{2,\,43} = 6.18$, $p = 0.004$), *P. annua* ($F_{2,\,43} = 5.43$, $p = 0.0079$) and *P. cookii* ($F_{2,\,40} = 9.46$, $p = 0.0004$). Both *P. annua* and *A. stolonifera* showed a significant effect of fertilisation on δ^{15}N ($F_{1,\,43} = 14.9$, $p < 0.001$ for *A.*

stolonifera; $F_{1, 43}$ = 15.13, p < 0.001 for *P. annua*). Only *P. annua* showed a significant effect of warming on $\delta^{15}N$ ($F_{1,43}$ = 4.15, p = 0.048). Data are logged (due to negative values, $\delta^{15}N$ + 10 were logged) to improve visual display, due to large variation. Letters indicate significant differences between treatments from an emmeans post-hoc test at the α = 0.05 significance level.

3.4.4. Soil N and P and microbial C, N, and P under warming and increasing TOC

The heating cable from the incubation experiment heated soil cores above the control temperature, where the mean control and two heated temperatures were 5.10 ± 0.16°C, 8.03 ± 0.18°C and 11.10 ± 0.54°C, respectively (Fig. S5b). There were differences in TOC between the sites where the soils were collected from (p < 0.001, Fig. S9a). The soils were maintained at a high SWC for the duration of the experiment, and all but one site (‘Low’) showed no difference in soil core mass between the start and end of the experiment (Fig. S9b). While there were strong site differences on SWC (p < 0.001), there was no evidence for an effect of warming, indicating that warming had not dried soil cores (Fig. S9c).

There was a significant positive relationship between soil iN and TOC (p < 0.05) and a negative relationship between soil PO_4^{3-} and TOC (p < 0.001), but oN showed no evidence for a relationship with TOC (Fig. 5). There was no evidence for a warming effect at either +3°C or +6°C relative to the controls for these variables. While microbial biomass significantly increased with TOC (p < 0.01), this relationship remained unaffected by the increased temperature. There was no evidence for a relationship between microbial N and TOC, and no effect of warming (Fig. 5). Microbial P showed a significant interaction between TOC and the +6°C temperature treatment, increasing P immobilisation at the higher temperature (Fig. 5). The R^2 values for the significant relationships with TOC were very low, indicating high variation and that TOC alone is not a good predictor variable for soil or microbial N and P.

To determine whether excessive SWC was a confounding factor in the experiment, SWC (%) was regressed against soil nutrients. There was a positive relationship between SWC and soil NH_4^+ but a negative relationship between SWC and soil NO_3 (p < 0.05), and thus no relationship between SWC and iN (Fig. S10). Furthermore, oN and PO_4^{3-} had a positive relationship with SWC (p < 0.05), but TOC showed no change (Fig. S10). The Adj. R^2 values for these regressions were very small (from 0.03 to 0.35 for significant relationships), indicating high variation in the data and that despite significant regressions, SWC was not a good predictor variable. Microbial C, N, and P all showed a positive relationship with SWC ($p < 0.001$), and SWC explained *ca*. 30 % of the variation in microbial C, N, and P.

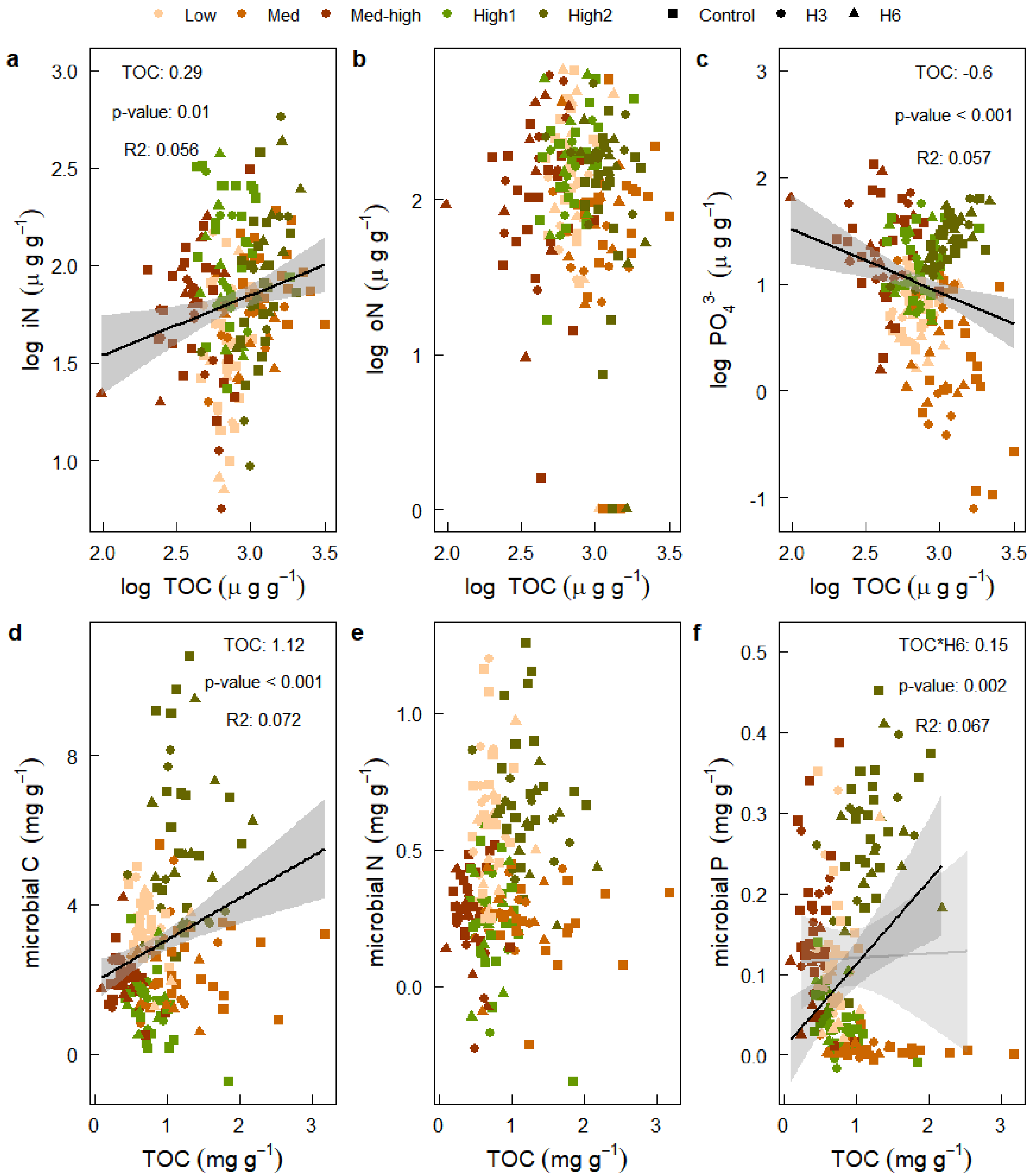

Fig. 5: Soil and microbial variables regressed against soil TOC. Point colours are according to the sites and their vertebrate influence, and shapes for different warming treatments (■ control, ● +3°C ("H3") and ▲ +6°C ("H6")). Where a significant effect was detected, model coefficients and linear regression lines are presented on the plot. **a)** Soil iN, where there was evidence for a significant

relationship between soil iN and TOC ($F_{1,191}$ = 6.67, p = 0.01). There was no evidence for an effect of warming. **b, e)** Soil oN and microbial N with TOC. These parameters showed no evidence for a relationship between the respective dependent variables and TOC, and no effect of warming. **c**) Soil PO_4^{3-}, which showed a negative relationship with TOC ($F_{1, 195}$ = 11.38, p < 0.001) but no effect of warming. **d**) Microbial C showed a positive relationship with TOC ($F_{1, 195}$ = 14.55, p < 0.001) but no effect of warming. **f**) Microbial P, where there was evidence for a significant interaction between TOC and the +6°C treatment ($F_{3, 192}$ = 3.55, p = 0.016). Grey line shows the control, and black line the +6°C treatment. Where necessary to meet model assumptions (normality and homoscedasticity), the continuous data were logged. R^2 values presented are marginal R^2.

3.5. Discussion

Contrary to the hypotheses and evidence from the literature, soil warming resulted in marginal or non-significant grass biomass and soil nutrient and microbial responses. However, while the responses recorded in the current study were relatively small, warming did stimulate soil [iN] release in the potted experiment, increase leaf [N], and increase biomass for *P. annua*. While most short-term studies document significant increases in plant biomass and microbial activity (e.g., Rustad et al. 2001; Bai et al. 2013), limited or non-significant responses to warming have been reported. For example, the three tundra sites in Bai et al. (2013) resulted in no significant soil [iN] response, despite the overall increase in soil iN pools (by 20%) when sites from lower latitudes were included. Thus, the data presented here showing only a 12% increase in NH_4^+ in the potted experiment and no change in the incubation experiment contributes to the evidence for limited increases in microbial mineralisation under warming. This is further shown by the limited responses in microbial biomass and N, which did not show a detectable warming response despite the large temperature increase. The increased leaf [N] and *P. annua* biomass with warming, although significant, were relatively small, suggesting that temperature is not the only limiting factor for both plant and microbial activity.

The substantially higher increases in biomass and leaf [N] in fertilised as opposed to unfertilised plants suggests that despite soil warming and therewith increased soil [iN], there were nutrient limitations to plant growth. When NPK was added, all species increased biomass,, and by a far larger margin than the warming-induced increases (e.g., 449% for fertilisation compared to 24% for warming across all species, respectively). Larger fertiliser- than warming-induced biomass increases have been reported previously, although usually only following long-term warming and may thus be attributed to the transitory nature of stimulating mineralisation (e.g., Jonasson et al. 1999; Graglia et al. 2001; Rinnan et al. 2007). These results show that even at shorter timescales the use of fertilisation as a positive control may overestimate plant warming responses over both long and short timescales. Furthermore, although increased leaf [N] under warming treatments is interpreted as indirect evidence for increased soil N-release (Bai et al. 2013; Sistla et al. 2013), the lack of concomitant increases in biomass may indicate limitations of other nutrients. Indeed, this experiment documented increased soil [iN] but not [PO_4^{3-}], and decoupling between N and P mineralisation with warming have been shown elsewhere (Geng et al. 2017). Therefore, warming-induced increases in soil N were either too low to support significant changes in biomass, or the lack of simultaneous increases in P or K (which the fertiliser provided) restricted most species from increasing biomass production. Therefore, nutrient fertilisation under warming should not be assumed, and the use of fertiliser treatments to approximate warming effects should be interpreted with caution.

Contrary to the hypothesis, soil warming and increases in [iN] did not alter the N-form predominantly used by the plants with warming. In cold ecosystems, the higher concentrations of bioavailable oN than iN result in higher plant oN acquisition (Kielland 1994, 2001), and thus large alterations in the iN:oN ratio are presumed necessary to alter the predominant N-form acquired. In the current study, a 12% increase in soil NH_4^+ may not have strongly altered the relative available fractions; mean iN relative to oN in the potted experiment was 48 ± 5.2 and 193 ± 27 µg g^{-1} respectively (Table S7). Therefore, it is relatively unsurprising that no change in N-form acquisition was detected. Furthermore, differences in N-form use following warming have been shown when plants are grown in competitive mixtures or in the natural environment (Kuster et al. 2016; Jiang et al. 2018). This may suggest that alterations in N-form acquisition depend on the presence of competition for a resource, in line with the hypothesis that resource partitioning by N-form allows for co-existence (Weigelt et al. 2005; Kahmen et al. 2006; Miller et al. 2007a; Tian et al. 2020).

The determination of intact oN acquisition with amino acid isotopes is a challenging measurement, due to possible microbial mineralisation and plant respiration, for both the use of single-labelled (^{15}N) and dual-labelled ($^{13}C^{15}N$) isotopes (Warren 2012). The current study only had access to single-labelled ^{15}N-oN. Given the low bacterial activity and microbial mineralisation rates for the island (Grobler et al. 1987; Smith and Steenkamp 1992a) and low concentrations of ^{15}N-glycine provided relative to the high soil [oN], mineralisation of ^{15}N glycine was assumed to be low. The limitations of single-labelled isotopes were taken into consideration with the interpretation of the ^{15}N data; however, given the lack of evidence for a significant effect of warming on the ^{15}N-form acquired, the use of single-labelled isotopes had limited effects on the interpretation of these results.

Short-term soil warming in the incubation experiment did not affect soil nutrients or microbial biomass and N, despite the large temperature increases (+3°C and +6°C). The lack of a detectable increase in soil [iN] contrasts evidence from the literature (e.g., Hobbie 1996; Rustad et al. 2001, Salazar et al. 2020) and the results from the planted experiment which found a significant increase in NH_4^+, which may suggest that the experiment duration was too short (42 d compared to five months) to detect differences. Furthermore, despite other evidence on the contrary (e.g., Nadelhoffer et al. 1991; Jonasson et al. 1999; Rinnan et al. 2007; Brzostek et al. 2012; Barnard et al. 2020), soil oN and PO_4^{3-} and microbial C and N did not change with warming, adding to the evidence for limited warming effects on decomposition. Despite the short generation time for microorganisms and evidence for increased microbial biomass with warming (Pendall et al. 2004), other studies have documented a lack of temperature response in microbial biomass (Jonasson et al. 1999). This suggests some level of microbial population resilience to short-term warming, and indeed, evidence shows increased microbial biomass and altered community composition are detected only following 15 years of warming and altered vegetation composition (Rinnan et al. 2007).

In line with the hypothesis, soil TOC had a significant relationship with microbial biomass and soil nutrients. Microbial biomass increased with soil TOC, in line with the hypothesis that increased organic substrates allow for higher microbial biomass. Other evidence from MI shows higher microbial activity and diversity at sites with high vertebrate influence (French and Smith 1986; Grobler et al. 1987). Soil inorganic nutrients N and P had significant but opposite relationships with TOC, where iN increased but PO_4^{3-} decreased. The differences between iN and PO_4^{3-} with warming and soil TOC present an interesting result and may suggest a decoupling between these nutrients under future warming scenarios. Indeed, microbial P increased with TOC and warming (+6°C), which may explain the lack of increased bioavailable PO_4^{3-} in the soil and indicate an increasing P limitation for plants under warming scenarios (Geng et al. 2017). Should these results persist, and not represent a transitory response in microbial mineralisation, it may affect plant-available N and P ratios which may have far-reaching consequences on plant nutrition and species composition (Güsewell 2004). It must be noted that the relationship between TOC, temperature, and microbial P was weak, indicating the presence of other important but unaccounted for variables that may explain microbial P immobilisation. Limited P availability has been linked to reduced plant biomass responses to increased N (Madan et al. 2007), and this evidence suggests the possibility of a decoupled relationship between N and P with warming in the MI ecosystem.

The limited response of soil and microbial variables to warming was unexpected, given the evidence for strong short-term responses and the high temperature increase implemented. Studies often cite high SWC as a limiting effect on soil and microbial warming responses, as both low and excessive moisture limits microbial responses to warming (Allison and Treseder 2008; Beier et al. 2008; Falloon et al. 2011). However, most variables (NH_4^+, oN, PO_4^{3-}, microbial C, N and P) showed positive (but weak) relationships when regressed against SWC, which may suggest that sub-Antarctic microbial communities are adapted to the high SWC of the environment. Moreover, the high variance between samples may be an effect of the high heterogeneity within the soil cores. Soils represent a highly heterogeneous environment, and microbial activity occurs in small oases of activity (Classen et al. 2015). The high variance in TOC and lack of detectable temperature responses may thus be an effect of the scale at which this experiment was conducted. For example, while one soil core represents the environment utilised by an individual plant, cores have been estimated to hold $10^6 – 10^8$ microbes, representing meta-communities (Classen et al. 2015). Other studies have found that the high spatial heterogeneity, even on seemingly small scales (e.g., within one soil core), renders difficulties in detecting short-term changes in microbial nutrient content (Schmidt et al. 2002). Overall, however, these results show that temperature may not be the only limitation to plant-soil interactions in cold ecosystems, and highlight the importance of other variables.

The planted experiment showed evidence for species-specific responses to warming. In this study, *P. annua* increased in biomass under warming and *A. stolonifera* showed similar positive RGR in unfertilised treatments. However, *P. magellanicus* and *P. cookii* decreased in biomass over time in the unfertilised treatments. This may indicate insufficient nutrients to promote plant growth in the soils used in this experiment, and a low tolerance for disturbance by the two native and slow-growing species (Mathakutha et al. 2019). The invasive species, however, are ruderal, with high disturbance tolerance (Mathakutha et al. 2019) which may have allowed for growth following transplantation. Furthermore, *P. annua* has been shown to have a high capacity for nutrient acquisition in otherwise limiting environments (Cavieres et al. 2018; Rudak et al. 2019), thus allowing it to increase biomass under warming. *Poa annua* is a highly invasive grass with a cosmopolitan distribution and is currently the only invasive vascular plant on the Antarctic continent (Molina-Montenegro et al. 2016, 2019). The evidence for a high capacity for resource acquisition under warming by *P. annua* warrants further attention, as it may lead to increased competition with native species. Indeed, a high resource acquisition capacity may contribute to the documented range expansion for *P. annua* and other invasive species (le Roux et al. 2013).

The current study largely focussed on the indirect effects (N-release) of warming on plant biomass. However, soil warming may have directly influenced plant responses. For example, the documented increase in leaf [N], while often interpreted as indirect evidence for increased soil N (e.g., Bai et al. 2013; Sistla et al. 2013), may be due to increased root biomass, which could increase plant nutrient acquisition (BassiriRad 2000; Yin et al. 2013). Furthermore, the increased biomass in *P. annua* may be due to increased root growth and nutrient absorption, increasing not only N uptake but also contact with other nutrients. *Poa annua* has shown heightened photosynthetic responses to increasing temperature (Ripley et al. 2020) and has high plasticity for biomass allocation under limiting conditions (Rudak et al. 2019); traits that may have contributed to the documented biomass increases. Although soil warming increased soil iN, the extent to which higher N promoted *P. annua* growth relative to possible interactive direct effects, including higher N diffusibility, uptake, and root growth (BassiriRad 2000; Qiao et al. 2016) requires further investigation.

3.5.1. Conclusion

Plant-soil relationships involve multiple complex and highly interactive processes. The limited results for increased biomass may suggest that the predictions for high N-release and productivity increases under warming (e.g., Kirschbaum 1995) may not have accounted for interacting and thus regulating processes within plant-soil interactions. The current study adds to the evidence that increased soil decomposition rates and productivity are not ubiquitous across sites or species. I suggest that increased N without concomitant increases in other macronutrients limits plant responses to soil warming, and that the mineralisation of nutrients such as P under warming warrant investigation. Furthermore, this study shows that fertiliser use does not necessarily represent the effects of soil warming. Importantly, however, the lack of significant results does not preclude the importance of temperature in cold ecosystems. Studies run over a longer term have identified mechanisms that may explain a lag in response to warming. For example, there is evidence that microbial biomass and community only changes following long-term vegetation changes (Rinnan et al. 2007), and indeed higher vegetation than temperature control over decomposition rates (Steinauer et al. 2015; Ward et al. 2015). This suggests the presence of confounding mechanisms that contribute to resilience in short-term climate perturbations, which may be especially relevant when investigating change on interactive processes.

Chapter 4

General discussion and synthesis

This thesis explored the hypothesis that sub-Antarctic MI grasses acquire organic N (oN) and that soil warming influences plant growth and nutrition through stimulating microbial inorganic N (iN) mineralisation and altering plant-available oN and iN fractions. Temperature-limited microbial mineralisation rates result in higher bioavailable oN than iN soil fractions, which may affect plant N-form acquisition and growth (Lipson and Näsholm 2001; Cambui et al. 2011). Furthermore, the low temperatures and thus N-limited productivity have resulted in predictions that climate warming will increase microbial iN mineralisation, indirectly stimulating plant productivity and altering the relative fractions of available N-forms (Kirschbaum 1995; Kuster et al. 2016). From this study, sub-Antarctic grasses were able to acquire oN but growth responses on oN were species-specific. Furthermore, while soil warming increased soil [iN] it did not alter the N-form acquired by grasses and had no effect on plant growth except for *P. annua* which significantly increased biomass under warming. These results show the importance of investigating prevailing assumptions about the functioning of cold ecosystem productivity and abiotic limitations in diverse contexts. This includes the current description of the sub-Antarctic soil N cycle (e.g., Smith 1977, 2008), and the interplay between abiotic drivers and plant-soil processes under global change. Drawing from the literature, I comment on the evidence presented in the data chapters in light of sub-Antarctic soil N-cycling, and plant-soil interactions on a broader scale. Furthermore, I discuss the evidence for species-specific responses to the different experimental manipulations and the evidence that nutrients other than N may limit plant responses to increased microbial mineralisation.

4.1. Plant oN uptake: where to now?

Although the physiological capacity for plant oN acquisition has long been established, the paradigm shift acknowledging and documenting its ecological relevance is relatively recent (Aerts and Chapin 1999; Schimel and Bennett 2004). There is a consensus among ecological studies that plants use oN directly, and that it forms an important N resource where it represents a substantial portion of the soil N pool (Näsholm et al. 2009; Paungfoo-Lonhienne et al. 2012; Moe 2013). However, as a relatively new avenue of research in plant-soil-N interactions, understanding the extent to which oN forms an N-resource and investigating widespread oN use has proven challenging. Furthermore, as evidence of a 'lag-effect' following the paradigm shift, some studies and natural systems have neglected to account for plant oN acquisition and thus its role in plant nutrition. For example, a recent study evaluating the extent to which microbes drive soil N availability assumed that iN is a critical control of ecosystem productivity without considering the role of oN (Li et al. 2019). The sub-Antarctic provides a good example of a system in which oN uptake has not been considered, despite the large fraction of oN and paucity of iN (Smith and Steenkamp 1992a). However, the evidence for high uptake and growth on oN presented in the current study suggests oN is an important resource for sub-Antarctic grass nutrition.

The limitations associated with quantifying oN availability and use *in situ* contribute to the challenges of this paradigm shift (Schimel and Bennett 2004). This includes limitations with the methods commonly used to document N-form use by plants, such as supplying plants with $^{13}C^{15}N$ isotopes, where results and interpretations can be misleading (see Jones et al. 2005a; Warren 2012). Methods to overcome the limitations of isotope tracers have been suggested, including decreasing time between isotope provision and harvest to minimise ^{13}C respiration or separate ^{13}C and ^{15}N uptake, and simultaneous collection of soil with plant material to determine plant and microbial $^{13}C^{15}N$ isotope signatures (Warren 2012; Wilkinson et al. 2015). Furthermore, investigations into soil oN components are still in their infancy (Warren 2014). Soil oN components are challenging to quantify as they include a diverse array of compounds that together constitute a large pool, and thus there are few methods suitable and widely available for oN characterisation (Jones and Kielland 2012; Moe 2013; Warren 2014). Therefore, although there is widespread evidence for plant growth on oN and its direct acquisition *in situ*, these limitations render most evidence for oN acquisition largely qualitative. To comprehend plant oN use quantitatively requires effort into the characterisation of soil oN pools (Warren 2014) and the widespread use of a rigorous and standardised approach to determining intact oN uptake (Jones et al. 2005a; Warren 2012). This is important, as describing the extent to which plants acquire oN will not only aid our understanding of plant nutrition, but also rhizosphere interactions such as plant-microbe competition and ultimately soil N cycling.

Quantification of oN and iN uptake *in situ* is particularly relevant considering the evidence that N-forms represent different ecological niches (Kahmen et al. 2006; Miller et al. 2007a) and that species have different growth responses on different N-forms. Plant biomass allocation when grown with glycine relative to NO_3^- provision was species-specific, and if these differences are sustained in the field, it may affect competition and uptake rates of N, and interspecies competition. However, field soil N exhibits high spatiotemporal heterogeneity, thus hydroponics experiments may not predict *in situ* biomass allocation responses (Hodge 2004, 2009). Plant growth responses to field oN compared to iN require investigation to determine whether there are ecologically relevant energetic consequences to N-form acquisition, as has been shown in laboratory experiments (Franklin et al. 2017). Indeed, this may further inform niche partitioning hypotheses and plant competition for various N resources. Under current trends of climate warming, increased decomposition or microbial community changes may affect the relative bioavailability of iN or oN, which may lead to alterations in plant N-form acquisition, particularly when in competition (Kuster et al. 2016; Jiang et al. 2018; Pang et al. 2019). However, determining the effects of oN relative to iN uptake under warming depends on an understanding of oN and iN use *in situ*, including plant growth and niche-partitioning. These questions ultimately rely on the accurate quantification of oN resources and their acquisition by plants.

4.2. Plant-soil interactions under global change

The responses of cold ecosystem flora to increasing temperatures are widely documented, however, the diversity of responses, sites investigated, and variables measured makes it difficult to identify overarching trends and underlying mechanisms driving cold-ecosystem change (Bjorkman et al. 2020). The predictions for the current study assumed that cold ecosystem productivity is indirectly limited by low temperatures and thus low N availability (Chapin 1983), anticipating microbially mediated N-release under soil warming to alleviate these limitations. To this end, there is evidence for cold ecosystem change under climate warming, with evidence for greening across the Arctic (Jia et al. 2003), changes in Arctic vegetation composition under experimental and climate warming (Elmendorf et al. 2012; Bjorkman et al. 2020), and range expansion and community reorganisation on MI linked to increased temperature (le Roux and McGeoch 2008b). However, the evidence from soil- or decomposition-based literature includes a wide variety of responses to warming, suggesting that while plant productivity responds to increasing temperatures, the extent to which this is driven indirectly through N-release remains unclear.

The evidence for varying responses of decomposition to warming suggests that the early predictions for a large indirect temperature influence on plant productivity through N-release did not account for potential confounding or regulating processes. For example, some cold ecosystem sites show no response in N mineralisation to warming, including sites in Rustad et al. (2001), Bai et al. (2013), and the present study, or evidence that initial warming-induced increases in microbial activity are transitory (Romero-Olivares et al. 2017). Furthermore, there is evidence for feedbacks between extracellular enzyme and microbial activity that limit microbial responses to warming, for example, the seasonal separation between hydrolytic and oxidative enzyme activity curtails net decomposition increases (Sistla and Schimel 2013). In the present study, short-term warming increased plant biomass and soil iN marginally, suggesting that large increases in microbial activity and plant biomass with warming are not ubiquitous should not be assumed.

While limited short-term responses may suggest that the interplay between plant and soil processes confer a level of resilience to climate perturbations, it does not suggest resilience to long-term climate warming. For example, a long-term warming experiment in Sweden documented no change in microbial biomass following five years (Jonasson et al. 1999; Schmidt et al. 2002) but a significant change in microbial biomass and community composition following changes in vegetation composition after 15 years (Rinnan et al. 2007). This suggests that rather than microbial activity and N-release driving plant responses to climate warming (see Kirschbaum 1995), warming-induced vegetation changes may be a prominent driving force to microbial communities (Classen et al. 2015). Indeed, Classen et al. (2015) posit that microbial responses to warming are mediated through direct effects on vegetation. This highlights the complexity of identifying the drivers of ecosystem change under climate warming, due to biotic interactions within and between plant and soil processes. Short-term responses to warming do not represent the long-term effects, and thus there is a need for long-term climate manipulations to identify the mechanisms driving change. Long-term ecosystem change will depend on how warming influences interactive and not individual processes, highlighting the need for an integrative approach to combine plant- and soil-level responses to warming.

4.3. Are plant responses to increased N limited by other nutrients?

The questions posed in this thesis investigate the relationship between soil N and plants, however, there was evidence that N is not the only limiting nutrient for plant growth. While Arctic plant growth is generally described as N-limited, other limitations such as P availability also impede productivity (Nadelhoffer et al. 1992). In the current study, when high [N] was added to an otherwise nutrient depauperate solution (mire water), or soil warming resulted in increased [iN] but not [PO_4^{3-}], plant growth was low. However, when N was supplied in hydroponics with higher nutrient concentrations (LA solution), and soil N increased concomitantly with P and K (NPK fertilisation), plant growth increased. Limited plant responses to increased N due to insufficient P (Madan et al. 2007) may be particularly important given the evidence for different trends in soil N and P and increased microbial P immobilisation under warming. While some studies have documented increased P mineralisation under warming (Jonasson et al. 1999; Rinnan et al. 2007), this appears to be site-specific (Nadelhoffer et al. 1991), and there is some evidence that P and N availability become decoupled with warming (Geng et al. 2017). This suggests that the long-term ramifications of N release relative to other important macronutrients (such as, but not limited to, P) require further investigation.

This study and several others (e.g., Graglia et al. 2001; Rinnan et al. 2007) have shown warming does not result in similar increases in plant biomass as NPK fertilisation. This may be due to differences in the amount of N increased under warming compared to that provided by fertilisation. Alternatively, it may indicate a decoupling between N, P, K, and other nutrient availability with warming, resulting in different nutrient ratios between warming and fertilisation treatments, e.g., higher N:P in the warming relative to fertilisation treatments. Concomitant increases of macronutrients in addition to N with warming are not ubiquitous across sites and so should not be assumed. Indeed, the lack of proportional increases in the availability of macronutrients may be a strong limiting factor to increases in plant productivity. Furthermore, fertiliser application does not account for the complexity of plant-soil interactions under warming. For example, stimulated microbial activity may be a transient response and decrease over time (Romero-Olivares et al. 2017), or long-term changes in microbial and vegetation community composition may occur (Rinnan et al. 2007), influencing nutrient release and demand. Indeed, the use of fertiliser treatments to approximate long-term warming effects largely follows the early predictions for strong indirect effects of warming on plant growth through microbial nutrient release (Kirschbaum 1995). However, the evidence for stronger vegetation than warming controls on microbial communities (Classen et al. 2015; Steinauer et al. 2015; Ward et al. 2015), and that nutrient release under warming is a transient, site-specific, and nutrient-specific response questions this prediction.

4.4. Species-specific responses to experimental manipulation

The evidence for species-specific responses to experimental manipulation may be relevant to sub-Antarctic (and particularly MI) terrestrial invasion ecology. The four species under investigation included the native species *Polypogon magellanicus* and *Poa cookii* with relatively limited distributions and the invasive, cosmopolitan species *Agrostis stolonifera* and *Poa annua*. Native and invasive MI vegetation differs in functional traits associated with resource acquisition capacity, such as higher specific leaf area and specific root length in the invasive flora (Mathakutha et al. 2019). Evidence from this study suggests that the four graminoids have different growth responses under N-form provision and limiting nutrient conditions. These growth responses are likely linked to the different life history strategies that the species exhibit, between the slow-growing natives and ruderal aliens. For example, in the warming potted experiment, both *P. cookii* and *P. magellanicus* had low relative growth rates compared to *A. stolonifera* and *P. annua*, suggesting a lower disturbance tolerance under nutrient-limiting conditions. Furthermore, there were species-specific responses to biomass partitioning and leaf [N] which may indicate different strategies for N capture and competition (Miller and Cramer 2004; Hodge 2009), where *P. annua* actively sourced N under low [N] conditions but *P. cookii* and *P. magellanicus* showed a larger response to the N-form. The different results between the invasive and native species to different forms of N (oN vs iN), [N], and limiting nutrient conditions call for further investigation and may contribute to the competitive abilities of the species under future climate scenarios, should warming lead to further range expansion.

Poa annua consistently showed high responses across the experiments. This species is highly invasive, with a near global distribution including the Antarctic continent (Molina-Montenegro et al. 2019; Govaerts et al. 2021). The traits associated with the high invasibility of the species are generally attributed to highly adaptive phenotypic plasticity (Molina-Montenegro et al. 2016). This includes rapid photosynthetic acclimation to higher temperatures compared to MI native grasses (Ripley et al. 2020), high biomass allocation plasticity allowing for high resource acquisition (Rudak et al. 2019), and high capacity for N capture allowing it to compete with native Antarctic and sub-Antarctic plants (Cavieres et al. 2018). Indeed, a high capacity for nutrient uptake, shown by root proliferation under low [N] and higher $\delta^{15}N$ enrichment in warming and hydroponics experiments compared to the other species, may explain how *P. annua* increased biomass under warming whereas all other species were likely limited either by the relatively low [iN] increase or by other macronutrients. The potential for *P. annua* to outcompete native species may be of concern under future climate scenarios. However, given that *P. annua* is a ruderal species, the likelihood of this annual outcompeting native species depends largely on the extent of physical disturbance the island has faced.

4.5. Marion Island and sub-Antarctic plant-soil interactions

Following the relatively recent paradigm shift in our understanding of soil N cycling and what constitutes plant-available N (Aerts and Chapin 1999; Schimel and Bennett 2004), a wealth of literature documenting plant oN uptake across a wide range of ecosystems has emerged. The use of oN by plants *in situ* is most prevalent in soils replete in oN but not iN (Jones et al. 2005b), as is typical for most cold ecosystem soils due to temperature-limited microbial mineralisation (Kielland 1995). The soils of sub-Antarctic islands have high oN but not iN, unless proximal to large vertebrate colonies (Smith 1978; Erskine et al. 1998). For example, the total N measured using the Kjeldahl method reported 26 mg g^{-1} N, of which only 0.06% was iN at a site uninfluenced by animals, compared to 1.8% iN near a Wandering Albatross nest or 13% iN in the mud surrounding a King Penguin colony, on MI (Smith 1978). The current study measured 0.5 M K_2SO_4-extractable N, resulting in lower but still dominant oN fractions (81%) at a site not exposed to vertebrate fauna. Despite this, the sub-Antarctic terrestrial N-model is largely based on work undertaken prior to or during this paradigm shift, assuming that only iN is ecologically relevant to plants (Smith 1977, 2007, 2008; Smith and Steenkamp 1992a), representing an example of a ‘lag effect’ following the paradigm shift. Revising our understanding of N-cycling in the sub-Antarctic is necessary, as previous work underestimated the total amount of plant-available N. This is particularly relevant given the threat of climate change in the sub-Antarctic (Pendlebury and Barnes-Keoghan 2007; le Roux and McGeoch 2008a) which may influence plant-soil interactions and N availability.

The evidence presented here for oN acquisition by sub-Antarctic grasses suggests that oN represents an important but previously unacknowledged contributor to sub-Antarctic plant N requirements. Early work investigating MI soil N cycling showed that microbial mineralisation rates were insufficient to account for plant N requirements (Smith 1988; Smith and Steenkamp 1992a). This traditional model showed that soil invertebrates, including (but not limited to) Oligochaeta (earthworms), *Pringleophaga marioni* (flightless moths), and *Ectemnorhinus* larvae (weevil), release inorganic nutrients through feeding on soil organic matter, the amount of which was assumed adequate to meet plant N requirements (Smith and Steenkamp 1992b, c, 1993; Smith 2007, 2008). Plant oN acquisition was not accounted for, and only one other study in a herbfield on Macquarie Island (Schmidt and Stewart 1999) provided evidence for sub-Antarctic plant oN acquisition. The large fraction of plant-available N despite the low iN and plant growth on oN show that the sub-Antarctic and MI terrestrial N-cycling requires re-evaluation.

There is evidence for significant climate change trends on MI (le Roux and McGeoch 2008a), and this study shows evidence for limited effects of soil warming on MI grass growth, contrasting the predictions for high MI nutrient release with increasing temperatures (Smith 2002). There is however significant climate warming on the island, and indeed, vegetation range expansion has been linked to temperature increases (le Roux and McGeoch 2008b). However, the limited responses of plant biomass to warming may suggest that N increases are largely meaningless without concomitant changes in other nutrients that support plant productivity. This could have important consequences for future predictions of plant range expansions on MI. Indeed, the respective nutrient ratios, such as N:P, may contribute to the edaphic limits to range expansion shown by Cramer et al (2022). Long-term plant responses to warming on MI will however depend on the effects of both above- and below-ground warming, where this study addressed below-ground warming. Indeed, above-ground biomass shows higher rates of response to warming than below-ground biomass (Liu et al. 2021). However, allocation responses to warming are species-specific (e.g., Hollister and Flaherty 2010). Furthermore, above-ground responses to warming include phenological as well as growth responses (Bjorkman et al. 2020; Liu et al. 2021), adding complexity to understanding how long-term warming affects plants on a larger scale. Therefore, even though the documented plant responses to warming in this study were low, this does not suggest a long-term resilience to climate warming. For MI and other oceanic islands, the limited number of biotic interactions results in lower resilience to climate-induced changes in the abundance or range of different functional groups compared to continental systems, and a change in one such group can have far-reaching consequences on the integrity of the system (Harter et al. 2015).

In addition to warming trends, MI has experienced a strong decrease in rainfall since 1950 (*ca*. 27%) (le Roux and McGeoch 2008a). The relationship between SWC, plant growth, and microbial activity across the island warrants attention, given the evidence that SWC limits microbial activity both when too low and when excessive (Beier et al. 2008). Furthermore, warming and drying on the island may have an interactive effect on microbial activity, and in the short term may stimulate mineralisation and nutrient release in waterlogged soils but suppress mineralisation in dry soils (Allison and Treseder 2008; Falloon et al. 2011). When regressed against SWC, both microbial biomass and nutrient immobilisation increased, which may suggest that microbial populations are adapted to the excessive water content on the island. However, little is known of how microbial activity and thus nutrient mineralisation would respond to drying events. Furthermore, little is known of the effects of decreased precipitation on MI vegetation. Reduced rainfall has negative effects on *Azorella selago* (le Roux et al. 2005), a keystone species to the sub-Antarctic environment. Considering the high rainfall that is typical for the island and thus water-logged environment that the species are adapted to, decreased precipitation may substantially impact the island's vegetation.

From a topical MI perspective, it is necessary to determine the extent to which plants meet their N requirements through oN, microbial- or invertebrate-released iN. In a complex history of terrestrial invasions (Cooper 2008; Greve et al. 2017), the introduced *Mus musculus* (house mouse, introduced in the 1800s; Watkins and Cooper 1986) preys on soil invertebrate populations, and has significantly reduced their abundance and thus role in soil iN release (Crafford 1990; Bergstrom and Chown 1999; McClelland et al. 2018). There are plans to eradicate mice from the island within the next five years (Parkes 2014; Birdlife South Africa 2023). Releasing soil invertebrates from this predation pressure will likely influence soil N cycling and iN release

(Bergstrom and Chown 1999; Smith 2002; McClelland et al. 2018), and may influence the relative fractions of oN and iN available for acquisition by plants. Furthermore, the introduction of invasive invertebrate species may impact nutrient release through litter decomposition. For example, the invasive slug *Deroceras panormitanum* alters C:N and N:P nutrient ratios released from litter decomposition compared to the indigenous caterpillar *P. marioni*, which may impact peat nutrient quality and nutrient cycling (Smith 2007). Moreover, climate warming may affect soil N contents through stimulating microbial (although only limited increases were documented here) and invertebrate activity (Aerts 2006). However, invertebrate predation by mice is thought to preclude warming-induced nutrient release (Smith 2002). Therefore, the effects of warming on invertebrate activity depend on the success of the mouse eradication, as there is evidence that MI mouse activity and thus ecological impact has increased with warming temperatures (McClelland et al. 2018). This further emphasises the need to re-evaluate the MI soil N cycle, including plant oN acquisition and invertebrate nutrient release both with and without mouse predation.

Marion Island plant nutrition is particularly interesting given the history of mammalian invasions. Upon annexation in 1947, Marsh (1948) described the island as a "jade jewel", remarking on the lush, green MI vegetation. However, more recent descriptions have a different perception: "… the island is now more brown than green" (Chown and Froneman 2008, pp 363). Indeed, comparisons with the neighbouring Prince Edward Island (PEI) support these (perhaps subjective) observations (Chown and Froneman 2008). Following annexation, *Felis catus* (domestic cats) were introduced to MI in 1949 to rid the weather station of mice, but subsequently became feral, devastating bird populations by 450 000 burrowing petrels a year (van Aarde 1980; Watkins and Cooper 1986). Although the cats were eradicated in the 1990s (Bester et al. 2002), bird populations show only a modest recovery, attributed to an increase in bird predation by mice (Dilley et al. 2017, 2018). The cats had large indirect effects on vegetation, effectively removing substantial allochthonous input of N, P, and other nutrients in bird guano. This has been linked to decreases in tussock grassland on the island (Gremmen and Smith 2008) and may to some extent explain the islands now 'brown' appearance. Neighbouring PEI was not invaded by cats or mice, and although this has not been explicitly tested, may account for the disparities in NDVI signal between the islands. Furthermore, allochthonous nutrient deposition by vertebrates adds a diversity of nutrients in addition to N, e.g., *P, cookii* stands associated with vertebrate presence have detectably higher leaf [N] but also leaf [P], [K], [Fe], and [Na] (Smith 1978). Therefore, given the high soil [oN] but low vegetation growth and ‘brown’ appearance, vertebrate presence across such sub-Antarctic islands may play a vital role in plant general nutrition in addition to N. The impending mouse eradication will presumably release both burrowing birds and soil invertebrates from predation pressure, and therewith may increase nutrient release, and it remains to be seen whether MI will be restored to the ‘jade jewel’ of tussock grasslands it was once described. However, separating the effects of future climate change such as warming and drying from biotic changes such as increased invasion frequency (Bergstrom and Chown 1999) and the impending mouse eradication leaves future predictions of MI vegetation and its nutrition difficult and largely speculative. The proximity of Prince Edward Island, with a largely similar cohort of native plant, invertebrate and vertebrate species represents a comparative control, and one of great value should we wish to fully understand the ecological history of the PEIs in the 19th and 20th century.

4.6. Moving forward

The results from the current study serve to inform future hypotheses investigating sub-Antarctic plant nutrition. The evidence presented here suggests that sub-Antarctic plants use oN and that oN relative to iN uptake affects plant growth. In addition to representing simple ecological systems for climate change investigations (Harter et al. 2015), oceanic islands such as MI may represent a good ecosystem to investigate the hypotheses for ecological niche partitioning by N-forms and how oN relative to iN uptake affects plant growth. For example, there are large differences between soil oN and iN fractions along coastal and inland soils due to high allochthonous N deposition by vertebrate fauna (Smith 1978). Transects between areas of high and low oN:iN soil ratios but in similar environmental niches could be used, e.g., proximity from seal or seabird colonies along the coast or further inland near burrowing petrel colonies, to test the hypothesis that oN acquisition is predominant in soils with a paucity of iN to be tested. Furthermore, due to the low floral diversity, plants such as *P. magellanicus* occur both along the coast and inland, providing a good study system to test whether N-form use changes with soil oN:iN ratios within a species, and whether this affects plant growth and allocation *in situ*. Therefore, investigations into MI plant oN use are not only important for re-evaluating the soil N cycle, but the MI system may represent a good site to investigate widespread questions regarding plant oN uptake and its ecological implications.

The limited investigations into sub-Antarctic plant-soil responses to climate warming result in an abundance of variables that have not yet been considered, despite their potential importance in regulating or promoting changes in plant and soil processes. For example, future investigations need to incorporate measurements of extracellular enzyme activity and how this affects N bioavailability for plant growth and nutrition. Extracellular enzyme activity represents an important step in the soil N-cycle, as it regulates nutrient availability for microbial activity (Schimel and Bennett 2004). Extracellular enzymes generally increase activity under warming, however, enzymes involved in different processes (e.g., those involved in C, N, or P cycles) may show different temperature sensitivities (Razavi et al. 2017) which if sustained over the long term may affect soil C, N, and P ratios. Therefore, understanding future trends in microbial activity, such as mineralisation, requires investigations into extracellular enzyme responses to warming (Brzostek and Finzi 2011; Sistla and Schimel 2013).

Another avenue that requires further research, particularly for sub-Antarctic systems, is mycorrhizal associations that influence plant nutrient uptake. Mycorrhizal associations have been identified for sub-Antarctic plants, including vesicular-arbuscular and dark septate mycorrhizae (Laursen et al. 1997; Frenot et al. 2005), however, there is limited literature investigating their role in sub-Antarctic plant nutrition. Mycorrhizal associations have been suggested to be a major pathway for *in situ* oN acquisition by plants (Jones et al. 2005a; Talbot and Treseder 2010), and their associations have been linked to increased plant oN acquisition (Moe 2013), although non-mycorrhizal plants have the capacity for intact oN uptake (Näsholm et al. 2009). Moreover, mycorrhizal associations improve plant P uptake (Hodge and Storer 2015), and thus may play an important role in plant nutrient acquisition should N and P mineralisation become decoupled under climate warming. Furthermore, there is evidence for increased mycorrhizal infection with warming, effectively increasing N uptake (Qiao et al. 2016), suggesting future investigations into sub-Antarctic plant-soil interactions under warming should account for mycorrhizal symbiont responses.

4.7. Conclusion

This thesis investigated sub-Antarctic plant-soil interactions under global change, and in the wake of a paradigm shift in our understanding of plant N acquisition. Both avenues of research present topical, stimulating questions regarding plant nutrition and growth under cold ecosystem climate warming. The evidence for sub-Antarctic grass oN acquisition and limited biomass responses to soil warming contributes to our understanding of sub-Antarctic plant-soil interactions. It also highlights the importance of investigating prevailing assumptions made about plant nutrition and growth under climate warming in the late 20th century, i.e., what constitutes plant-available N, and whether warming indirectly stimulates plant growth through N-release. Although it is more than two decades since the recognition that soil oN is an ecologically relevant N resource for plant nutrition, our understanding of the extent to which it is available and acquired *in situ* is still very limited. Moreover, the decades of research investigating climate warming in northern high latitudes signifies that plant responses to warming are highly diverse and not necessarily driven indirectly by N-release. Notably, this study highlights the potential importance of nutrients in addition to N that may limit or promote plant responses to warming. The complex interplay between plant and soil processes may confer a level of resilience to short-term climate perturbations. However, this does not preclude long-term responses to warming which may be driven by changes in vegetation community composition or range expansions or contractions.

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Appendix 1: Supplementary Material

Table S1: Summary of soil Total N, organic N, and inorganic N (NO_3^- and NH_4^+), as determined by various authors on Marion Island (presented either as mean ± SE, or as a range): 1.) investigating soil from different plant communities, and 2.) the influence of animals on soil N fractions.

1. Plant community	Total N (µg g^{-1})	NH_4^+ (µg g^{-1})	NO_3^- (µg g^{-1})	Source
Polypogon magellanicus drainage line	25000 ±1805	tr. - 29	0.0 - tr.	(Smith, 1976)
Poa cookii tussock grassland	16600 ±1054	9 - 84	0.0 - 10	(Smith, 1976)
Polypogon magellanicus mire	23400 ±1328	24 - 65	0.0 - 7	(Smith, 1976)
Mire-grassland peat	16000 ± 27000	7 - 120	0 - 22	(Smith and Steenkamp, 1992a)

2. Biotic (animal) influence	oN (µg g^{-1})	NH_4^+ (µg g^{-1})	NO_3^- (µg g^{-1})	Source
Uninfluenced soil	26000 ± 2277	14 ± 3.98	2 ± 0.76	(Smith, 1978)
Slope, near King Penguin colony	29000 ± 4330	143 ± 14.66	35 ± 22.17	(Smith, 1978)
Wandering albatross nest - occupied	30000 ± 1848	545 ± 322	5 ± 0.64	(Smith, 1978)
Wandering albatross nest - unoccupied	24000 ± 1652	102 ± 39.38	19 ± 6.58	(Smith, 1978)
Giant Petrel nest - occupied	28000 ± 4503	136 ± 71.94	35 ± 20.15	(Smith, 1978)
Gentoo Penguin	36000 ± 1674	425 ± 349.3	25 ± 23.04	(Smith, 1978)
King Penguin: mud inside colony	110000 ± 6178	13442 ± 560	2603 (± 508.4)	(Smith, 1978)

tr. = traces

Table S2: Soil N concentrations including total dissolved N (TDN) and iN for the three sites in the *in situ* ^{15}N-uptake experiment, from values provided by (Smith et al. 2001) for the three habitat types. The 'Biotic' site falls under Coastal Tussock Grassland, 'Slope' under Closed Fernbrake, and 'Fellfield' under Mesic Fellfield habitats. Values presented are means and medians in parentheses and were calculated on an oven dry soil mass basis (Smith et al. 2001).

Site	Vertebrate influence	Grasses present	TDN (%)	NH_4^+ (μg g^{-1})	NO_3^- (μg g^{-1})
Biotic	High	*P. magellanicus, A. stolonifera, P. annua, P. cookii*	3.7 (3.7)	20.8 (17.3)	15.1 (4.7)
Slope	Med	*P. cookii*	2 (2)	3.7 (0.7)	1.7 (0.1)
Fellfield	Low	*P. magellanicus, P. annua*	0.8 (0.7)	0.5 (0.3)	0.0 (0.0)

Table S3: Elemental composition (mM) and pH of the two hydroponics solutions. Results from the mire experiment show means (±SE) from elemental analysis, and the expected concentrations from the Long Ashton (LA) solution made for the LA experiment.

	Mire (mM)	Long Ashton (mM)
Ca^{2+}	0.025 ± 1.5e-18	4
Mg^{2+}	0.082 ± 0.01	1.5
K^{+}	0.043 ± 0.005	2
Na^{+}	0.73 ± 0.10	4.2
Cl^{-}	1.10 ± 0.11	8
SO_4^{2-}	0.038 ± 0.007	3.53
Cu^{2+}	0.0004 ± 0.0001	0.003
Mn^{+2}	0.0005 ± 6.6e-5	0.021
Zn^{2+}	0.0007 ± 0.0002	0.002
B^{3+}	0.0009 ± 0	0.14
Fe^{2+}	0.007 ± 0.002	0.09
PO_4^{2-}	0.032 ± 3.1e-18	2.17
NH_4^{+}	0.02 ± 0.005	0
NO_3^{-}	0.05 ± 0	4 or 0.4
pH	5.1 ± 0.7	5.5

Table S4: Site details from which soil cores (n = 20) were extracted for the soil incubation experiment. Predominant vegetation, faunal influence (ranked from low to high) and type is based on field observations (April 2019 – May 2020).

Site	Predominant vegetation	Faunal influence	Vertebrate presence	Invertebrate presence
Warming experiment site	Bryophyte and *Sagina procumbens* mat	Low	None / some ad-hoc visitation by *Chionis minor* (Black-faced Sheathbills)	Earthworms present
Slope complex *ca*. 400 m from the coast	*Austroblechum penna-marina*, some *P. cookii*	Medium	Inactive *Pterodroma macroptera* (Great-winged Petrel) nests from the previous breeding season	None
Biotic slope, ≥ 3 m away from a burrowing petrel nest	*Austroblechum penna-marina*	Medium-High	*Procellaria aequinoctialis* (White-chinned Petrel): recently active nest site	Earthworms present
Biotic slope, < 1 m from a burrowing petrel nest entrance	*Austroblechum penna-marina*	High (1)	*Procellaria aequinoctialis* (White-chinned Petrel): recently active burrow	None
Biotic site at the coast	Bryophyte mat	High (2)	High trampling and manuring by Southern Elephant Seal, Antarctic fur-seal, and some penguin species	Earthworms present

Table S5: Soil sites where iButtons were placed *in situ* on MI from (08-11-2019 to 10-04-2020), with coordinates and mean ± SE soil temperatures (le Roux and Schoombie, *pers. comm*).

Site name	Coordinates	Temperature (°C)
Kampkoppie	-46.895427, 37.611773	5.48 ± 2.66
Mixed Pickle	-46.873683, 37.637732	5.88 ± 2.71
Repettos	-46.844362, 37.766535	6.32 ± 2.96
East Cape	-46.897869, 37.899481	6.31 ± 3.24
Kildalkey	-46.952965, 37.842466	5.47 ± 2.88

Table S6: Results from the one-sample t-tests testing whether there was evidence for significant $\delta^{15}N$ enrichment (i.e., $\delta^{15}N$ enrichment > 0) in the planted warming experiment, after providing the four grass species in warming and fertiliser treatments with ^{15}N-glycine, ^{15}N-NH_4^+, or ^{15}N-NO_3^-. Adjusted p-values are those calculated following the Benjamini Hochberg correction for the increased Type 1 Error rate; p-values printed **bold** indicate statistical significance.

Species	Treatment	^{15}N-form	t-value	df	p-value	Adjusted p-value
P. magellanicus	C	Glycine	0.81	3	0.479	0.489
P. magellanicus	C	NH_4^+	0.17	2	0.882	0.882
P. magellanicus	C	NO_3^-	4.54	3	0.020	0.055
P. magellanicus	C+NPK	Glycine	1.23	3	0.307	0.327
P. magellanicus	C+NPK	NH_4^+	2.96	3	0.060	0.089
P. magellanicus	C+NPK	NO_3^-	14.88	3	0.001	**0.022**
P. magellanicus	H	Glycine	2.73	3	0.072	0.093
P. magellanicus	H	NH_4^+	1.91	3	0.153	0.167
P. magellanicus	H	NO_3^-	3.88	3	0.030	0.063
P. magellanicus	H+NPK	Glycine	3.05	3	0.055	0.089
P. magellanicus	H+NPK	NH_4^+	4.09	3	0.026	0.059
P. magellanicus	H+NPK	NO_3^-	4.85	3	0.017	0.053
P. cookii	C	Glycine	2.35	3	0.100	0.114
P. cookii	C	NH_4^+	6.85	2	0.021	0.055
P. cookii	C	NO_3^-	3.79	3	0.032	0.065
P. cookii	C+NPK	Glycine	2.54	3	0.085	0.107
P. cookii	C+NPK	NH_4^+	7.07	3	0.006	**0.029**
P. cookii	C+NPK	NO_3^-	11.88	3	0.001	**0.022**
P. cookii	H	Glycine	2.51	3	0.087	0.107
P. cookii	H	NH_4^+	6.93	2	0.020	0.055
P. cookii	H	NO_3^-	6.23	2	0.025	0.059
P. cookii	H+NPK	Glycine	2.44	3	0.093	0.108
P. cookii	H+NPK	NH_4^+	3.21	3	0.049	0.084
P. cookii	H+NPK	NO_3^-	11.12	3	0.002	**0.022**
A. stolonifera	C	Glycine	2.91	3	0.062	0.089
A. stolonifera	C	NH_4^+	3.23	3	0.048	0.084
A. stolonifera	C	NO_3^-	4.05	3	0.027	0.059

A. stolonifera	C+NPK	Glycine	1.06	3	0.368	0.384
A. stolonifera	C+NPK	NH_4^+	2.77	3	0.069	0.092
A. stolonifera	C+NPK	NO_3^-	3.67	3	0.035	0.067
A. stolonifera	H	Glycine	3.13	3	0.052	0.086
A. stolonifera	H	NH_4^+	2.89	3	0.063	0.089
A. stolonifera	H	NO_3^-	8.33	3	0.004	**0.029**
A. stolonifera	H+NPK	Glycine	2.46	3	0.091	0.108
A. stolonifera	H+NPK	NH_4^+	2.09	3	0.128	0.143
A. stolonifera	H+NPK	NO_3^-	2.81	3	0.068	0.092
P. annua	C	Glycine	6.34	3	0.008	**0.029**
P. annua	C	NH_4^+	6.23	3	0.008	**0.029**
P. annua	C	NO_3^-	8.48	3	0.003	**0.029**
P. annua	C+NPK	Glycine	7.27	3	0.005	**0.029**
P. annua	C+NPK	NH_4^+	6.70	3	0.007	**0.029**
P. annua	C+NPK	NO_3^-	6.48	3	0.007	**0.029**
P. annua	H	Glycine	10.57	3	0.002	**0.022**
P. annua	H	NH_4^+	2.97	3	0.059	0.089
P. annua	H	NO_3^-	3.53	3	0.039	0.071
P. annua	H+NPK	Glycine	4.05	3	0.027	0.059
P. annua	H+NPK	NH_4^+	7.40	3	0.005	**0.029**
P. annua	H+NPK	NO_3^-	6.80	3	0.006	**0.029**

Table S7: Mean ± SE inorganic N, organic N for each site in the soil incubation experiment.

Site	iN (μg g^{-1})	oN (μg g^{-1})
Low	48.4 ± 5.2	193.0 ± 27.0
Med	72.1 ± 6.1	95.2 ± 16.8
Med-high	71.5 ± 8.8	164.0 ± 23.9
High (1)	142.0 ± 15.0	207.0 ± 23.4
High (2)	115 ± 18.3	161.0 ± 17.5

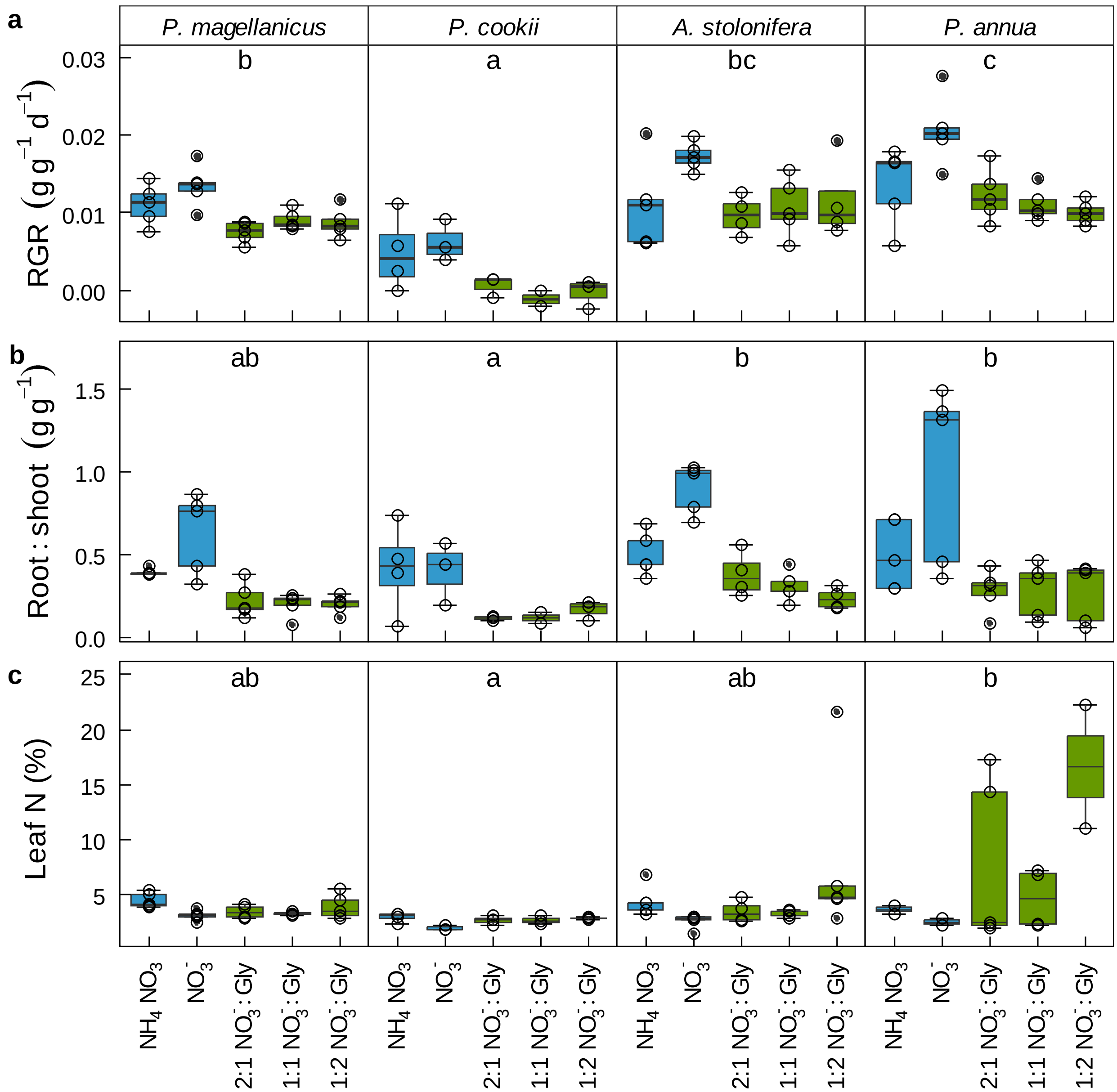

Fig. S1: Growth variables for the study species (*P. magellanicus*, *P. cookii*, *A. stolonifera*, and *P. annua*) with five different N-treatments: **a** RGR, **b** root:shoot ratio, and **c** leaf [N]. There was a significant difference between species in all response variables (RGR: $F_{3,\,80} = 39.85$, $p < 0.0001$, root:shoot ratio: $F_{3,\,80} = 7.55$, $p = 0.0002$, leaf [N]: $F_{3,\,70} = 5.25$, $p = 0.0025$), indicated by letters determined by an emmeans posthoc test. Open circles show individual data points, showing how replication decreased for some treatments, species, and variables. Letters indicate significant differences between species from an emmeans post-hoc test at the $\alpha = 0.05$ significance level.

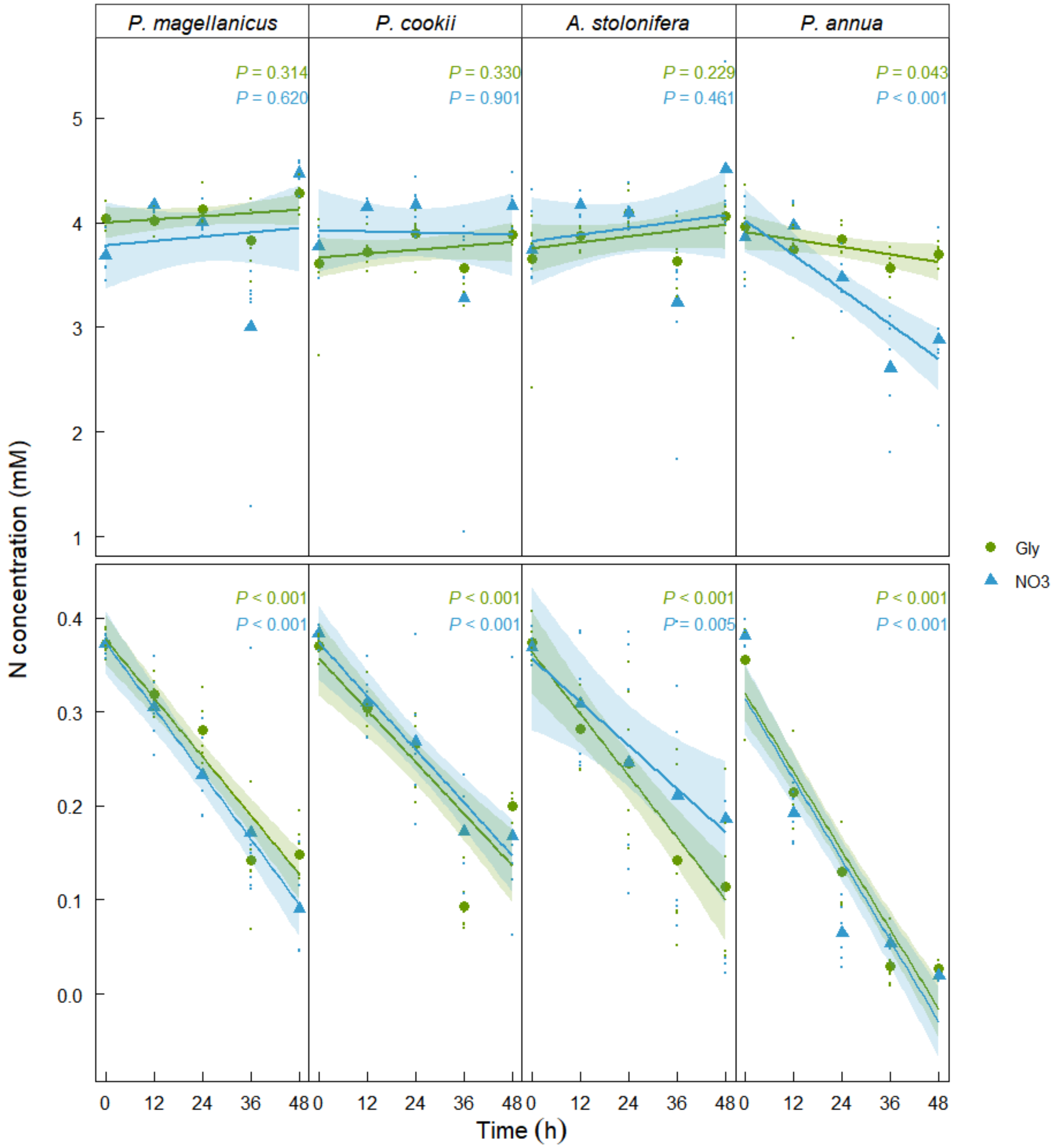

Fig. S2: Nitrogen-depletion (glycine (●, green) or NO_3^- (▲, blue)) over time for each species, in the 4 mM N (top row) and 0.4 mM N (bottom row). Points and ribbons show the individual data points and 95% CI ribbons. Diamonds and circles show mean concentrations at each time interval for NO_3^- and glycine, respectively. Plot text shows the respective *p*-value for linear models. There is a significant difference between the glycine (green) and NO_3^- (blue) slopes in the 4 mM treatment for *P. annua*, as the 95% CI's do not overlap.

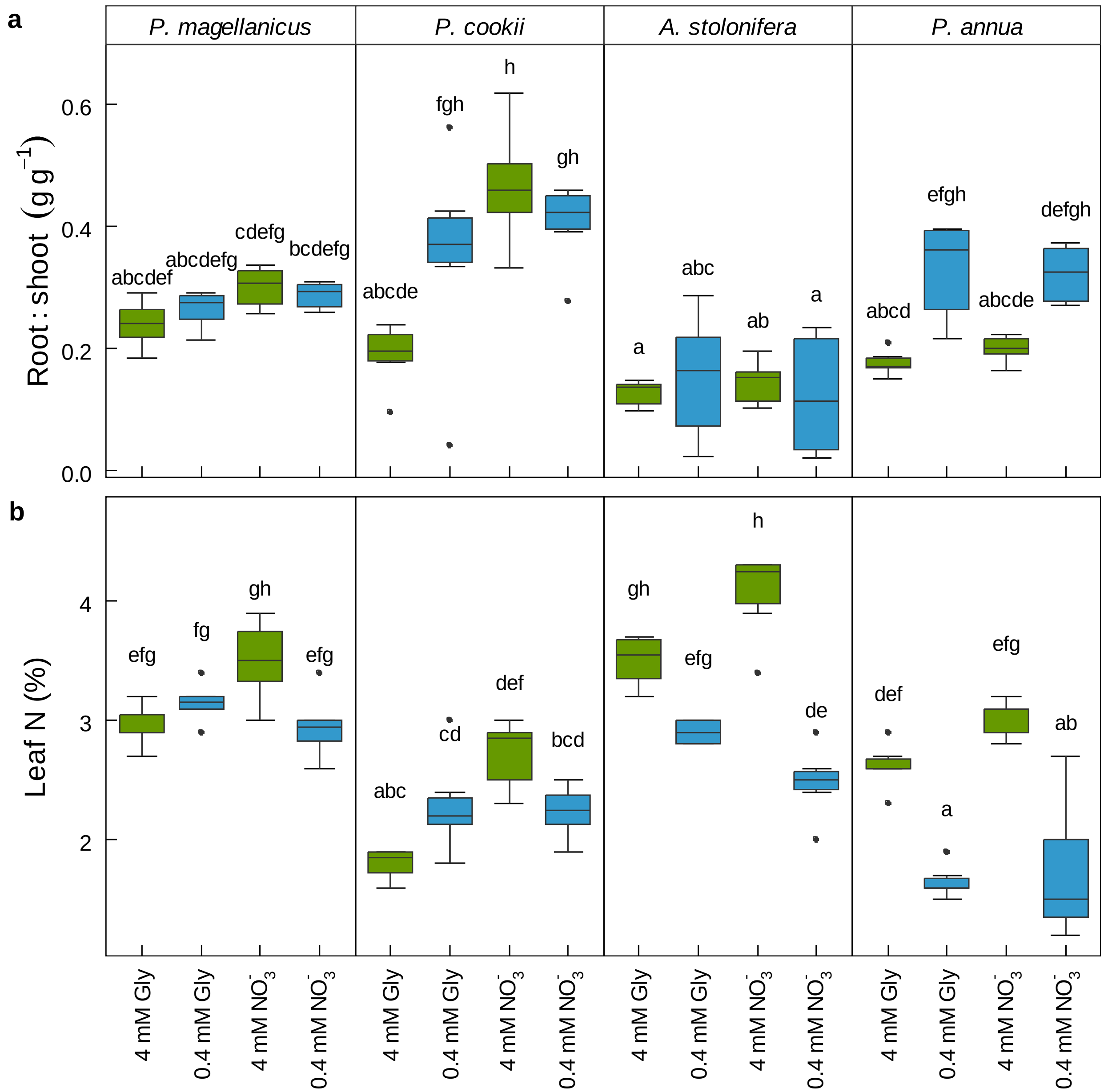

Fig. S3: Growth parameters for all study species in the four N-treatments in the LA growth experiment: a) root:shoot ratio, and b) leaf [N] concentration, displaying the results of an ANOVA including treatments and species. For root:shoot ratio and leaf [N], there was a significant interaction between species and N-treatments ($F_{3,\,78}$ = 4.25, p < 0.001; $F_{9,\,80}$ = 8.90, p < 0.001 respectively). Letters indicate significant differences from an emmeans post-hoc test at the α = 0.05 significance level.

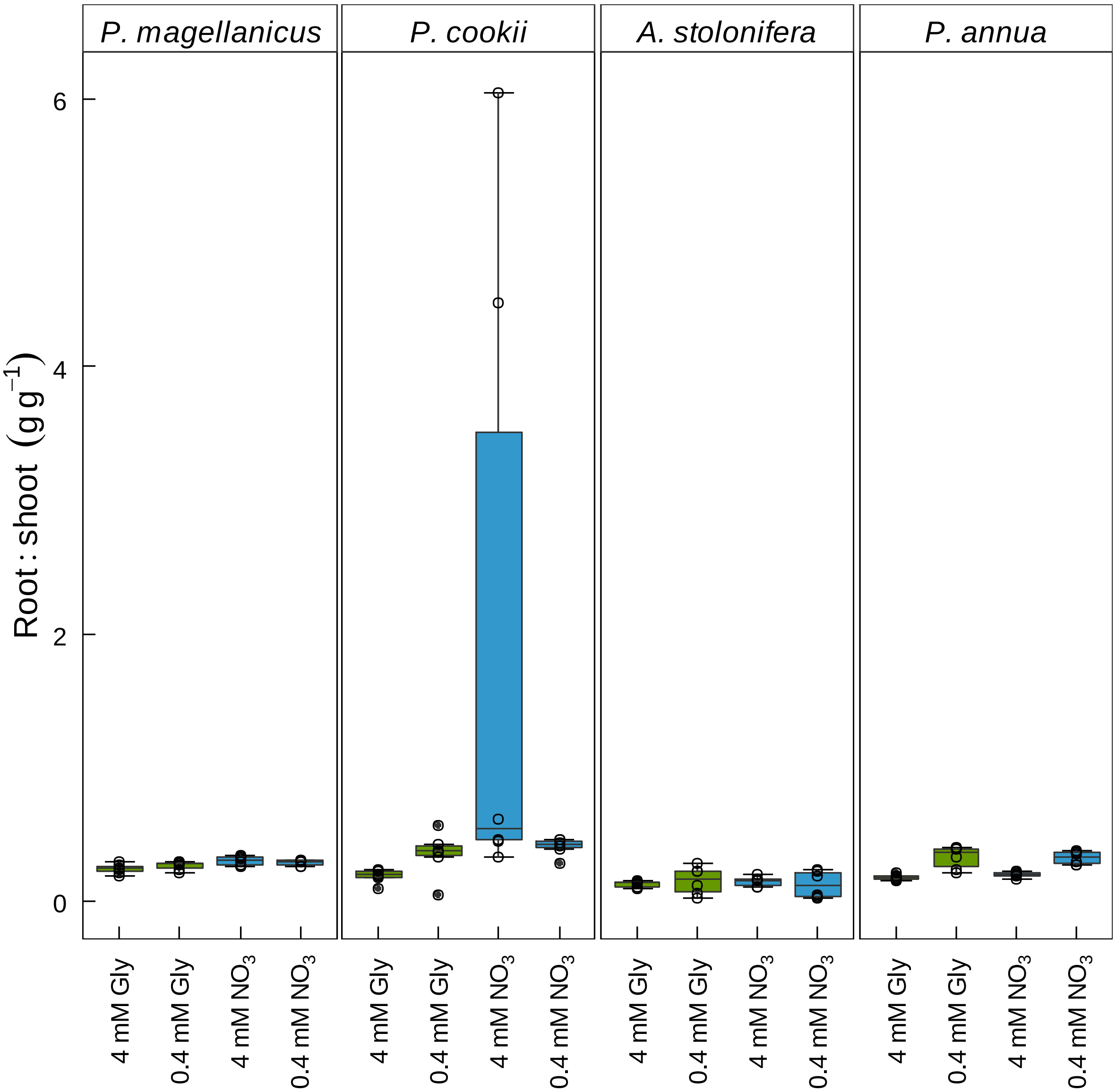

Fig. S4: Root:shoot ratio for grasses grown in Long Ashton solution with the four N-treatments of either glycine or NO_3^-, provided at 4 or 0.4 mM, including the two *P. cookii* replicates where the main tiller had died, leaving root fraction extremely high relative to the smaller, newly emergent tillers. Points show individual data points.

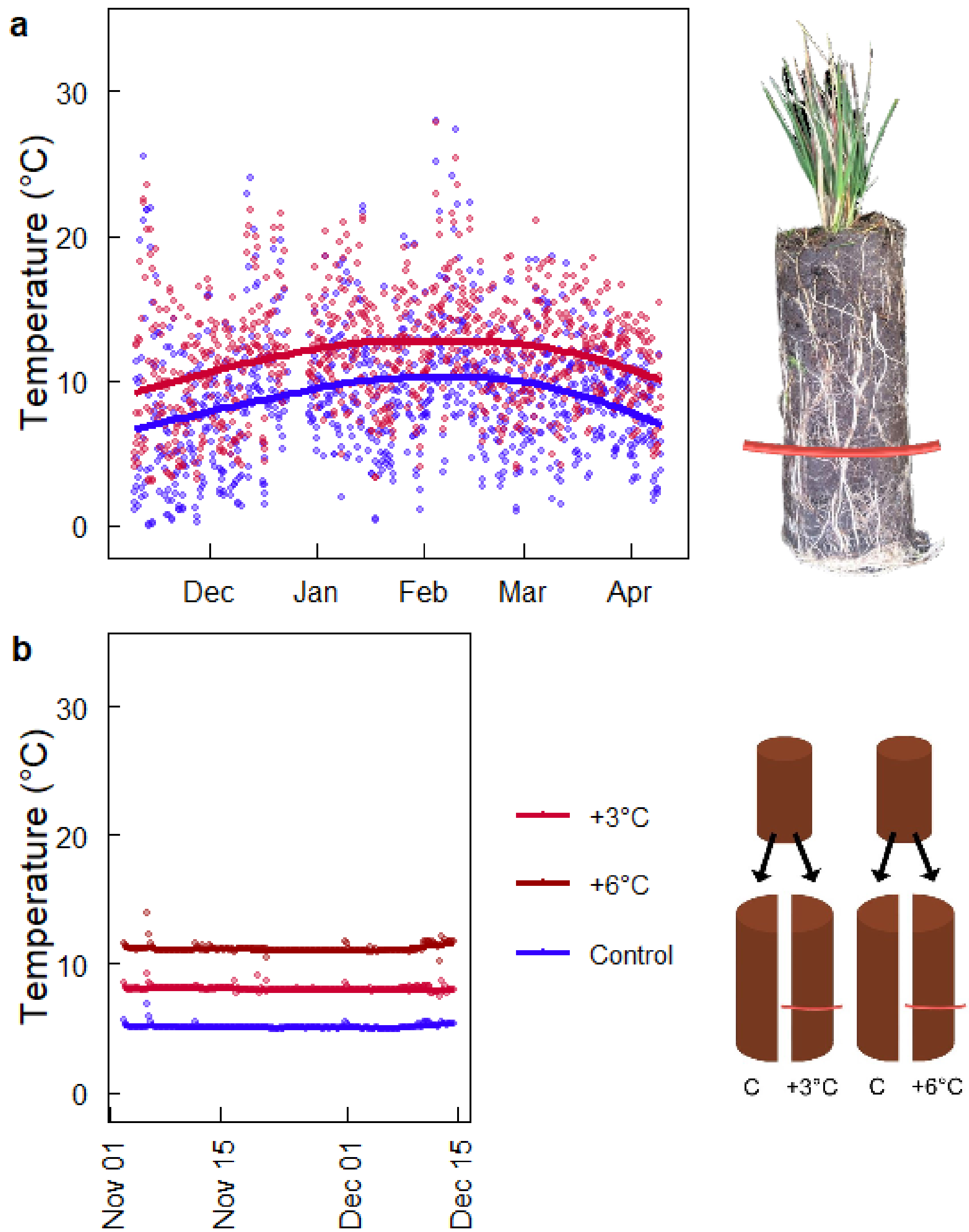

Fig. S5: Temperature per hour over time showing the control temperature (blue) and heated temperature (red), with points showing the mean temperature per hour, and lines plotted with ‘loess’ model fitting: **a** in the MI potted experiment, and **b** for the soil incubation experiment. There was an unexpected power outage for five days during late December in the MI potted experiment, resulting in no heating and no data records for that period. The red lines through the soil cores represent the position of the heating cable.

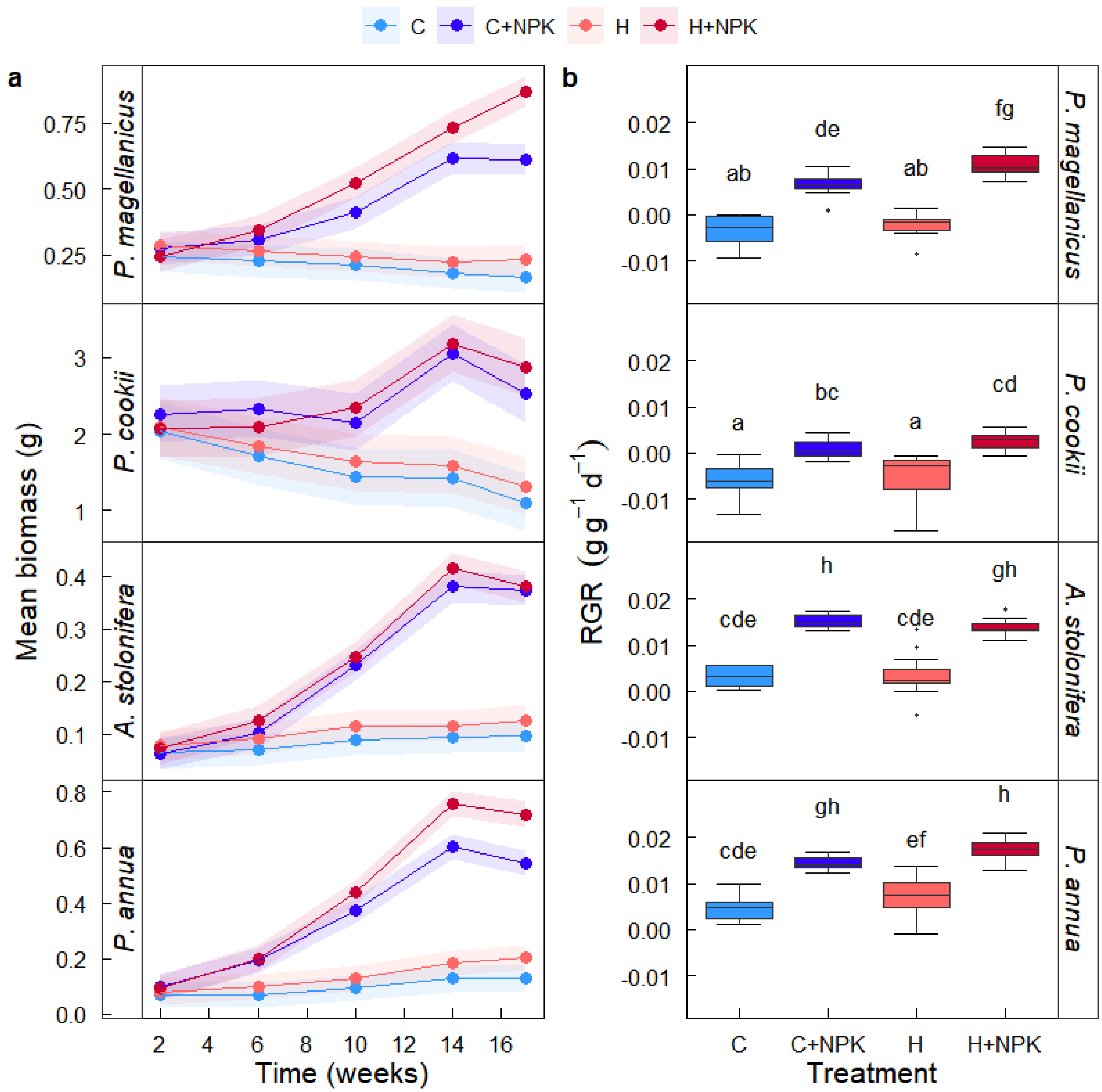

Fig. S6: Plant growth over the first 17 weeks of the experiment on the four treatments for the four species. Approximate biomass was determined as the sum of the leaf and flower mass ($P_{lant} = M_{leaf} + M_{flower}$), where $M_{leaf} = N_{tiller} * M_{dev} + [(N_{leaf} - N_{tiller}) * M_{exp}]$ and $M_{flower} = N_{inflorescence} * M_{inflorescence}$. In this equation, N_{tiller}, N_{leaf} and $N_{inflorescence}$ is the number of tillers, leaves, and inflorescences counted during the biomass assessment, and M_{dev}, M_{exp} and $M_{inflorescence}$ the mean mass of developing leaves, expanded leaves and inflorescences (respectively) from a sub-sample of n = 10 individual plants of each species (Ibrahim 2007). **a** Approximate biomass over time at each biomass assessment. Points show the mean approximate biomass and ribbons the 95% CI calculated with emmeans. There is a significant difference between heated and control treatments with NPK for *P. magellanicus* and *P. annua* at week 17, as seen by the lack of overlap in the 95% CI's. **b** Relative growth rate (RGR) for each species in each treatment. While there was no evidence for a significant interaction between the two treatments, there was evidence for a significant interaction between warming treatment and species ($F_{3, 176} = 2.96$, $p = 0.034$) and NPK treatment and species ($F_{3, 176} = 4.03$, $p = 0.0084$). Letters indicate significant differences from an emmeans post-hoc test at the $\alpha = 0.05$ significance level.

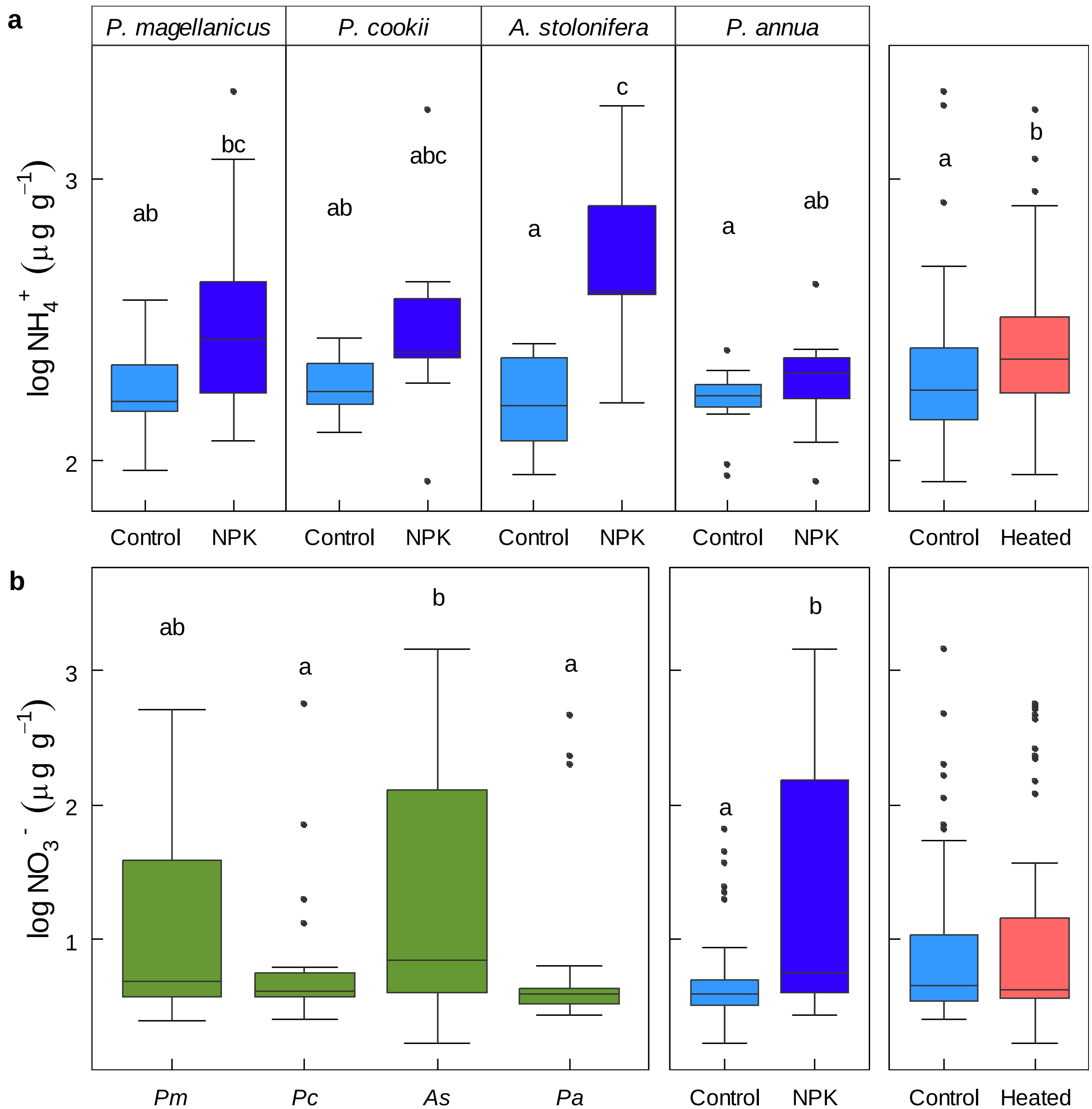

Fig. S7: Soil NH_4^+ and NO_3^- in the MI warming experiment, after five months of soil warming. **a** Soil NH_4^+, where there was evidence for a significant interaction between fertilisation treatment and species ($F_{3,\,87} = 3.55$, $p = 0.018$), and evidence for a significant effect of warming ($F_{1,\,87} = 3.99$, $p = 0.049$), with significantly higher NH_4^+ in the warmed treatments. **b** Soil NO_3^-, where there was evidence for a significant effect of species and fertilisation treatment ($F_{3,\,90} = 3.47$, $p = 0.019$; $F_{1,\,90} = 21.07$, $p < 0.001$, respectively). Letters indicate significant differences from an emmeans post-hoc test at the $\alpha = 0.05$ significance level.

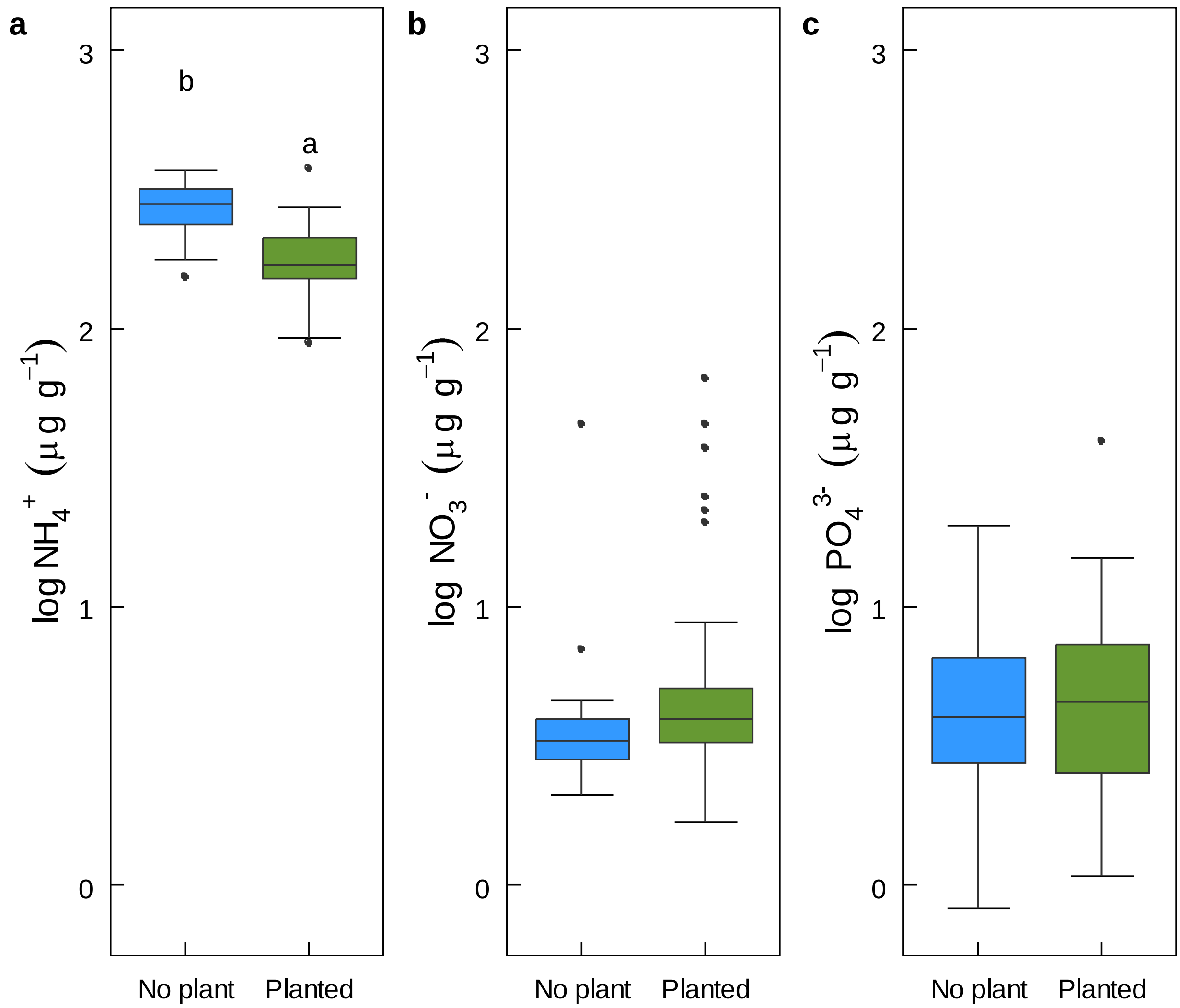

Fig. S8: Soil inorganic nutrient (N and P) concentrations following five months of warming. Data are logged to improve visual display, due to large variabilities. **a-c** NH_4^+, NO_3^- and PO_4^{3-} concentrations between pots with ("Planted") and without ("No plant") plants. There was only evidence for a difference between planted and unplanted pots for soil [NH_4^+] ($F_{1, 57} = 18.18$, $p < 0.001$). **d-f** NH_4^+, NO_3^- and PO_4^{3-} concentrations between control and warmed pots. There was no evidence for a significant response in soil inorganic nutrients to warming. Letters indicate significant differences from an emmeans post-hoc test at the $\alpha = 0.05$ significance level.

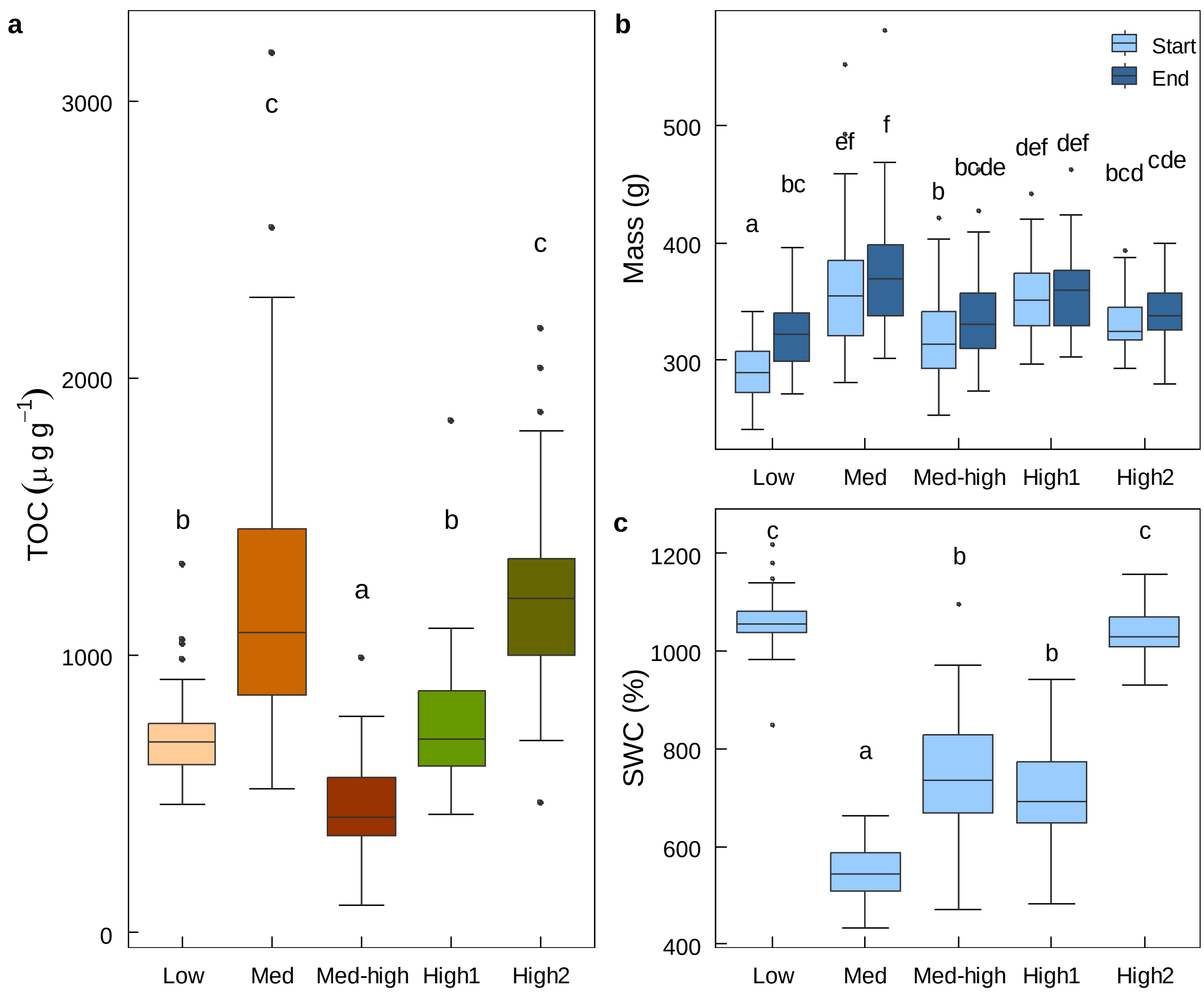

Fig. S9: Soil TOC and SWC for the incubation experiment. **a** TOC in the three warming treatments; Control, +3°C (H3) and +6°C (H6). There was no evidence for an effect of warming on soil TOC, but evidence for a strong effect of site ($F_{4,\,174}$ = 69.8, p < 0.001). **b** Soil core masses at the start and end of the experiment. While there was no evidence for an interaction between temperature (control, +3°C, +6°C) and time (start versus end), there was evidence for an interaction between site and time ($F_{4,\,369}$ = 2.66, p = 0.032). **c** SWC (%) at the end of the experiment. There was no evidence for a significant effect of temperature on SWC, but evidence for a significant site effect ($F_{4,\,174}$ = 296.35, p < 0.001). Letters show significant differences between site and time (**b**) and site (**a**, **c**) from an emmeans post-hoc test at the α = 0.05 significance level.

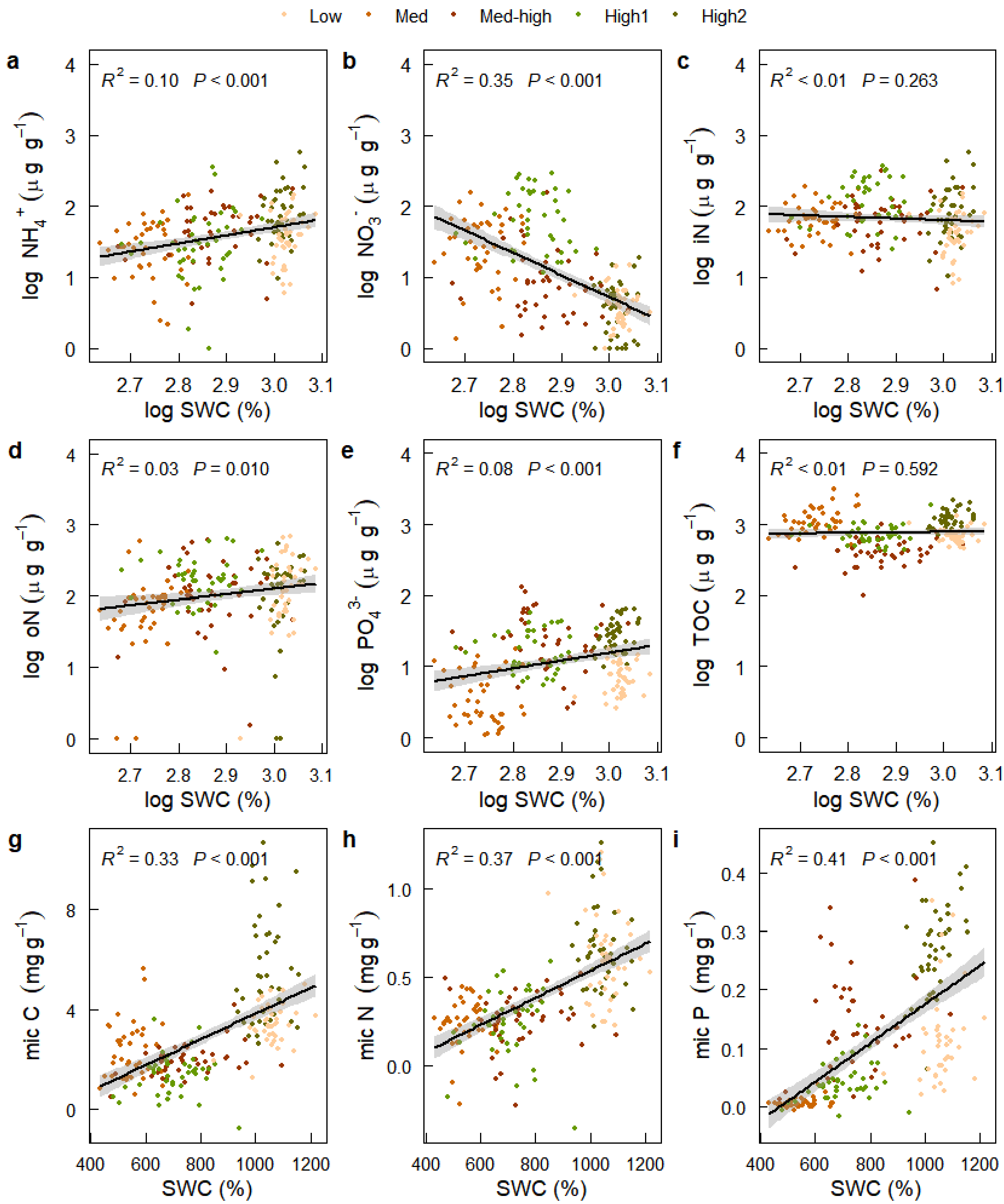

Fig. S10: Linear regressions between SWC (%) and soil nutrient variables (µg g^{-1} dry weight), where all sites and warming treatments are pooled. The respective multiple R^2 and p-values are presented at the top of each plot. There was evidence for a significant increase in soil NH_4^+ (t =

4.84, $p < 0.001$, adj. $R^2 = 0.10$, **a**), oN ($t = 2.43$, $p = 0.016$, adj. $R^2 = 0.02$, **d**) and PO_4^{3-} ($t = 3.83$, $p < 0.001$, adj. $R^2 = 0.06$, **e**) with increasing SWC, and a significant decrease in soil NO_3^- with SWC ($t = -10.93$, $p < 0.001$, adj. $R^2 = 0.37$, **b**). There was no evidence for a significant relationship between soil iN or TOC and SWC ($p > 0.05$, **c** and **f**). There was evidence for a significant increase in microbial C ($t = 9.96$, $p < 0.001$, adj. $R^2 = 0.33$, **g**), N ($t = 10.81$, $p < 0.001$, adj. $R^2 = 0.37$, **h**) and P ($t = 11.67$, $p < 0.001$, adj. $R^2 = 0.40$, **i**) with increasing SWC. Point colours (‘Low’, ‘Med’, ‘Med-high, ‘High1’, ‘High2’) refer to the sites soil cores were collected from and their respective vertebrate influence.