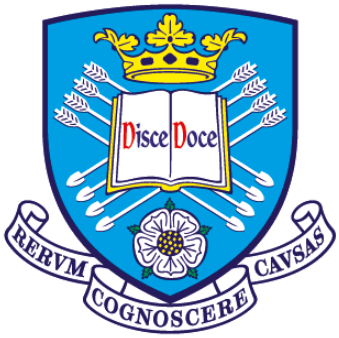


The University Of Sheffield.

# Consequences of Climate Change for Upland and Boreal Ecosystems: Assessing the Role of Soil Microbes in Carbon Cycling.

Jasmine Roha Wakefield

*A thesis submitted in the partial fulfilment of the requirements for the degree of Doctor of Philosophy*

Submission date

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In dedication to The People of Palestine,

and in loving memory of the more than 18000 Palestinians killed while I wrote these words

*Enough for me to die on her earth*

*be buried in her*

*to melt and vanish into her soil*

*then sprout forth as a flower*

*played with children from my country.*

*Enough for me to remain*

*in my country's embrace*

*to be in her close as a handful of dust*

*a sprig of grass*

*a flower.*

Palestinian poet, **Fadwa Tuqan** (1917-2003)

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## Author's declaration

I, Jasmine Roha Wakefield, confirm that this thesis is entirely my own work. I am entirely aware of the universities guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not been previously presented for an award at this, or any other, university.

## Thesis Summary

The burning of fossil fuels and land use changes have substantially altered the composition of the atmosphere. Consequently, the climate is changing into a warmer more volatile state. This thesis investigates how the soil microbial cycling of carbon changes under climate change. The second chapter investigates how trend climate change ($CO_2$ fertilisation), combined with nitrogen deposition and the alleviation of phosphorus limitation, alters soil microbial biodiversity and communities. In this chapter, two phosphorous limited grasslands' response is assessed over a year. Elevated $CO_2$ appeared to strongly influence soil microbial communities, particularly bacterial communities, and to a greater extend in the acidic grassland compared to the limestone. The two grasslands appeared to have opposing soil organic carbon storage trends under $eCO_2$. There was also evidence that $eCO_2$ can extend summer communities into autumn when they would usually undergo change at that time. The third chapter is a methods chapter and aims to improve understanding about how common soil sample storage methods alter the measured soil properties commonly used in research. This chapter illustrates that soil sample storage should be avoided where possible and analysis should be conducted on fresh soil, apart from for $\delta^{13}C$ of organic carbon as it is stable under all storage methods tested. The fourth chapter investigates how extreme event damage, investigated by removing aboveground plant material mimicking high plat mortality, can strongly influence soil respiration but that soil organic carbon appeared unaffected. In contrast, site level effects such as soil moisture (drought) and time of year appeared to strongly alter soil organic carbon.

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# Chapter 1. General introduction

The year I was born there was 362 ppm of carbon in the atmosphere, the year I started this PhD there was 412 ppm and as I write this there is 421 ppm. The increase in $CO_2$ in the atmosphere is driven by the anthropogenic combustion of fossil fuels and land use changes, and the consequences of these (IPPC, 2018). The effects of climate change span, the global air currents that mediate our weather systems (Stendel *et al.*, 2021), to the microscopic organisms inhabiting the world bellow our feet (Bardgett, Freeman and Ostle, 2008). In our lifetime, and at all scales, the change in the chemical composition of the atmosphere is affecting life.

## 1.1. Trend climate change

The ability of terrestrial ecosystems to sequester additional carbon from the atmosphere is dependent on photosynthesis and on the biogeochemical cycling of carbon through soil ecosystems. In particular, soil microorganisms mediate the transfer of photosynthate-fixed carbon to soil and back to the atmosphere through root-associated processes and through the decomposition of soil organic matter. Furthermore, soil microorganisms can boost plant productivity by releasing inorganically and organically bound plant nutrients, feeding back into ecosystem productivity and therefore carbon sequestration (Jacoby *et al.*, 2017). However, plant transfer of carbon to free-living and symbiotic soil microorganisms can also stimulate the decomposition of soil organic matter (priming), leading to the release of $CO_2$ from soils to the atmosphere (Kuzyakov, Friedel and Stahr, 2000; Hartley *et al.*, 2012; Koranda *et al.*, 2013; Lindahl and Tunlid, 2015).

Trend climate change ($CO_2$ fertilisation) can increase the above ground growth of plants due to increased photosynthesis and enhanced photosynthetic efficiency (Ainsworth and Long, 2005, 2021; Elmendorf *et al.*, 2012), reduced evapotranspiration due to greater stomatal closure with greater atmospheric $CO_2$ and so also enhanced soil water availability (Ainsworth and Long, 2005) and increased root production (Hungate *et al.*, 1997). Roots contribute the formation and turnover of

SOM by storing carbon in the soil (Trumbore, 2000; Gielen *et al.*, 2005). A review on the effects of roots under $eCO_2$ found increases in total root biomass (+28.8%), root length (+26.0%) and root diameter (+8.4%), expansions of root biomass deeper into the soil, and increases root respiration (+58.9%), rhizodeposition (+37.9%) and fungal colonisation (+3.3%) (Nie *et al.*, 2013). Plants increased growth response to $eCO_2$ (elevated $CO_2$) is often constrained by nutrient limitation (Reich *et al.*, 2006), to which plants can (as well as increasing root production) increase carbon allocation belowground to encourage the microbial mining of nutrients (Dijkstra *et al.*, 2013). However, this can promote both ecosystem carbon sequestration or loss (Kuzyakov *et al.*, 2019).

Belowground carbon allocation by plants, under $eCO_2$, is increased even in the absence of increased above ground biomass (Körner and Arnone, 1992). In addition to the quantity of plant derived carbon, the quality of litter and root derived products is reported to change (King, Thomas and Strain, 1997; Zak *et al.*, 2000; Pendall, Mosier and Morgan, 2004). Most of the indirect effects on soil communities with $eCO_2$ is driven by changes in carbon availability but soil processes are complex, which makes predicting how they change difficult. Under elevated $CO_2$, a reduction in soil decomposition has been found, where authors suggested that increased plant nitrogen uptake had increased microbial nitrogen limitation reducing their capacity to degrade soil organic matter (Hu *et al.*, 2001). However, under $eCO_2$ soil microorganisms are also found to limit plant growth by increasing the microbial immobilisation of nutrients (Keane *et al.*, 2023). There is a greater understanding of how plants respond to $eCO_2$ but the mechanisms behind how soil microbial communities react is less understood.

Another effect of increased $CO_2$ in the atmosphere is warming. I will briefly cover how warming effects the balance between ecosystem photosynthesis (removal of $CO_2$ from the atmosphere) and ecosystem respiration (input of $CO_2$ to the atmosphere), however there are a many other effects including plant phenological (Parmesan and Yohe, 2003; Root *et al.*, 2005) and microbial cellular responses (Bradford, 2013), to name two. Increased temperatures can release cold constraints on photosynthesis (Elmendorf, Henry, Hollister, Björk, Bjorkman, *et al.*, 2012). Warmer temperatures promote increased photosynthesis by plants; however, they similarly stimulate soil respiration

(assuming no water limitation) which limits soil carbon sequestration (Parmentier *et al.*, 2018). The balance between terrestrial ecosystem respiration and carbon fixation by plants could be disrupted by warming, as warming more positively increases respiration rates compared to photosynthetic rates (Ise *et al.*, 2010; Mahecha *et al.*, 2010; Yvon-Durocher *et al.*, 2010; Smith and Dukes, 2013). The effect of this imbalance is incorporated into the Intergovernmental Panel on Climate Change (IPCC) coupled climate-carbon cycle models where by 2100 it contributes ~2 °C of global mean annual warming (IPCC, 2018). Warming by itself, strongly effects carbon cycling in environments, although not assessed in this thesis, it drives substantial changes to carbon cycling and warrants further investigation. In particular, research on the combined impact of other global change drivers and warming on soil microbial communities is essential, given their crucial role in soil organic matter (SOM) decomposition (Bardgett, Freeman and Ostle, 2008), and specifically, as the influence of warming on soil microorganisms remains relatively understudied (Crowther and Bradford, 2013) .

## 1.2. Climate driven extreme events

Extreme events are another symptom of climate change, and can often produce contrasting ecosystem responses compared to trend climate change. Extreme events are defined in different ways ecologically and climatologically, but are typically marked by their rareness (Gutschick and BassiriRad, 2003) and/or strong impact and comparably short duration. Some can be limited to only a particular set of ecosystems, such as extreme winter warming events that appear in arctic and boreal ecosystems only (Bokhorst *et al.*, 2011) (Bokhorst *et al.*, 2009), while others may effect more ecosystems and regions such as heatwaves or pest outbreaks (IPPC, 2007). However, despite the large diversity in the types of extreme events, common factor for many is their ability to cause high plant mortality (Bokhorst *et al.*, 2011) and hence have a major effect on ecosystem $CO_2$ balance, reducing the carbon sink capacity of the ecosystem (Treharne *et al.*, 2019). For example, in 2012, northwest Scandinavia experienced a record low in terrestrial productivity and 14 different anomalous weather events were linked to driving it (Bjerke *et al.*, 2014).

Future climate models predict more variability, including increased extreme heat and heightened occurrences of intense precipitation and drought (Easterling *et al.*, 2000; Orlowsky and Seneviratne, 2012). Warming trends are anticipated to enhance plant growth in temperate regions, increasing carbon sequestration (Bonan, 2008; Piao *et al.*, 2009). However, extreme heat, such as heat waves, typically reduces primary production and plant growth in ecosystems (Reichstein *et al.*, 2013). In 2003, a mega heat-wave in Europe led to a 30% decrease in gross primary productivity, illustrating the impact of extreme heat events on ecosystem functioning (Cias, 2003). The 2005 drought in Amazonian rainforests caused a decline in plant production, accompanied by increased tree mortality and reduced growth (Phillips *et al.*, 2009). Aside from drought and heat, precipitation event intensity is expected to rise globally, irrespective of changes in total annual precipitation for most regions (Tebaldi *et al.*, 2006), likely putting plants under increased stress. The 1999 extensive flooding of the River Rhine, for instance, resulted in significant damage and mortality of adult trees (Kramer, Vreugdenhil and van der Werf, 2008). Therefore, broadly extreme events are found to largely negatively affect plants whereas trend climate change is largely reported to boost plant growth (when not constrained by nutrient limitation).

### *1.2.1. Belowground impacts of extreme events*

Few studies have assessed the belowground impacts of extreme climatic events. Microorganism activity, at the ecosystem level, influences the uptake and release of $CO_2$ (Bardgett, Freeman and Ostle, 2008). Extreme climatic events are likely to interfere with the microbial cycling of carbon and nutrients, especially given the abrupt cut-off of carbon supply from plants due to severely reduced primary productivity (Treharne *et al.*, 2019). In addition, root growth can be reduced, for instance, in by up to 72% and 63% following one and three consecutive winters of extreme warming events (Bokhorst *et al.*, 2011). A reduction in root growth suggests a reduction in ecosystem respiration (less $CO_2$ released to the atmosphere), as roots are major contributors to respiration. Furthermore, a global review on drought impacts on carbon cycling found that drought decreased average soil organic carbon content (-3.3%), primarily attributed to a reduction in plant litter input (-8.7%) and reduced

litter decomposition (-13.0%) across all three global ecosystem types (grassland, forests and shrublands) (Deng *et al.*, 2021). This indicates strong controllers of plants health on soil carbon and nutrient cycling. There is a need, therefore, to understand the underlining mechanisms associated with how carbon cycling changes –especially belowground- after events, to create more accurate global carbon models. This is especially important in terms of assessing how highly organic soils could convert to a climatic carbon source.

## 1.3. Atmospheric nitrogen deposition

Through land use changes, particularly agricultural ones, and the burning of fossil fuels, humans have also increased global nitrogen deposition (Schlesinger, 2009). Nitrogen deposition was historically first a major problem in northern Europe, although rates have now started to decline (Tomlinson *et al.*, 2020). However, global nitrogen emission scenarios project elevated rates of atmospheric nitrogen deposition in most regions by 2030 (Dentener *et al.*, 2006). The cumulative loading represents a large input of nitrogen into ecosystems that is substantially greater than natural forms (Fowler *et al.*, 2013). A global review of nitrogen deposition effects on soil microorganisms revealed consistent impacts across all terrestrial ecosystems (Zhang, Chen and Ruan, 2018). This suggests similar responses in different ecosystems, supporting the extrapolation of nitrogen deposition microbial results from one ecosystem to infer potential effects in others. The authors of the review reported that nitrogen deposition reduced microbial biomass, microbial respiration and relative abundance or arbuscular mycorrhiza (Zhang, Chen and Ruan, 2018). This indicates that nitrogen deposition can confer substantial alterations in microbial functioning and the cycling of carbon and nutrients. This is of particular concern as arbuscular mycorrhiza are important phosphorus acquisition plant partners (Smith *et al.*, 2011; Jiang *et al.*, 2021) and as nitrogen deposition can increase the demand for phosphorus in some environment (Phoenix *et al.*, 2004).

Nitrogen deposition can alleviate nitrogen limitation increasing carbon sequestration (Janes-Bassett *et al.*, 2021) and cause soil acidification (Tian and Niu, 2015) which can retard microbial decomposition

of SOM (Li *et al.*, 2018). Nitrogen deposition can increase soil acidification by increasing plant uptake of $NH_4^+$ increasing $H^+$ ions release into the soil, $NO_3^-$ leaching when $NO_3^-$ availability exceeds biotic demands (Lucas *et al.*, 2011), and by depleting available base cations in the soil ($Ca_2^+$,$Mg_2^+$ and $K^+$) and increasingly non-base cations ($Mn_2^+$ and $Al_3^+$) (Tian and Niu, 2015). Furthermore, soil acidification likely strongly affects soil microbial community composition indirectly as pH is one of the strongest drivers of community composition across geographical scales (Bahram *et al.*, 2018). In addition, the mobilisation of non-base cations ($Mn_2^+$ $Al_3^+$ and $Fe_3^+$), due to a loss of buffering base cations, have the potential to be toxic to plants (Delhaize, Physiology and 1995, 1995; Van Den Berg *et al.*, 2005). Therefore, nitrogen deposition can have strong effects on carbon cycling and the biotic and abiotic soil environment.

Nitrogen deposition is also found to stimulate the decomposition of SOM in tropical forests by altering the trophic structure of fauna communities, promoting decomposition through increased abundances of predators and detritivores (Yin *et al.*, 2022). Nitrogen deposition can further indirectly effect carbon cycling through affecting plant species abundance. Nitrogen deposition can strongly reduce aboveground biodiversity (Sala *et al.*, 2000; Payne *et al.*, 2017). Over time, shifts in vegetation composition, especially ones concerning the predominant plant functional type or growth forms, may alter litter quality and decomposability (Hobbie, Schimel, Trumbore and Randerson, 2000; Saleska *et al.*, 2002; Quested *et al.*, 2003). Plant litter containing more labile carbon compounds and high nutrient content positively correlate with faster decomposition rates; whereas, litter containing more recalcitrant structural carbon and secondary metabolite compounds are negatively related with decomposition rates (Meentemeyer, 1978; Taylor, Parkinson and Parsons, 1989; Kurokawa and Nakashizuka, 2008; Coq et al., 2010; Makkonen et al., 2012; Jackson, Peltzer and Wardle, 2013; García-Palacios et al., 2016). Therefore, the effects of climate change and nitrogen deposition are likely to interact in unpredictable ways to affect ecosystem carbon cycling.

## 1.4. Soil microorganisms

Soil microorganisms are a highly biodiverse complex community. In one gram of soil thousands to millions of different species exist (Torsvik, Øvreås and Thingstad, 2002; Gans, Wolinsky and Dunbar, 2005). The majority of soil microorganisms are not cultured (Whitman, Coleman and Wiebe, 1998) so the sequencing of soil microorganism will help our understanding how they respond to global change and further our understanding of ecosystem functioning. Microbial activity and abundance is globally generally enhanced in the rhizosphere compared to the bulk soil (Berendsen, Pieterse and Bakker, 2012), while diversity is reduced because of dominance of few taxa in the rhizosphere (Philippot *et al.*, 2013); however, arctic rhizosphere microbial diversity is reported to be higher than the bulk soil (Tam *et al.*, 2001; Kumar *et al.*, 2016).

Symbiotic mycorrhizal associations are important for plant productivity in nutrient-poor environments (Smith and Read, 2008). In exchange for supplying host plants with nitrogen and phosphorus, hosts provide mycorrhizal fungi with labile forms of carbon (Read, 1991). However, their role in carbon flux is not well defined (Selosse and Roy, 2009). They are broadly defined into two groups, reflecting colonisation strategy, the ectomycorrhizas (ECM) and endomycorrhizas (Smith and Read, 2008). Endomycorrhizas colonise inside the root cells, and are further divided into orchid, ericoid and arbuscular mycorrhizas (AMs). AM fungi are a more widespread fungal symbiont (more than 80% of land plants) (Smith and Read, 2008), belonging to the phylum Glomeromycota a divergent group from the same ancestor as Basidiomycetes and Ascomycetes (Schwarzott, Walker and Schubler, 2001). ECM colonises the root intercellular spaces and often form associations with trees and deciduous shrub species; In particular, ECM species of Basidiomycetes and Ascomycetes form associations the tree families *Pinaceae, Fagaceae, Dipterocarpaceae and Caesalpinoidaceae*, shaping forests (Smith and Read, 2008). Ericoid mycorrhiza (ERM) form symbiosis with ericaceous plant species, which include dominant tundra and heathland species (Kytöviita, 2005) such as *Calluna, Empetrum, Vaccinium and Cassiope* genera known to be damaged by extreme events (Bokhorst *et al.*, 2009; Treharne *et al.*, 2020b).

In the conventional view on carbon cycling, mycorrhizal fungi were primarily a vector for the transfer of carbon from plants to the soil, which promotes carbon sequestration. However, there is accumulating evidence that mycorrhizal fungi can act as decomposers, either directly or indirectly, contributing to the release of carbon from SOM (soil organic matter) (Talbot, Allison and Treseder, 2008; Vowles and Björk, 2019). No evidence for mycorrhizas acting as decomposers has been found for AM fungi (Talbot, Allison and Treseder, 2008). Although, ERM can act as decomposers (Talbot, Allison and Treseder, 2008), more recent evidence from boreal forests suggests they contribute to carbon sequestration in soils (Clemmensen *et al.*, 2015). ERM cell walls are melanised, slow to decompose, and contribute to a build-up of fungal necromass in soils (Clemmensen *et al.*, 2015; Fernandez and Kennedy, 2018). It is unclear how a reduction or increase in symbiotic fungi biomass may affect net carbon sequestration. However, it is clear that a change carbon allocation belowground will affect mycorrhizal biomass (Lipson *et al.*, 2006; Parker *et al.*, 2017, 2020), thus due to the role mycorrhizal fungi play in carbon and nutrient cycling, there is likely to be an effect on carbon sequestration.

Bacterial communities directly mediate the breakdown of soil organic matter. Bacteria process nitrogen in the soil through nitrification ($NH_4^+ \rightarrow NO_3^-$) which involves two steps, where bacteria and archaea oxidise $NH_4^+$ into $NO_2^-$, and $NO_2^- \rightarrow NO_3^-$ (Blaud *et al.*, 2015). Denitrification (anaerobic reduction of $NO_3^- \rightarrow N_2$) involves several steps (Blaud *et al.*, 2015). Soil phosphorus is usually classified into three forms in the soil, phosphate ions in solution (generally of low concentration), inorganic phosphorus (e.g. sorbed on Fe and Al oxides, insoluble metallophophsates, calcium phosphates), and organic phosphorus (e.g. sugar phosphates, phosphomonoesters, phopshodiesters, and phytate) (Shen *et al.*, 2011). The enzyme mainly used by bacteria to mineralise organic phosphorus is phophomonoesterase (Nannipieri *et al.*, 2011). Heterotrophic soil microorganisms are primarily limited by carbon and secondly by nutrients (Soong *et al.*, 2019). Therefore, either an increase, change, or decrease in carbon availability in the soil will likely effect the bacterial cycling of nutrients.

## 1.5. Sensitive ecosystems in the context of this thesis's research

Some ecosystems can be considered particularly vulnerable to the effects of trend and extreme event climate change. During the past 50 years, the Arctic has warmed at a faster rate than any other region globally (AMAP, 2017). Consequently, climate models project a higher frequency of climate extremes in the Arctic (Beniston *et al.*, 2007). This concentration of climate change at the poles, along with the large amounts of carbon (Ping *et al.*, 2008; Schuur *et al.*, 2008) stored in arctic soils makes them an important area of study. Similarly, upland heathlands at lower latitudes sequester substantial amounts of atmospheric carbon in soils (Dise, 2009) which is relatively stable due to the recalcitrant plant matter (ericaceous species e.g. *Calluna vulgaris)* and acidic soils which slow decomposition rates (Anderson and Hetherington, 1999; Berg and Laskowski, 2006). Heathlands in Europe are sensitive to long-term historical nitrogen deposition which is found to alter carbon storage and nutrient dynamics (Southon *et al.*, 2013). Experimental nitrogen deposition, in a lowland heathland, increased nitrogen uptake by plants, soil decomposition rates and nitrogen cycling (Power, Ashmore and Cousins, 1998), and in an upland heathland greater carbon sequestration was recorded (Field *et al.*, 2017). A survey of both upland and lowland heathlands in the UK found that greater ambient nitrogen deposition strongly reduces species diversity, with a concurrent increase in nitrophilous plant species, across spatial and climatic gradients (Southon *et al.*, 2013). Other ecosystems, such as grasslands, are important ecosystems to study as they are the most extensive ecosystem globally (Ali *et al.*, 2016). Therefore, representing a massive carbon sequestration potential. Temperate grasslands are able to sequester additional carbon under $eCO_2$ but this response is constrained when grasslands are limited by nitrogen or phosphorus (Soussana and Lüscher, 2007). However, when an increase in the input of carbon belowground in grasslands under $eCO_2$ is found, it could be accompanied by greater turnover limiting net carbon sequestration (Cardon *et al.*, 2001). Soil respiration increases, stimulated by $eCO_2$, are enhanced by nitrogen deposition depending on the amount of nitrogen supplied (enhanced in the low treatment but not the high) (Gao *et al.*, 2020). This suggests not linear responses to the combined effects of $eCO_2$ and nutrient availability, which may indicate complex interactions with combined drivers of global change and climate change effects.

## 1.6. Thesis chapter rational

*Chapter 2. Soil microbial communities biodiversity responses to elevated $CO_2$, nitrogen deposition and the alleviation of phosphorus limitation, in two phosphorus limited grasslands*

In soil ecosystems, microorganisms are crucial in the role of carbon and nutrient cycling, and their influence is assumed to be significant in shaping how terrestrial ecosystems respond to global change. Phosphorus limited grasslands can be important reservoirs for above ground biodiversity (Phoenix *et al.*, 2020). Lots of research on plant biodiversity has indicated that increased plant biodiversity can boost ecosystem carbon sequestration (Fornara and Tilman, 2008; Steinbeiss *et al.*, 2008; Cong *et al.*, 2014) by increasing plant biomass (Tilman, Hill and Lehman, 2006) and reductions in plant biodiversity can reduce soil organic carbon storage (Cardinale *et al.*, 2012). Soil communities generally have high levels of taxonomic and functional diversity (De Deyn and Van Der Putten, 2005).

Nevertheless, there is a gap in the literature on how global change drivers such as $eCO_2$ and nitrogen deposition affect soil biodiversity, especially in phosphorus limited ecosystems including grasslands. As outlined above $eCO_2$ will likely increase carbon partitioning belowground and soil microorganism are likely sensitive to this change. Understanding how $eCO_2$ and nutrient manipulation affects soil microbial biodiversity and changes communities, in phosphorus limited grasslands, is important in disentangling how these ecosystems will respond in the future. This is especially interesting since phosphorus limited grasslands of differing soil pH (limestone and acidic) and aboveground plant species composition have responded to $eCO_2$ by both increased and decreased aboveground plant biomass (Keane *et al.*, 2023).

Therefore, the FACE chapter in this thesis aims to investigate how 16S and ITS soil microbial biodiversity and communities, of two contrasting soil pH phosphorus limited grasslands, which have undergone >25 years of nitrogen treatment (N deposition simulation) and phosphorus treatment

(alleviation of phosphorus limitation) respond to $eCO_2$. A time series approach in analysis was taken where four time points that coincided with spring, summer, autumn and winter were sampled over a year. This was done as research suggests that climate change may be altering the timings of the seasons (Sparks and Menzel, 2002) and soil organic priming activity (stimulated by $eCO_2$) can contribute to climate feedbacks outside of the growing season (Keidel, 2019). In past work, sampling has usually taken place only in the growing season. Shannon diversity, Chao species richness and beta diversity for treatments was measured from the 16S and ITS sequencing data for each time point (season). Furthermore, measurements of soil pH and total soil organic carbon are conducted to explore variations in organic carbon storage and assess the effects of the treatments on soil acidification. This additional information aims to offer insights into potential reasons for microbial biodiversity and community changes

*Chapter 3. Quantification of storage effects in analysis of upland soil samples*

Due to the COVID-19 pandemic, I missed my initial Arctic field season, prompting a delay until the following summer (I thought at the time). Utilizing the additional time, I focused on better preparation by investigating how the storage and transportation of samples could influence measured soil properties I was interested in assessing. The experiment aimed to assess the impact of soil storage treatments and the duration of sample storage on measured properties compared to fresh soil.

Fresh soil, sourced from accessible moorland in The Peak District National Park, UK, featuring vegetation cover functionally similar to Arctic region (*Calluna vulgaris)*, underwent initial property measurements (*extractable total nitrogen, nitrate, ammonium & phosphorus; microbial biomass, dissolved inorganic and organic carbon, total organic carbon and* $\delta^{13}$ *C of organic carbon; and activity of five soil enzymes (BG, NAG, POX, PER, PHO)).* Subsequently, soil storage preservation techniques were applied, including freeze-drying, refrigeration (4 °C), air drying, and freezing (-18 °C). Measurements were then taken on the treated soil after one month and again after three months, with the relative change in each property from the fresh soil calculated and similarity was

assessed. The resulting alterations in measured soil properties of the stored soil were compared to the fresh soil to draw inferences for optimizing storage methods during remote (e.g. arctic) fieldwork.

### *Chapter 4. Consequences of plant--to-soil carbon cut-off following an extreme climatic event simulation*

With the additional time that the COVID-19 pandemic provided, due to a missed arctic field season, a proxy extreme event (EE) was set-up on *C. vulgaris* dominated moorland. Extreme events vary in their effects on ecosystems but a common theme is the damage and mortality of plant cover. Plants allocate a substantial amount belowground via rhizodeposition (Nguyen, 2003; Jones, Nguyen and Finlay, 2009), which is an important carbon source for soil microorganism (Weintraub and Schimel, 2003; Blagodatskaya and Kuzyakov, 2008). The removal of plant carbon input into the soil, by the clipping vegetation cover, offers a way to investigate how plant activity and the distribution of photosynthate carbon belowground controls microbial carbon cycling.

An EE proxy was set-up on matched plot pairs (clipped vegetation plot neighbouring control plot) trenched around the boarder. The experimental treatment was called a 'proxy' rather than a 'simulation' because it simulated the photosynthate flow cut-off resulting from the EE, rather than simulating the EE itself. Sampling was conducted through a time-series, starting with time points closer together to capture the initial response, and then further apart, to monitor the response over time (up to X days after the EE proxy). Throughout the time series microbial carbon cycling was investigated by monitoring soil respiration, dissolved organic carbon, total soil organic carbon and microbial biomass. Soil temperature and soil moisture was also measured to investigate the effects of the physical loss of canopy, which could indirectly affect microbial carbon cycling. After a year (X days), an intensive sampling was conducted which included more response variables, to investigate carbon and nutrient cycling. This included measuring the response of five key soil enzymes involved in carbon, nitrogen and phosphorus cycling. Soil extracellular enzyme ratios were calculated from these to investigate the relative investment of the microbial community into nitrogen vs phosphorus

acquisition and recalcitrant vs labile carbon sources. In addition, extractable total nitrogen, nitrate and ammonium, and extractable phosphorus were also measured to investigate nutrient availability.

### *1.6.1. Thesis aims*

The aims of this thesis is to investigate soil microbial carbon cycling and diversity under climate change. One experimental chapter investigates how $eCO_2$, likely boosting rhizosphere carbon availability, combines with nutrient manipulations, effects soil microbial biodiversity and community change over a year. In addition, the work was undertaken to investigate how potential microbial changes may coincide with changes to soil pH and total organic carbon in the two grasslands. Another experimental chapter, investigates how removing rhizosphere carbon through an EE proxy (clipping of above ground material), affects the microbial cycling of carbon and nutrients, initially, over time, and a year afterwards. In addition, the work was carried out to investigate how the microbial community processes soil carbon, in the absence of fresh photosynthate carbon, through investment into microbial growth, respiration, or resource acquisition. Both increased and decreased rhizosphere carbon can theoretically lead to soil carbon sequestration and loss, from and to the atmosphere. Therefore, disentangling the drivers that control the storage and release of carbon from soils, in these two scenarios, is of the upmost important to our understanding of how ecosystems will and are changing in the Anthropocene.

# Chapter 2. Soil microbial communities biodiversity responses to elevated $CO_2$, nitrogen deposition and the alleviation of phosphorus limitation, in two phosphorus limited grasslands

## 2.1. Abstract

Human activities are increasing atmospheric $CO_2$ levels and the rates of nitrogen deposition onto ecosystems. While more is known about the impacts on above ground biodiversity, the impacts below ground on soil microorganisms is much less clear. Grasslands are the most extensive ecosystem globally, and phosphorus limited grasslands are often associated with high plant biodiversity. Nutrient limitation is reported to constrain gains in plant growth under $eCO_2$. Plants can, as a mechanism to overcome relative nutrient limitation, increase carbon allocation belowground with one of the consequences being to stimulate the microbial decomposition of soil organic matter to increase plant access to nutrients in the soil. However, there is limited investigation into how phosphorus limited grasslands soil microbial biodiversity and community structure will change with $eCO_2$. To assess community responses to global change, it's crucial to consider the impacts of elevated $CO_2$ (eCO2) and nitrogen deposition, as these changes co-occur. In this study, the addition of a phosphorus treatment alongside $eCO_2$ helps disentangle the drivers of $eCO_2$ responses in phosphorus-limited ecosystems. Soil microbial biodiversity and community changes in two phosphorus-limited grasslands (limestone and acidic) using a long-term mini-FACE setup with $eCO_2$ and N or P nutrient manipulations, was investigated. A seasonal time-series (spring, summer, autumn, winter) explored the impacts of $eCO_2$, nitrogen treatment, and phosphorus treatments on bacterial and fungal communities. Elevated $CO_2$ reduced bacterial Shannon diversity and spring bacterial species richness (acid Grassland), and fungal and bacterial species richness (limestone grassland).This was attributed to a relative increase in dominance of certain species driven by the alleviation of carbon limitation via greater plant carbon allocation belowground. Spring and summer bacterial alpha diversity was lower than autumn and winter. These reductions were additionally attributed to a dominance effect from

greater belowground carbon allocation by plants during these seasons. More seasonally dynamic bacterial communities appeared more sensitive to $eCO_2$. Fungi communities were relatively resistant to $eCO_2$ but relatively more sensitive to nutrient manipulation. There was evidence, in the acidic grassland, that $eCO_2$ extended summer microbial communities into autumn, possibly due to greater plant exudation or a longer exudation season under $eCO_2$. Elevated $CO_2$ effects soil microbial communities, particularly bacterial communities and more so in the acidic grassland.

## 2.2. Introduction

The burning of fossil fuels, land use changes and intensive agriculture have increased global $CO_2$ (carbon dioxide) and regional nitrogen availability, altering the stoichiometry and functioning of ecosystems (Peñuelas *et al.*, 2012, 2013). Terrestrial ecosystems' abilities to remove $CO_2$ from the atmosphere, as $CO_2$ levels continue to rise, is driven by the stimulation of photosynthesis with photosynthate carbon stored in biomass and soils. This $CO_2$ induced increase in ecosystem carbon sequestration, is the most important biogeochemical feedback mitigating anthropogenic climate change (IPCC and Intergovernmental Panel on Climate Change, 2023). However, the ability of ecosystems to increase carbon sequestration is constrained by nutrient availability (Reich *et al.*, 2006; Zavalloni *et al.*, 2012; Reich and Hobbie, 2013; IPCC and Intergovernmental Panel on Climate Change, 2023).

Along with increased $CO_2$ many ecosystems have had increased nitrogen deposition, which has been identified as the third greatest threat to biodiversity globally (Sala *et al.*, 2000; Payne *et al.*, 2017). Although, nitrogen deposition is thought to alleviate relative nitrogen limitation induced by $eCO_2$, potentially increasing the carbon sequestration capacity of ecosystems (Schlesinger and Andrews, 2000), nitrogen deposition is found to reduce soil pH (Tian and Niu, 2015) which strongly controls the distribution of microbial communities (Bahram *et al.*, 2018). The combined response of soil microbial communities to nitrogen deposition and $eCO_2$ is of great importance to our understanding of ecosystems scale carbon cycling.

Microorganisms that live in the soil play an important role in ecosystem carbon and nutrient cycling. Soil microorganisms, such as bacteria and fungi, decompose organic matter, breaking it down into simpler compounds. This process releases carbon dioxide ($CO_2$) back into the atmosphere and converts complex organic materials into simpler forms that can be utilized by plants and other soil organisms (Van Der Heijden et al., 2008; Six et al., 2006). Their activity can boost plant growth by increasing the bioavailability of soil nutrients (Van Der Heijden, Bardgett and Van Straalen, 2008). For example, they transform organic nitrogen into ammonium ($NH_4^+$) and nitrate ($NO_3^-$), making it accessible for plant uptake (Paul, 2014; Schimel and Bennett, 2004). Phosphate-solubilizing bacteria release phosphorus from insoluble compounds, making it available to plants (Rodríguez and Fraga, 1999; Vessey, 2003). Nitrogen-fixing bacteria, such as Rhizobium species, form nodules on the roots of leguminous plants, converting atmospheric nitrogen ($N_2$) into a form that plants can use (Zahran, 1999; Franche et al., 2009)

Mycorrhizal fungi form symbiotic relationships with plant roots, enhancing the plants' ability to absorb nutrients like phosphorus from the soil. In return, plants provide the fungi with carbohydrates produced through photosynthesis (Smith and Read, 2008; Brundrett, 2002). Fungi can even pick up and relocate bacteria, via their hyphae networks, in the soil environment to optimise soil organic matter decomposition (Kohlmeier *et al.*, 2005). Microorganisms are responsible for the majority of the breakdown of plant material as they are the only organism capable of producing recalcitrant carbon degrading enzymes, like those that break down lignin (Romaní *et al.*, 2006; Masai, Katayama and Fukuda, 2007). Some microorganisms contribute to carbon sequestration by stabilizing organic carbon in the soil. This can occur through the formation of soil aggregates that protect organic matter from rapid decomposition (Jastrow et al., 2007; Lal, 2004).

Soil microorganisms do not interact solely with each other to influence soil carbon cycling; a cascade of plant, bacterial, fungal, soil faunal interactions, along with abiotic conditions, shape and influence the cycling of carbon and nutrients. As different soil types, with different abiotic factors such as soil pH, nutrient level, soil age, climate and season are assumed to contain specific microbial communities

(Fierer and Jackson, 2006), they will functionally cycle carbon and nutrients differently in these different ecosystems. Elevated $CO_2$ has been found to have different effects on microbial diversity, including reported increases, decreases and no change (discussed further on in more detail) (Janus *et al.*, 2005; Lipson *et al.*, 2014; Tu *et al.*, 2015). The different effects reported could be due to different ecosystems responding uniquely to additional plant-carbon availability.

Research has demonstrated global patterns in microbial communities that correlate with environmental factors such as salinity (Lozupone and Knight, 2007; Auguet et al., 2010), pH (Lauber et al., 2009), and habitat type (Dinsdale et al., 2008; Nemergut et al., 2011; Fierer et al., 2012), and links between microbial community and ecosystem processes have been observed within numerous individual studies (van der Heijden et al., 1998; Torsvik and Øvreås, 2002; Bardgett and van der Putten, 2014; Wagg et al., 2014). Therefore, it is important for studies to assess the effects of $eCO_2$ on a variety of ecosystems to ensure a comprehensive understanding of the future effects of $eCO_2$.

In the soil, even when a large range of microorganisms are present only a small proportion will be adapted to the main source of organic resources present (Swift, Heal and Anderson, 1979), the rest will likely be in a state of dormancy. The functional redundancy in soil microbial populations allows the community to dynamically respond to changes in resource availability. This effect is commonly reported in microbial studies investigating changes in microbial communities during the stages of decomposition, due to differing degradation rates of chemical compounds (Fontaine, Mariotti and Abbadie, 2003).

High soil biodiversity contributes to the resilience and stability of soil ecosystems. Diverse microbial communities are better able to adapt to environmental changes and disturbances, maintaining soil functions under varying conditions (Griffiths and Philippot, 2013; Loreau and Mazancourt, 2013). Soil biodiversity, particularly the diversity of bacteria and fungi, enhances nutrient cycling by providing a wide range of enzymatic activities necessary for breaking down organic matter and releasing nutrients in forms accessible to plants (Torsvik and Øvreås, 2002; Delgado-Baquerizo et al.,

2016). Moreover, soil biodiversity can enhance plant resistance to pathogens through competitive exclusion and the production of antimicrobial compounds (Wagg et al., 2014).

Plants exhibit a variety of ecological interactions with soil microorganisms from competitive, to exploitative, neutral, commensal and mutualistic. Through root exudates, plant species are thought to select specific microbial populations (Berg and Smalla, 2009), and shape the microorganism communities around their roots to benefit their own health (Berendsen, Pieterse and Bakker, 2012). Plants can allocate around 11 – 17% of their net carbon fixation to rhizodeposition, although this varies considerably depending on plant species and age, soil type and nutrient availability (Nguyen, 2003; Jones, Nguyen and Finlay, 2009), but is reported to increase with $eCO_2$ (Darrah, 1995; Pendall, Mosier and Morgan, 2004; Fransson and Johansson, 2010). Rhizodeposition is therefore a major carbon source for the microbial production of extracellular enzymes that break down SOM (soil organic matter) (Schimel and Weintraub, 2003; Blagodatskaya and Kuzyakov, 2008).

The effects of $eCO_2$ (elevated $CO_2$) on plant growth include gains in plant biomass (Curtis and Wang, 1998), greater fine root production (Hungate *et al.*, 1997) and altered carbon allocation belowground (Pregitzer *et al.*, 1998; Hu *et al.*, 2001). Increased exudation (Phillips, Finzi and Bernhardt, 2011) and belowground plant biomass, with $eCO_2$, will increase the flow of carbon to soil microorganisms. Recent advancements suggest that heterotrophic soil microorganisms are primarily carbon limited and secondly by nutrients (Soong *et al.*, 2019). Therefore, the microbial community is likely sensitive to the effects of increased or changed carbon availability driven by plant responses to $eCO_2$. Changes in soil microbial populations are in turn likely to influence plant nutrient availability due to the microbes role in biogeochemical cycling (Bardgett *et al.*, 2013).

A major issue of the Anthropocene is the global trend for a reduction in plant and animal biodiversity (IPBES, 2020). Whether, this holds true for microorganisms is a profound unknown question (Thaler, 2021). Classical ecological theory predicts that biodiversity has strong effects on ecosystem functioning by contributing to ecosystems stability, productivity, nutrient dynamics and invaisability

of ecosystems (Tilman, 1999). Elevated $CO_2$ affects plant biodiversity through multiple indirect effects: altering the stoichiometry availability of nutrients, water availability and altering competition between plants (Potvin *et al.*, 2007). A change in aboveground biodiversity is likely to influence soil microbial communities, particularly as invasive plant species are known to have major effects on microbial communities (Van Der Putten, Klironomos and Wardle, 2007). Above ground grassland species evenness is reported to increase with $eCO_2$ (Zelikova *et al.*, 2014), but no change in biodiversity is also reported (Taylor *et al.*, in press). Furthermore, $eCO_2$ has been found to reduce the loss in plant diversity caused by nitrogen deposition (Reich, 2009). In an open air long-term (10 years) grassland $eCO_2$ and nitrogen deposition factorial experiment, nitrogen deposition reduced species richness by 16% at ambient $CO_2$ but only by 8% at $eCO_2$ (Reich, 2009). The authors suggested that $eCO_2$ and nitrogen deposition had multiple effects on plant traits and soil resources that led to changes in competitive interactions among plant species, and that $eCO_2$ mitigated the adverse effects of nitrogen enrichment on species richness (Reich, 2009). An in depth understanding of how above and belowground biodiversity is affected by $eCO_2$ and nitrogen deposition can help to explain how ecosystems as a whole are responding, particularly as greater above ground and belowground biodiversity have been found to jointly exploit phosphorus resources more competitively than less diverse ones (Oelmann *et al.*, 2021).

Globally grasslands are the most extensive terrestrial ecosystem (Ali *et al.*, 2016). In temperate regions species rich grasslands hold the greatest biodiversity (Carbutt, Henwood and Gilfedder, 2017). Along with their often high biodiversity, they are important global carbon sinks (Jones and Donnelly, 2004). Plant species which inhabit phosphorus limited grasslands have adapted various belowground evolutionary approaches to cope with phosphorus limitation, reducing plant competition leading to greater plant biodiversity (Phoenix *et al.*, 2020). In phosphorus limited systems, the availability of a low mobility nutrient like phosphorus (Tinker and Nye, 2000) is highly dependent on microbial processing. Plants in low nutrient ecosystems likely develop stronger relationships with microbes, either through direct symbiosis or nutrient-recycling feedback mechanisms (Aerts and Chapin, 1999). Symbiotic mutualist relationships between plants and microbes increase nutrient availability and

facilitate niche partitioning, promoting diversity among plant species (Van Der Heijden *et al.*, 2006; Van Der Putten, 2017). If global change drivers such as $eCO_2$ and nitrogen deposition influence soil microbial diversity, there could be a knock on effect on nutrient cycling, which could in turn influence plant biodiversity.

There is some evidence that aboveground and belowground biodiversity is positively correlated (Liu *et al.*, 2020). Higher aboveground biodiversity may enhance belowground biodiversity by changing the input of plant detritus into the soil. This detritus, characterized by diverse chemical and physical properties, creates a greater number of distinct niches for specialized decomposers (Hooper *et al.*, 2000; Gessner *et al.*, 2010). Alternatively, more biodiverse plant communities may be more productive (Tilman, Isbell and Cowles, 2014; Lange *et al.*, 2015), providing greater resource input that fosters greater microbial diversity (Hättenschwiler, Tiunov and Scheu, 2005; Bardgett and Van Der Putten, 2014). However, in a global review of the links between aboveground and belowground biodiversity, soil pH was the major factor found to negatively associate with the positive plant-microbe biodiversity correlation (Liu *et al.*, 2020). The authors suggested this was likely due to the extremes of pH limiting microbial fitness and survival (Tripathi *et al.*, 2018) and therefore the plant-microbe relationships. Such feedback mechanisms underscore the complexity of ecological relationships and emphasize the need to consider the many interactions within ecosystems. Therefore, understanding how above and belowground species are effected by $eCO_2$, nutrient alleviation and limitation is needed to disentangle how ecosystems will respond to global change in the future.

How soil microbial communities' biodiversity changes in response to global change could help disentangle how ecosystems are responding as a whole, and if they occur, uncover potential drivers of climate-feedbacks involving carbon sequestration. Some soil species are known to be sensitive to anthropogenic global change (Scheu and Schulz, 1996; Eggleton *et al.*, 2002; Bardgett *et al.*, 2005; Parrent, Morris and Vilgalys, 2006; Briones, Ineson and Heinemeyer, 2007). In particular, in a semi-arid shrubland FACE experiment the effects of $eCO_2$ increased fungal species richness, evenness and fungal biomass but bacteria responded relatively little to $eCO_2$ (Lipson *et al.*, 2014). It was suggested

that in this ecosystem, both greater fungal abundance and biodiversity were closely linked to greater plant carbon allocation belowground and fine root production (Lipson *et al.*, 2014). This contrasts reports from an agricultural (Anderson, Heinemeyer and Weigel, 2011) and grassland experiment, where $eCO_2$ tended to more dramatically affect the bacterial community (He *et al.*, 2010) but microbial biodiversity was not assessed. In another study, Aspen seedlings grown under $eCO_2$, had greater fungal and bacterial species richness of communities associated with roots, compared to seedlings grown under ambient $CO_2$ (Janus *et al.*, 2005). Whilst no discernible effects of $eCO_2$ on bacterial biodiversity have also been reported (Lipson *et al.*, 2006). In an Australian grassland FACE and warming experiment, bacterial diversity was unresponsive to $eCO_2$ or warming unlike fungal communities (Hayden *et al.*, 2012). Furthermore, microbial community structure has been reported to change with $eCO_2$ (Castro *et al.*, 2010; He *et al.*, 2012; Yu *et al.*, 2018) but also be resistant to change (Ebersberger *et al.*, 2004; Guenet *et al.*, 2012). No clear effect of $eCO_2$ on soil microbial biodiversity and community structure is found, and appears to be highly ecosystem specific. Therefore, how phosphorus limited grassland microbial biodiversity and community structure will be affected by $eCO_2$ is unknown.

There are limited studies assessing the effects of combined nitrogen deposition and $eCO_2$ on soil microbial diversity. One study that investigated the effects of $eCO_2$ and nitrogen fertilisation (separately and combined) on forest soil, at different depths, found that $eCO_2$ alone reduced fungal richness at 2-5cm but $eCO_2$ combined with nitrogen fertilisation increased fungal richness to similar levels found in ambient $CO_2$ soil (Weber, Vilgalys and Kuske, 2013). The response of microbial biodiversity to nitrogen deposition alone appears to be consistent among ecosystems and relatively well studied. In a steppe ecosystem nitrogen addition experiment, ammonium availability significantly reduced bacterial species richness and the resulting acidification indirectly changed bacterial community composition (Zeng *et al.*, 2016). In a semi-arid grassland in China, simulated nitrogen deposition significantly reduced both bacterial and fungal alpha diversity, with the authors noting soil acidification as the likely cause (Wei *et al.*, 2020). A review into the effects of nitrogen deposition found that the decreases in fungal and bacterial diversity found across ecosystems, is likely related to

reductions in microbial biomass (Wang, Liu and Bai, 2018). This indicates that soil acidification reduces the abundance of soil microbes in the soil, with some taxa or life histories being especially sensitive to acidification also resulting in the reported reduced microbial biodiversity. The impact of $eCO_2$, on the observed trend of decreased microbial diversity with nitrogen deposition, remains uncertain given a notable gap in the literature investigating their combined effects. Considering the future co-occurrence of both global change drivers, it is crucial for research to explore their combined effects to provide a more accurate representation of future conditions.

Consequently, an investigation into the effects on belowground biodiversity was conducted on a long-term nutrient and $eCO_2$ factorial designed mini free air carbon dioxide enrichment experiment (mini-FACE system). Soil monoliths from two contrasting phosphorus limited grasslands (a limestone and an acidic grassland), which had undergone over 20 years of nitrogen and phosphorus nutrient treatments, were transplanted to the facility for three years (2018). The soil microbial community, ITS and 16S, was sequenced four times over a year to investigate the effects of $eCO_2$, nitrogen deposition and the alleviation of phosphorus limitation over the four seasons; along with soil organic carbon and soil pH. The seasonal sampling was undertaken to fully evaluate the effects of treatment in the times of year with higher (spring and summer) and lower (autumn and winter) plant activity. Furthermore, grassland soil microbial communities are known to seasonally change (Habekost *et al.*, 2008), so sampling across seasons for a year provides a more complete understanding of the $eCO_2$ response on microbial communities.

It was hypothesised that:

1. Elevated $CO_2$, nitrogen deposition and the alleviation of phosphorus limitation would change the 16S and ITS community structure, but the effects of nutrient manipulations would be stronger than the $eCO_2$ effect due to the relationship between nitrogen deposition and soil acidification, and release of phosphorus limitation promoting greater aboveground biodiversity.

2. ITS and 16S species richness and biodiversity would increase under $eCO_2$ due to greater rhizodeposition increasing carbon availability, reducing the microbial carbon limitation, allowing a greater number of microbial niches to be occupied in the soil.
3. The effects of $eCO_2$ on soil microbial communities will be greater in summer as a greater rate of plant photosynthesis is expected, increasing the rate of plant C transfer belowground. Periods of greater rhizodeposition will change the microbial community present in the soil and likely effect ITS to a greater extent than 16S as plants are expected to have a closer relationship with symbiotic fungal partners compared to free living bacteria.

## 2.3. Methods

### *2.3.1. Field site description*

The Wardlow Hay Cop long-term experiment, situated within the Derbyshire Dales National Nature Reserve, UK, encompasses both acidic and limestone grasslands (Morecroft, Sellers and Lee, 1994). These grasslands are naturally phosphorus-limited (Carroll *et al.*, 2003). The limestone grassland (National Vegetation Classification Festuca-Avenula, CG2d; Rodwell, 1992) consists of a shallow ranker soil (Fig. 2.1. right image) with a pH of approximately 6.8 (Horswill *et al.*, 2008) extending around 10 cm from the limestone bedrock (Keane et al., 2020). This grassland supports diverse and largely calcicolous plant community. The acidic grassland community (NVC *Agrostis-Galium*, U4e; Rodwell, 1992) displays lower diversity and is more calcifugous. It consists of a deep (> 70 cm) cryptic podzol with a pH of about 4.4 (Horswill *et al.*, 2008; Keane *et al.*, 2020). The acidic soil is divided into two layers (Fig 2.1. left image): an organic-rich A horizon (approximately 7 – 10 cm depth), which houses the majority of biological activity, and a mineral horizon that extends to the underlying bedrock (Keane et al., 2020). Cattle grazing occurs on the site from the middle of May until December, with the addition of sheep in May and June. The maximum density of grazing animals is 20 cattle and 20 ewes with their lambs, distributed across an area of approximately 20

hectares (Carroll *et al.*, 2003). The site has a history of significant nitrogen deposition, with this peaking around 25 to 30 kg N $ha^{-1}$ $yr^{-1}$ (Phoenix *et al.*, 2004; Horswill *et al.*, 2008).


Figure 2. 1. Examples of the soil profiles of the acidic (left) and limestone (right) grasslands at Wardlow. The two distinct layers of the acidic; organic and mineral are clearly visible, and split according to their colour. The limestone soil represents a single organic-rich humic horizon, with a shallow profile. Image credit: Chris Taylor

Experimental plots for the nutrient addition long-term study were commenced in 1995 on replicate 9 $m^2$ plots. The experimental plots received one of three nutrient treatments: (i) distilled water control (0N), (ii) high nitrogen treatment (HN) to simulate high rates of N loading with an application rate of 14 g N $m^{-2}$ $yr^{-1}$, and (iii) a phosphorus treatment (P) aimed at alleviating phosphorus limitation with an application rate of 3.5 g P $m^{-2}$ $yr^{-1}$. The nutrient solutions were applied at canopy height, using $NH_4NO_3$ for nitrogen treatment and $NaH_2PO_4$ for phosphorus treatment as solutions in distilled water, administered using a fine spray via a backpack sprayer.

### *2.3.2. FACE $CO_2$*

Comprehensive descriptions of the MiniFACE system and the $CO_2$ experiment set-up can be found in Keane *et al.*, (2020). In 2017, 80 intact soil-turf monoliths (35 cm by 35 cm) were extracted from both grasslands at The Wardlow Hay Cop long-term experiment, with ten replicate monoliths obtained from each nutrient treatment and controls. To encompass the full soil profile, the monoliths from the limestone grassland were taken to a depth of 10 cm, while those from the acidic grassland were excavated to 20 cm, which included the main rooting zone and part of the mineral horizon (refer to figure 2.1. for an image of the contrasting soil profiles) (Keane et al., 2020).

These intact-turf monoliths were transplanted to free-draining polypropylene mesocosm boxes. In the limestone grasslands of Wardlow, the soil profile is small, and plant roots come into contact with the bedrock. To mimic this, limestone chips quarried from the same Derbyshire Carboniferous limestone as the bedrock were placed in the bottom of the mesocosms. The nutrient treatments in these mesocosms in the FACE experiment were continued with monthly applications.

The miniFACE experiment is situated at Bradfield Environmental Research Station in The Peak District, UK. It is located outdoors at a similar elevation and experiences comparable climatic conditions to Wardlow (approximately 360 m a.s.l and 390 m a.s.l, respectively). To maintain normal thermal buffering (Keane *et al.*, 2020), the mesocosms were placed into the ground flush with Bradfield soil and vegetation. Additionally, mesh was used to cover drainage holes at the base of the mesocosms, preventing plant roots from coming into contact with Bradfield soil.

Mesocosms from the 0N (control), HN (high nitrogen), and P (phosphorus) nutrient treatments in both grasslands were assigned to either an ambient $CO_2$ treatment (approximately 410 ppm) or an elevated $CO_2$ treatment (target 600 ppm). The elevated $CO_2$ fumigation at the miniFACE experiment began in April 2018 and has continued until the present day (2023). Fumigation is applied during daylight hours from the start of April to the end of October each year to encompass the growing season for

grassland vegetation at Bradfield. Note that there is limited grassland growth well into May some years.  (refer to figure 2.2. for an image of the experimental set-up of the miniFACE).


Figure 2. 2. Experimental set up for a block of $eCO_2$ mesocosms (top image). $CO_2$ enters the ring by the supply pipe and is supplied to the grasslands through laser-drilled holes in the miniFACE ring. The $CO_2$ sensor reads the $CO_2$ concentration of the middle of the ring and feeds back to the central computer to determine how much $CO_2$ to release to meet the target concentration (600 ppm). This is adjusted to account for wind speed and direction from a weather station on site. The bottom image shows an $eCO_2$ block during the active experiment, with the automated $CO_2$ flux chamber visible. Image credit: Ben Keane with annotations done by Chris Taylor

### *2.3.3. Soil sampling for soil microbial assessments*

Soil sampling was carried out at four time points over a year to create a one-year time series. The initial sampling (T1) was conducted on the 26th of April 2021, coinciding with spring and the commencement of the growing season, after the $CO_2$ fumigation had begun. The second sampling (T2) took place on the 29th of June 2021, timed to align with peak growing season conditions when sunlight hours reached their maximum. For the third sampling (T3) on the 22nd of October 2021, autumn was chosen as it marks the end of the growing season. Fumigation of $CO_2$ continued to the 31st of October but vascular plant uptake had likely been falling gradually since September, with likely minimal uptake at the third sampling. Lastly, the fourth sampling (T4) occurred on the 31st of January 2022, during mid-winter conditions when plant activity is presumed to be at its lowest point within the time series.

During each harvest, a 2cm soil corer was used to collect four randomly located sub-samples from each mesocosm down to a depth of 10cm, therefore sampling soil from the rooting zone and main area of biological activity as the experiment is focussed on capturing plant-soil interactions changed by $eCO_2$. For the acidic grassland, where this included collection of some mineral horizon, the mineral horizon soil was discarded. Between mesocosms, the soil corer and hands were cleaned with 100% ethanol to reduce cross-contamination. Sub-samples were pooled and sieved using a 5 mm mesh size sieve to remove stones, large roots, and ensure a well-mixed composite. Between samples, the sieves were washed thoroughly with Virkon, dried in a 50 °C oven, sprayed down with 70% ethanol, reducing cross-contamination between samples. The soil sieving process took place in a fume cupboard to minimize soil spillage around the workspace. Additionally, hands and the workspace were wiped down with 70% ethanol to ensure cleanliness and reduce any potential contamination.

### *2.3.4. Soil microbial analysis*

#### 2.3.4.1. Microbial community analysis

DNA was isolated from approximately 250 mg of soil using the DNeasy® PowerSoil® Pro Kit (QIAGEN, 2023), following the manufacturer's provided instructions. Afterwards, the DNA was quantified through UV-VIS spectrometry (Nanodrop 8000 Thermo Scientific) and preserved at -20°C for subsequent analysis.

Table 2. 1. Primer names and genetic sequences used in the two rounds of PCR for the ITS targeted operon and the one round of PCR for the 16S targeted operon (White et al., 1990; Gardes and Bruns, 1993; Ihrmark *et al.*, 2012; Apprill *et al.*, 2015; Parada, Needham and Fuhrman, 2016).

| Primer name | Primer sequence |
|---|---|
| ITS1f | CTTGGTCATTTAGAGGAAGTAA |
| ITS4 | TCCTCCGCTTATTGATATGC |
| ITS4-ill | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC |
| gITS7-ill | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGANNNHNNNWNNNHGTGARTCATCGARTCTTTG |
| 515F-ill | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGANNNHNNNWNNNHGTGCCAGCMGCCGCGGTAA |
| 806r-ill | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGACTACHVGGGTWTCTAAT |

For the identification of both total fungi and total bacterial species, primer sets targeting the rRNA operon were employed (refer to Table 2.1. for primer sequences). Amplification of the bacterial community was accomplished using 515F–806R primers, specifically targeting the V4 region of the 16S SSU rRNA, through a polymerase chain reaction (PCR) process (refer to Table 2 for PCR reaction mixture, settings, and primer sequences). Meanwhile, the total fungi community was amplified using a nested PCR approach with ITS1f/ITS4 primers followed by gITS7/ITS4 primers (Ihrmark *et al.*, 2012) to amplify the ITS region (refer to Table 2.2 for PCR reaction mixture, settings, and primer sequences). Amplicons were cleaned using AMPure beads (Agincourt) following the

manufacturer's instructions. gITS7-ill/ITS4-ill amplicons and 515F-ill/806r-ill amplicons underwent Nextera (Illumina) barcoding and sequence library preparation. 300 bp paired end read libraries were run on the Illumina MiSeq platform. Bacteria were sequenced by a commercial provider (Novogene) and fungi were sequenced by the Technology Facility at York (though The University of Leeds by arrangement).

Table 2. 2. The PCR mixtures and cycles used in each round of PCR for the ITS and 16S targeted operons. PCR cycles and temperatures are matched horizontally to the PCR reagents used.

| **ITS full length PCR round** | Each plate (uL) | PCR cycle temperatures | PCR cycle durations |
|---|---|---|---|
| x5 clear buffer [mg = 1.5mM] | 1164 | Initial denature 94 °C | *2 minutes* |
| 10mM dNTP | 116.4 | 20 cycles: | |
| 10um ITS4 | 116.4 | 94 °C | 30 seconds |
| 10um ITSIF | 116.4 | 57 °C | 30 seconds |
| Go Taq (5 units / uL) | 36.375 | 72 °C | 30 seconds |
| 5% blotto | 232.8 | *72 °C* | *5 minuets* |
| PCR water | 3455.625 | | |
| **ITS nested** | Each plate (uL) | PCR cycle temperatures | PCR cycle durations |
| x5 clear buffer [mg = 1.5mM] | 2910 | Initial denature 94 °C | *2 minutes* |
| 10mM dNTP | 291 | 35 cycles: | |
| 10um ITS7-ill | 291 | 94 °C | 30 seconds |
| 10um ITS4-ill | 291 | 58 °C | 30 seconds |
| Go Taq (5 units / uL) | 36.375 | 72 °C | 30 seconds |
| PCR water | 10439.63 | *72 °C* | *5 minuets* |
| **16S** | Each plate (uL) | PCR cycle temperatures | PCR cycle durations |
| x5 clear buffer [mg = 1.5mM] | 2910 | Initial denature 94 °C | *5 minutes* |
| 10mM dNTP | 291 | 30 cycles: | |
| 10um 515-ill | 291 | 94 °C | 30 seconds |
| 10um 806-ill | 291 | 57 °C | 30 seconds |
| Go Taq (5 units / uL) | 36.375 | 72 °C | 30 seconds |
| PCR water | 10148.63 | *72 °C* | *5 minuets* |

Primers were removed from sequence data using Cutadapt 4.6 (Martin, 2011). Sequences were processed R in Rstudio (R Core Team, 2023) following the dada2 pipeline (Callahan *et al.*, 2016). Reads were inspected for quality visually and appropriate filtering and trimming parameters were

selected. For the fungal sequences, the standard parametric error model was used. The Novoseq bacterial sequence data had binned quality scores, so error rates were estimated via four different methods and the best was selected visually by plotting the learned and observed error rates. The *learnErrors* function typically used for Miseq-style 0-40 quality stores has about $1/10^{th}$ of the information to learn the appropriate error function for Novoseq data. Option 1: alter loess arguments (weights and span and enforce monotonicity). Option 2: enforce monotonicity only. Option 3: alter loess function (weights only) and enforce monotonicity. Option 4: alter loess function arguments (weights and span and degree, also enforce monotonicity). Option 3: was visually identified as the best method in this case. The dereplication, sequence inference and merging of paired-end reads followed where sequence information was shared between samples to ensure sensitivity to rare taxa. Chimeras were removed and taxonomy was assigned using the Silva db v138 database for 16S sequencing and UNITE db for ITS sequences.

2.3.4.2. Downstream processing

To ensure that OTU's (operational taxonomical units) were representative, as close as possible, to different species and to limit the over assigning of species variants, OTU's were clustered at a 97% similarity level. OTU sequences were aligned using Muscle (MUltiple Sequence Comparison by Log-Expectation) (Edgar, 2004) computer programing software and a phylogenetic tree was constructed using FastTree2 (Price, Dehal and Arkin, 2010). The phylogenetic tree was imported back into R and using the *tip_glom* function from the *SpeedySeq V0.2.0* package (McLaren, 2020). After clustering OUT's at 97% similarity, any OTU reads assigned taxonomically down to species level with the same names were also clustered together. In addition, the clustered OTU table was ordered by percentage cumulative frequency, the OTUs and the bottom 1% tail of reads was removed. Mitochondria and chloroplast reads were identified via BLASTing against the NBCI database and removed. Similarly, anything identified as just fungi and just bacteria taxonomically were blasted and removed if sequences could not be verified against the NBCI database, as they were assumed to be non-specific PCR products. A new phylogenetic tree was created using the above methods, and the filtered and

clustered OTU table, taxonomy table and phylogenetic tree were imported back into *Phylosec* (McMurdie and Holmes, 2013) in RStudio.

16S data were rarefied down to 60000 and for ITS data reads were rarefied down to 10000, the rarefication depth was selected visually from rarefication curves, and depth was selected on a plateaued section of the curve which balanced the loss of samples to a minimum. Clustered OTUs sequenced from the 16S data are referred to as bacteria, and OTUs from the ITS data are referred to as fungi, for brevity (although, it is understood that they will not capture the full bacteria and fungi profiles of the soil, and the bacteria profile will include some archaea). Shannon diversity, Chao species richness and beta diversity measurements were performed on the clustered rarefied OTU data using the *Phylosec* package (McMurdie and Holmes, 2013). The effects of $CO_2$, nutrient treatment and time on Shannon and Chao species richness was tested for significance using a mixed model using the *lme4* package (Bates, Maechler, B. Bolker, *et al.*, 2015) to account for repeated measures, sample loss during PCR and rarefication, leading to unbalanced design. Residuals were checked for normality and in all cases were normal. Post hoc *tukey* test was performed with the *emmenmeans* package (Lenth, 2022). Beta diversity PCoA ordination plots were created using a weighted Unifrac distribution to account for phylogenetic based differences. The effects of $CO_2$, nutrient treatment and time on beta diversity was tested for significance using PERMANOVA (Oksanen *et al.*, 2022) on a weighted Unifrac distance matrix (McMurdie and Holmes, 2013) in all cases, as beta diversity dispersion did not significantly differ. To test for within group level significance a *pairwise.adonis* post hoc test was used when a main term was significant.

### *2.3.5. Organic carbon content*

Additionally, soil taken from the consolidated pooled sub-samples, was oven dried at 75°C until a consistent weight was achieved (approximately 3 days). The resulting dried soil was finely ground using a tissue grinder. Subsequently, 60 mg of the dried soil was weighed into a 1.5 ml Eppendorf tube, after which carbonates were removed from the sample utilizing the acid stripping technique of

Hedges and Stern (1984). This involved the gradual addition of approximately 700 µL of 6M HCl to the soil, with careful stirring using a blunt needle. Additional HCl (~100 µL) wasintroduced to samples that exhibited continued effervescence, guaranteeing complete removal of carbonates. The soil was left in contact with the acid for a 24-hour period to ensure complete reaction, after which the tubes were placed within a Techne DB 200/3 Dri-Block at 105°C for an additional 24 hours, to evaporate the acid. Next, the resulting pellets were finely powdered prior to analysis within an isotope-ratio mass spectrometer (ANCA GSL 20-20 Mass Spectrometer, Sercon PDZ Europa), following the methodology established by Harris, Horwáth and van Kessel (2001). The use of IRMS for measuring organic carbon (acid stripped soil) provides extreme precision and accuracy in detecting small changes over time (Santoiemma, 2018).

### *2.3.6. Soil pH*

Additional soil, taken from the consolidated pooled sub-samples, was mixed well in a 1:1 ratio with miliQ water, left for 30 minutes to come to room temperature and for the sediment to settle. The pH meter was calibrated with a pH 4, 7 and 10 buffer prior to use. The pH meter was held in the soil water solution until a consistent reading was displayed. In between samples, the meter was cleaned with miliQ water and wiped with a sterile tissue.

### *2.3.7. Additional analysis that was unsuccessful – quantitative spike*

After DNA extraction from the soil, a quantitative *Thermus thermophiles* plasmid spike was added to the extracts in a known concentration. This plasmid contained base sequences that would have been amplified by both the 16s and ITS amplification. The aim of this was to assess changes in the 16S and ITS community size through time. Unfortunately, the spike was not amplified successfully in all sequencing batches so was not successful and was discarded from the experiment.

## 2.4. Results

### *2.4.1. Alpha diversity*

#### 2.4.1.1. Acidic grassland bacterial Shannon diversity

Overall in the acidic grassland, $eCO_2$ reduced bacterial Shannon diversity (LME, df = 1, 37.42, F = 14.01, $p < 0.001$). Specifically, $eCO_2$ reduced spring ($t(106) = 2.83$, $p = 0.0056$), summer ($t(115) = 3.26$, $p = 0.0015$) and autumn ($t(113) = 0.012$, $p = 0.0122$) bacterial Shannon diversity. Over the year, $eCO_2$ reduced Shannon diversity in the control nutrient treatment ($t(39.4) = 2.9$, $p = 0.007$) and near-reduced it in the phosphorus treatment ($t(38.2) = 1.90$, $p = 0.06$)

Bacterial Shannon diversity varied depending on the time of year (LME, df = 3, 86.16, F = 154.72, $p < 0.001$). Autumn ($t(88.65) = 10.60$, $p < 0.001$) and winter ($t(85.93) = 11.52$, $p < 0.001$) increased in Shannon diversity compared to spring and summer (full contrast available in supplementary material S1.1). There was no $CO_2$ *x* time of year interaction (LME, df = 3, 86.54, F = 1.41, $p = 0.245$).

The was an overall effect of nutrient treatment on bacterial diversity (LME, df = 2, 33.93, F = 3.21, $p = 0.053$). Specifically, bacterial Shannon diversity in the nitrogen treatment was lower compared to in the phosphorus treatment ($t(39.1) = -2.16$, $p = 0.09$). There was no $eCO_2$ *x* nutrient treatment interaction (LME, df = 2, 40.45, F = 1.23, $p = 0.331$).

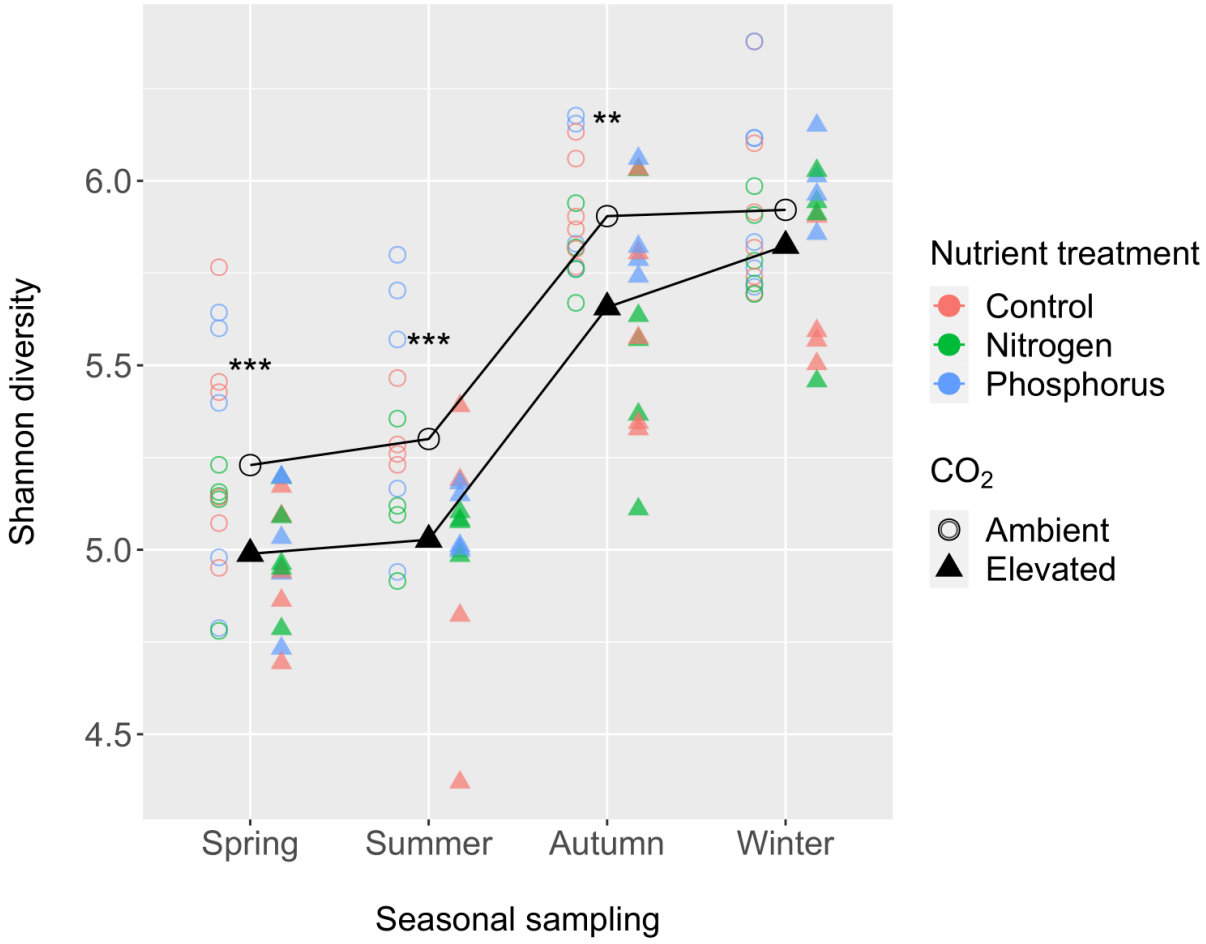


Figure 2. 3. The Acidic grassland's 16S Shannon diversity over a year. Hollow circles indicate ambient $CO_2$ and filled triangles indicate elevated $CO_2$ mesocosm results. Mean ambient and elevated $CO_2$ results for each seasonal time point are indicated in black and joined with a black line to show trends over the year (though the straight lines are not intended to signify a linear change between time points). Nutrient treatments are colour coded: red = control nutrient treatment, green = nitrogen nutrient treatment, blue = phosphorus treatment. (*) indicate significant difference at time point and number of stars indicate significance level (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, (+) indicates near significant difference $p < 0.07$.

2.4.1.2. Acidic grassland fungal Shannon diversity

Fungal Shannon diversity was unaffected by $eCO_2$ (LME, df = 1, 32.33, F 0.13, p = 0.72332). Fungal diversity varied depending on the time of year (LME, df = 3, 93.09, F = 2.24, p = 0.089). There was no $eCO_2$ *x* time of year interaction (LME, df = 3, 94.69, F = 0.70, p = 0.5567). Nutrient treatment (LME, df = 2, 31.30, F = 2.44, p = 0.10344) and the $eCO_2$ *x* nutrient treatment interaction did not affect fungal Shannon diversity (LME, df = 2, 34.20, F = 0.33, p = 0.72269).

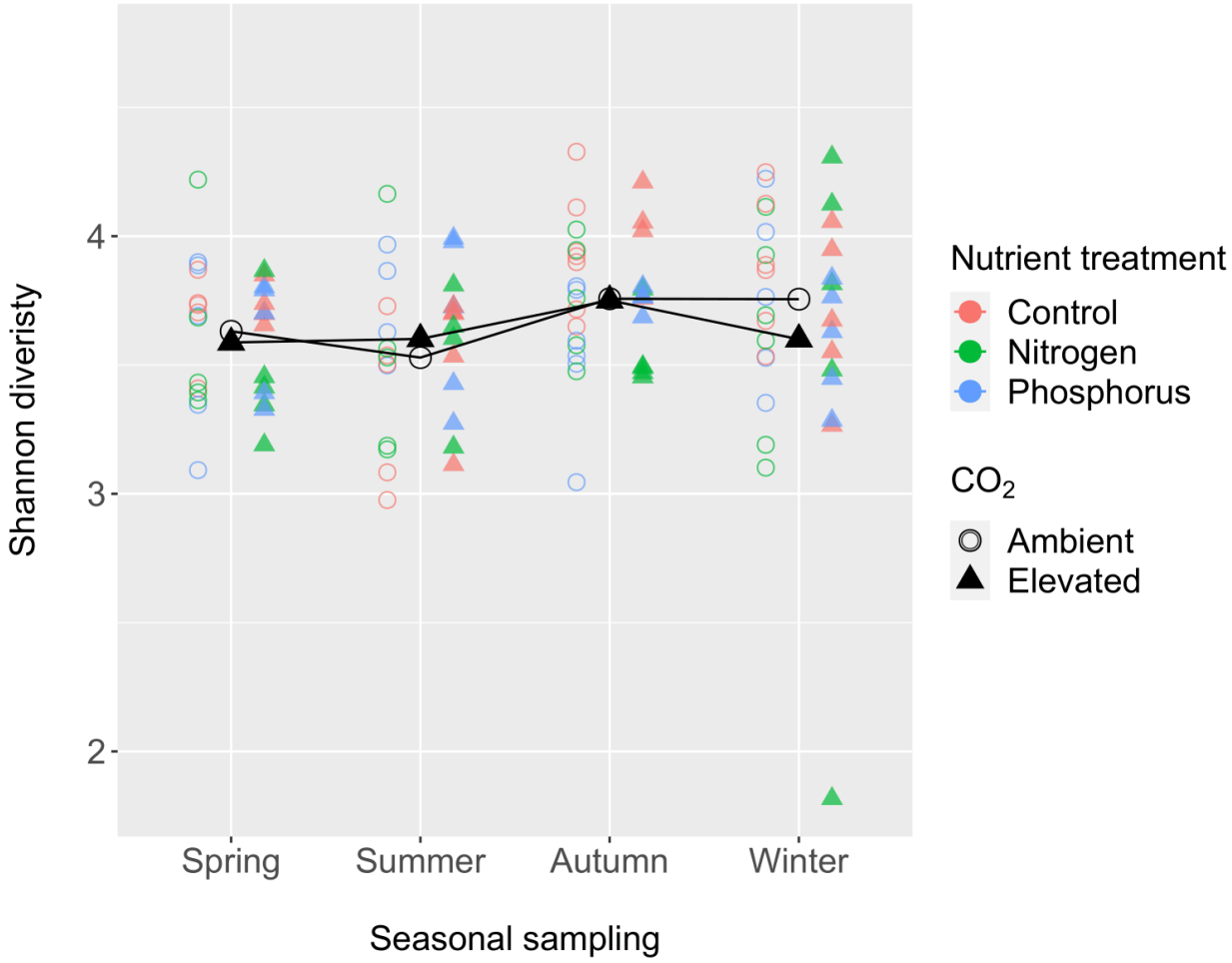


Figure 2. 4. The acidic grasslands ITS bacterial Shannon diversity over a year. Mean ambient and elevated $CO_2$ results for each seasonal time point are indicated in black and joined with a black line to show trends over the year (though the straight lines are not intended to signify a linear change between time points). Nutrient treatments are colour coded: red = control nutrient treatment, green = nitrogen nutrient treatment, blue = phosphorus treatment. (*) indicate significant difference at time

point and number of stars indicate significance level (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, + $p < 0.08$.

2.4.1.3. Acidic grassland bacterial Chao species richness

Overall, bacterial species richness was unaffected by $eCO_2$ (LME, df = 1, 31.67, F = 1.99, p = 0.1680) but a reduction was found in spring (t(127) = 2.27, p = 0.0247). Bacterial species richness changed depending on the time of year (LME, df = 3, 85.62, F = 13.21, p < 0.001). Autumn (t(90.48) = 2.32, p = 0.0231) and winter (t(84.15) = 2.06, p = 0.0426) had greater bacterial species richness compared to spring and summer (full contrast available in supplementary material S1.2).

There was no overall time of year *x* $eCO_2$ interaction (LME, df = 3, 86.99, F = 1.86, p = 0.1416) but there was an interaction effect between $eCO_2$ *x* winter (t(85.37) = 2.31, p = 0.0229) on bacterial species richness. Elevated $CO_2$ bacterial species richness crosses above ambient richness in winter only (Fig. 2.5).

Nutrient treatment did not affect bacterial species richness (LME, df = 2, 29.37, F = 2.19, p = 0.1299) and neither was there a nutrient treatment *x* $eCO_2$ interaction (LME, df = 2, 32.91, F= 0.07, p = 0.9326).

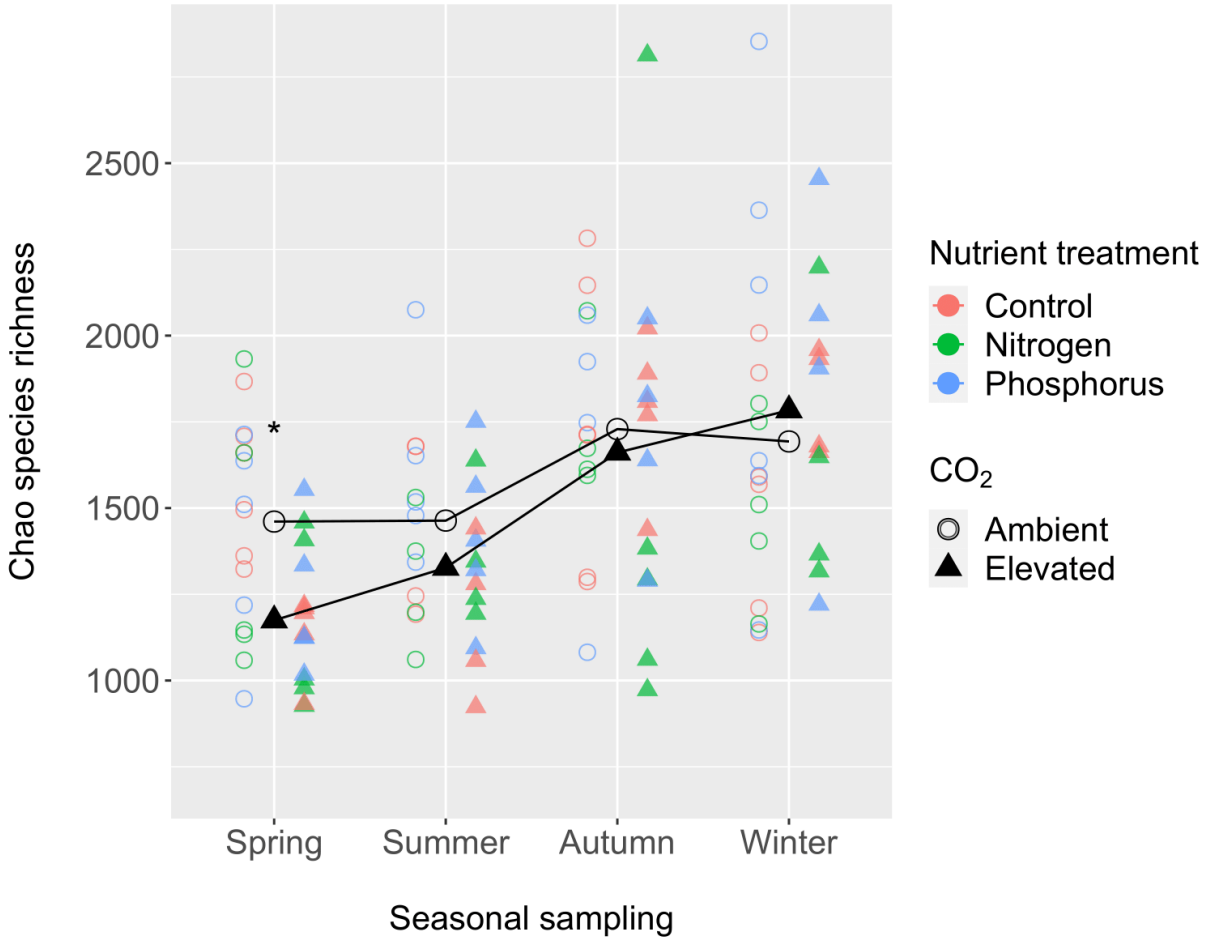


Figure 2. 5. The acidic grasslands 16S Chao species richness. Mean ambient and elevated $CO_2$ results for each seasonal time point are indicated in black and joined with a black line to show trends over the year (though the straight lines are not intended to signify a linear change between time points). Nutrient treatments are colour coded: red = control nutrient treatment, green = nitrogen nutrient treatment, blue = phosphorus treatment. (*) indicate significant difference at time point and number of stars indicate significance level (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, + $p < 0.08$.

2.4.1.4. Acidic grassland fungal Chao species richness

In the acidic grassland, fungal species richness was not affected by $eCO_2$ (LME, df = 1, 30.47, F = 2.31, p = 0.1390) or the time of year (LME, df = 3, 91.15, F = 0.46, p = 0.7081), nutrient addition (LME, df = 2, 29.45, F = 2.27, p = 0.1211) and neither were there a $eCO_2$ *x* time of year interactions

(LME, df = 3, 92.77, F = 0.021, p = 0.8901), or a nutrient treatment *x* $eCO_2$ interaction (LME, df = 2, 32. 33, F = 0.25, p = 0.7768).

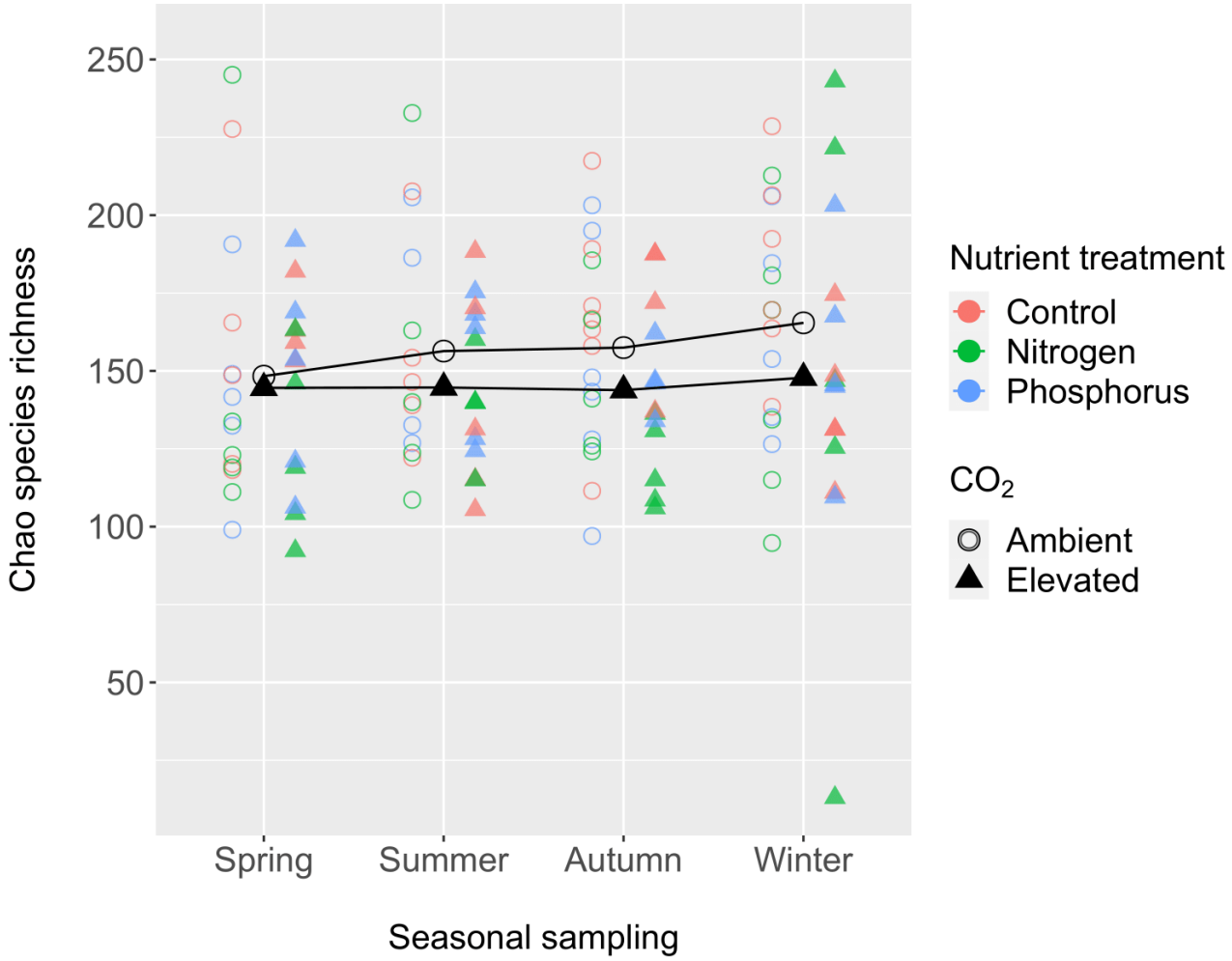


Figure 2. 6. The acidic grasslands ITS Chao species richness over a year. Mean ambient and elevated $CO_2$ results for each seasonal time point are indicated in black and joined with a black line to show trends over the year (though the straight lines are not intended to signify a linear change between time points). Nutrient treatments are colour coded: red = control nutrient treatment, green = nitrogen nutrient treatment, blue = phosphorus treatment. (*) indicate significant difference at time point and number of stars indicate significance level (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, + $p < 0.08$.

2.4.1.5. Limestone grassland bacterial Shannon diversity

In the limestone grassland, $eCO_2$ had no effect on bacterial Shannon diversity (LME, df = 1, 30.94, F = 0.24, p = 0.630810) but it did vary with the time of year (LME, df = 4, 80.23, F = 20.13, $p < 0.001$).

Autumn (t(79.53) = 2.17, p = 0.032697) and winter (t(79.52) = 3.97, p < 0.001) had greater bacterial Shannon diversity compared to spring and summer (full contrast available in supplementary material S1.3). There was no $eCO_2$ *x* time of year interaction (LME, df = 3, 80.23, F = 0.91, p = 0.435662).

Nutrient treatment affected bacterial Shannon diversity (LME, df = 2, 36.88, F = 7.20, p < 0.001). The nitrogen addition treatment had lower Shannon diversity compared to the phosphorus treatment (t(39.6) = -3.40, p = 0.0043). Over the year there was no $eCO_2$ *x* nutrient treatment interaction (LME, df = 2, 36.88, F = 0.91, p = 0.435662). However, there was a positive contrast within the model, where $eCO_2$ *x* phosphorus treatment interacted to increase bacterial Shannon diversity (t(39.99) = 2.14, p = 0.038738), differing to the $eCO_2$ trend for reduced diversity in the other nutrient treatments.

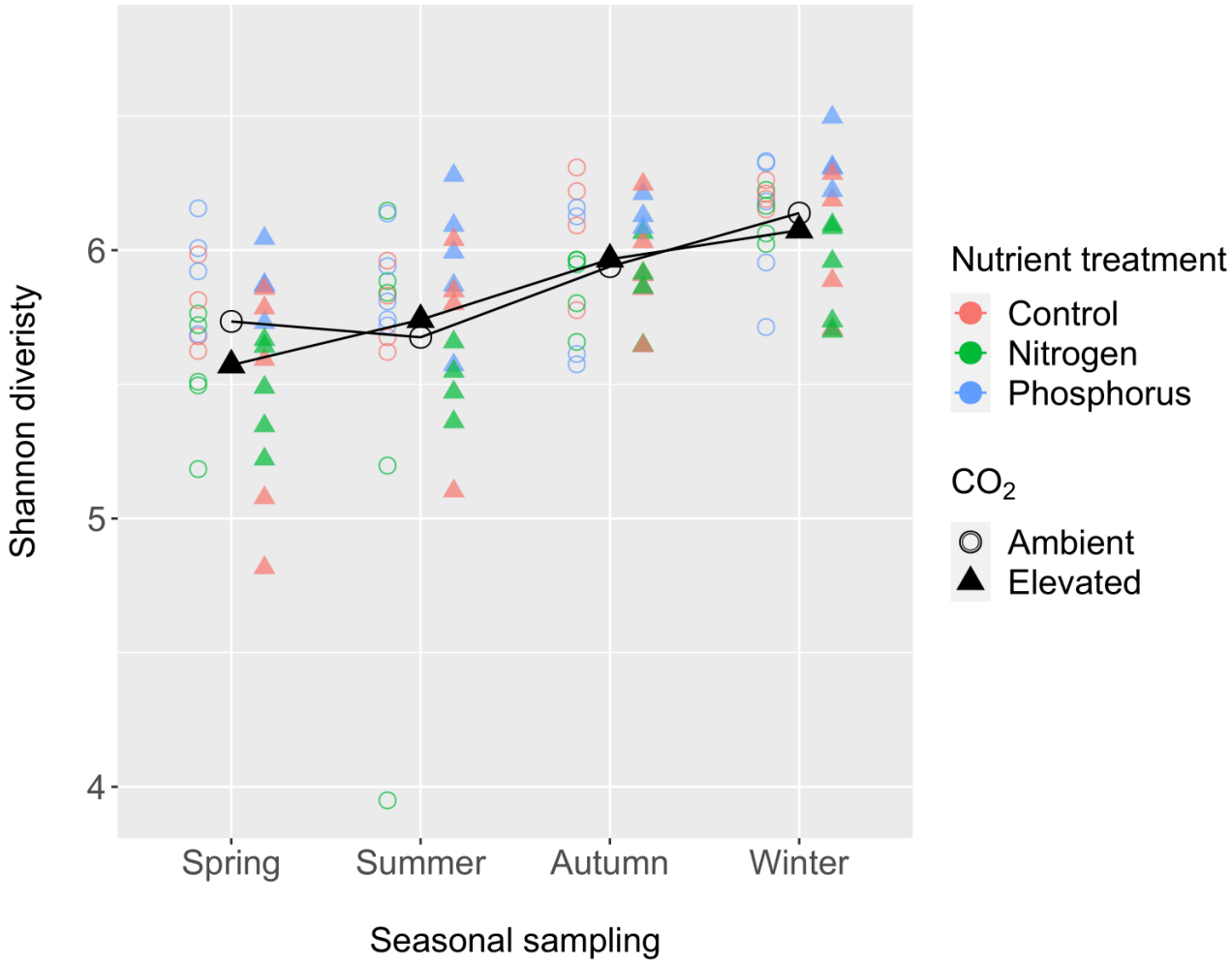


Figure 2. 7. The limestone grasslands 16S Shannon diversity over a year. Mean ambient and elevated $CO_2$ results for each seasonal time point are indicated in black and joined with a black line to show trends over the year (though the straight lines are not intended to signify a linear change between time points). Nutrient treatments are colour coded: red = control nutrient treatment, green = nitrogen nutrient treatment, blue = phosphorus treatment. (*) indicate significant difference at time point and number of stars indicate significance level (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, + $p < 0.08$.

2.4.1.6. Limestone grassland fungal Shannon diversity

Fungal Shannon diversity was unaffected by $eCO_2$ (LME, df = 1, 109, F = 2.51, p = 0.116), by the time of year (LME, df = 3, 109, F = 0.02, p = 0.995), or an interaction between them (LME, df = 3, 109, F = 0.03, p = 0.993).

However, nutrient treatment affected fungal Shannon diversity (LME, df = 2, 109, F = 5.63, p = 0.005). The phosphorus treated limestone grassland had greater fungal Shannon diversity (t(28.8) = -3.07, p = 0.0126) compared to the nitrogen treated grassland. There was no $eCO_2$ *x* nutrient treatment interaction (LME, df = 2, 109, F = 0.21, p = 0.813).

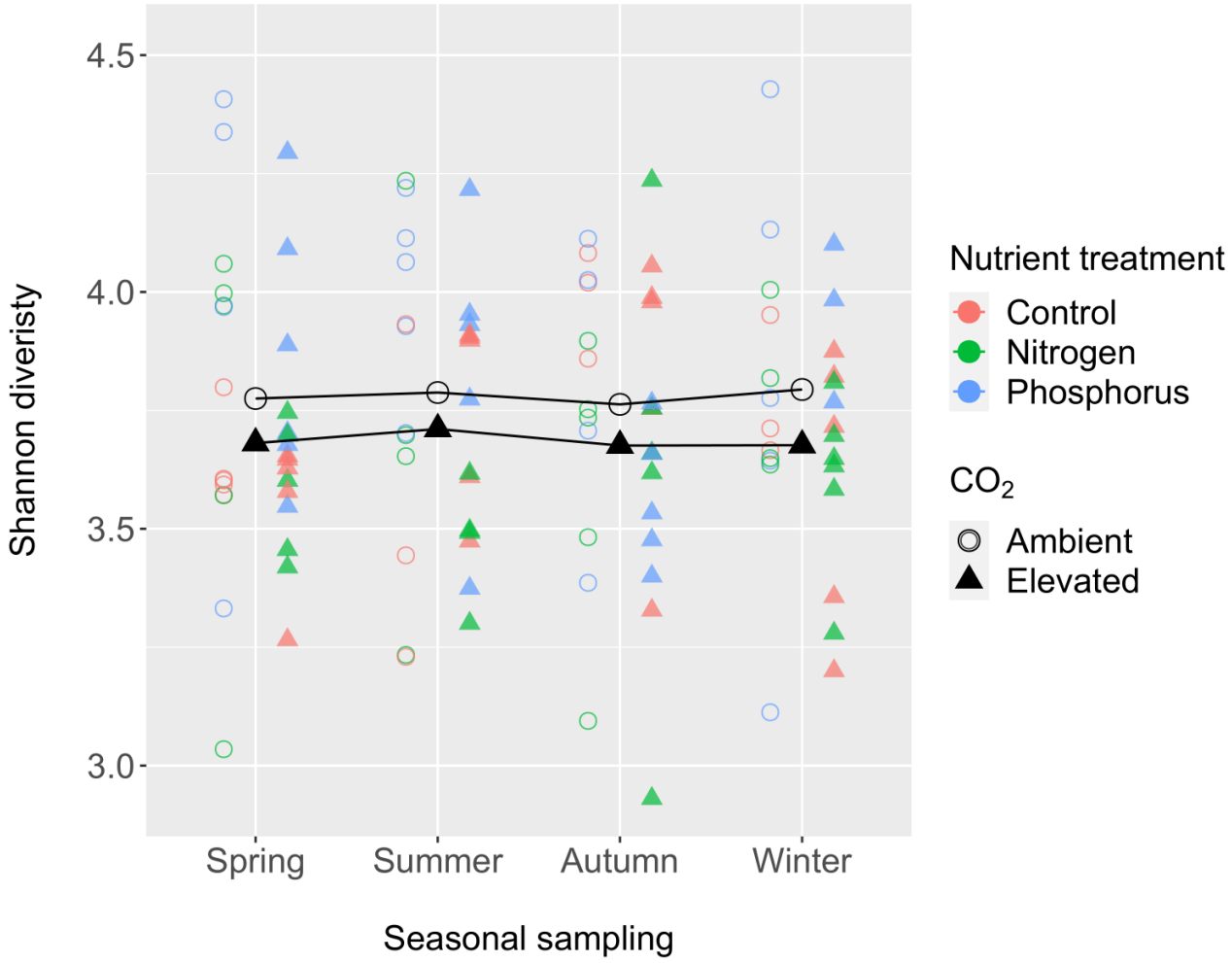


Figure 2. 8. The limestone grasslands ITS Shannon diversity over a year. Mean ambient and elevated $CO_2$ results for each seasonal time point are indicated in black and joined with a black line to show trends over the year (though the straight lines are not intended to signify a linear change between time points). Nutrient treatments are colour coded: red = control nutrient treatment, green = nitrogen nutrient treatment, blue = phosphorus treatment. (*) indicate significant difference at time point and number of stars indicate significance level (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, + $p < 0.08$.

2.4.1.7. Limestone grassland bacterial Chao species richness

Overall bacterial species richness in the limestone grassland, was unaffected by $eCO_2$ (LME, df = 1, 105, F = 0.15, p = 0.69520) but it varied depending on the time of year (LME, df = 3, 105, F = 8.25, p < 0.001). Winter bacterial species richness was greater than spring (t(95.3)=-3.834, p = 0.0004) and summer (t(105) = 2.71, p = 0.0013). There was no $eCO_2$ *x* time of year interaction (LME, df = 3, 105, F = 0.52, p = 0.66760).

Nutrient treatment over the year affected bacterial species richness (LME, df = 2, 105, F = 2.73, p = 0.07). Nitrogen amendment reduced bacterial species richness compared to the control nutrient treatment (t(31.1) = 2.14, p = 0.097). Elevated $CO_2$ *x* nutrient treatment interacted to affect species richness (LME, df = 2, 105, F= 4.74, p = 0.011). In the limestone grassland, with no nutrient enrichment, $eCO_2$ reduced (t(30.8) = 2.17, p = 0.0375) and in the phosphorus treatment $eCO_2$ increased (t(34.2) = -1.89, p = 0.067) bacterial species richness.

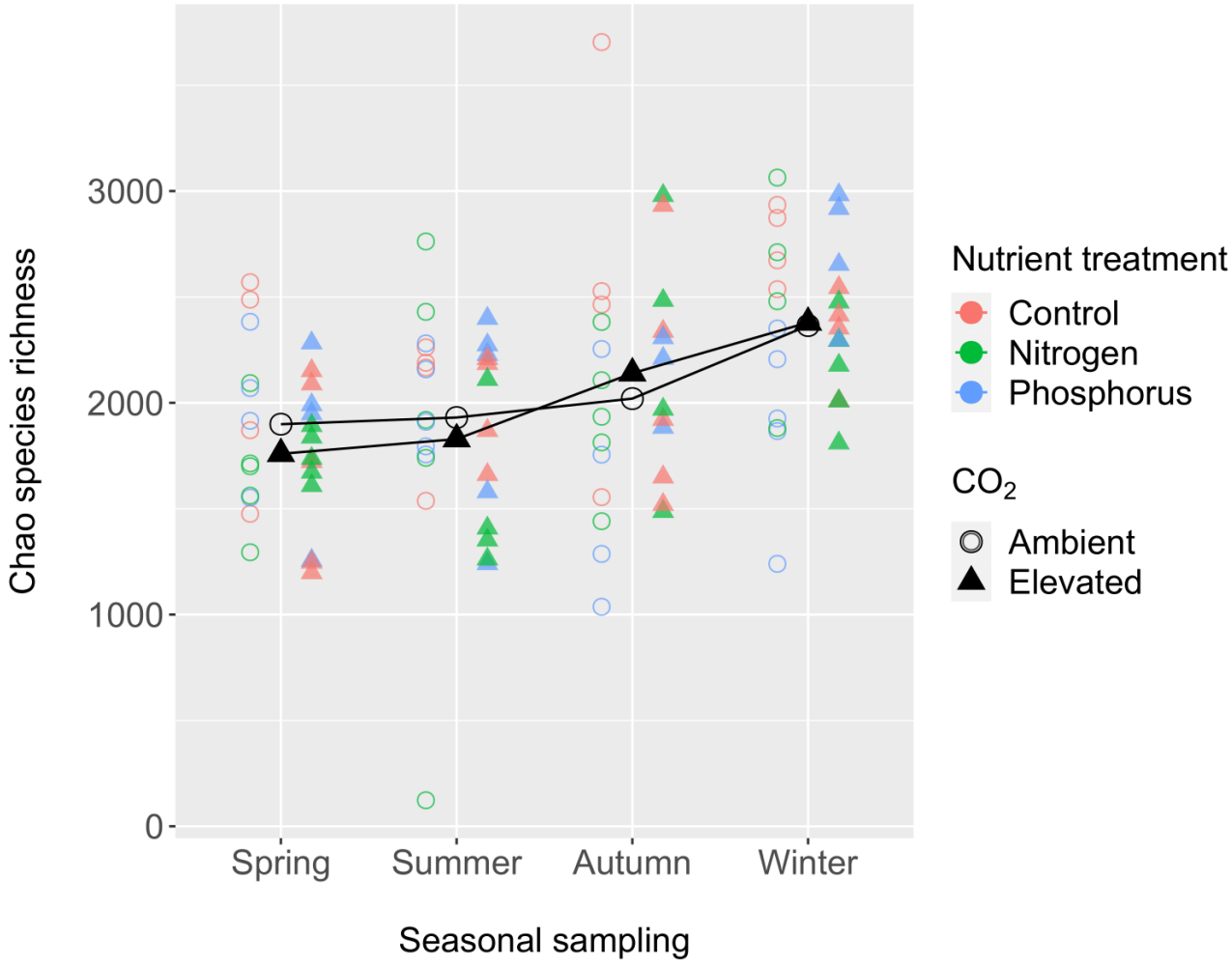


Figure 2. 9. The limestone grasslands 16S Chao species richness over a year. Mean ambient and elevated $CO_2$ results for each seasonal time point are indicated in black and joined with a black line to show trends over the year (though the straight lines are not intended to signify a linear change between time points). Nutrient treatments are colour coded: red = control nutrient treatment, green = nitrogen nutrient treatment, blue = phosphorus treatment. (*) indicate significant difference at time point and number of stars indicate significance level (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, + $p < 0.08$.

2.4.1.8. Limestone fungal Chao species richness

Overall, limestone fungal species richness was reduced by $eCO_2$ (LME, df = 1, 109, F = 6.56, p = 0.01177) but $eCO_2$ reduced spring (t(121)= 1.88, p = 0.06) and autumn (t(112) = 1.74, p = 0.08)

richness. Fungal species richness was unaffected by the time of year (LME, df = 3, 109, F = 0.50, p = 0.68583) and there was no $eCO_2$ *x* time of year interaction (LME, df = 3, 109, F = 0.60, p = 0.623).

The fungal species richness, was unaffected by nutrient treatment (LME, df = 2, 109, F = 1.64, p = 0.199) and there was no $eCO_2$ *x* nutrient treatment interaction (LME, df = 2, 109, F = 0.39, p = 0.681).

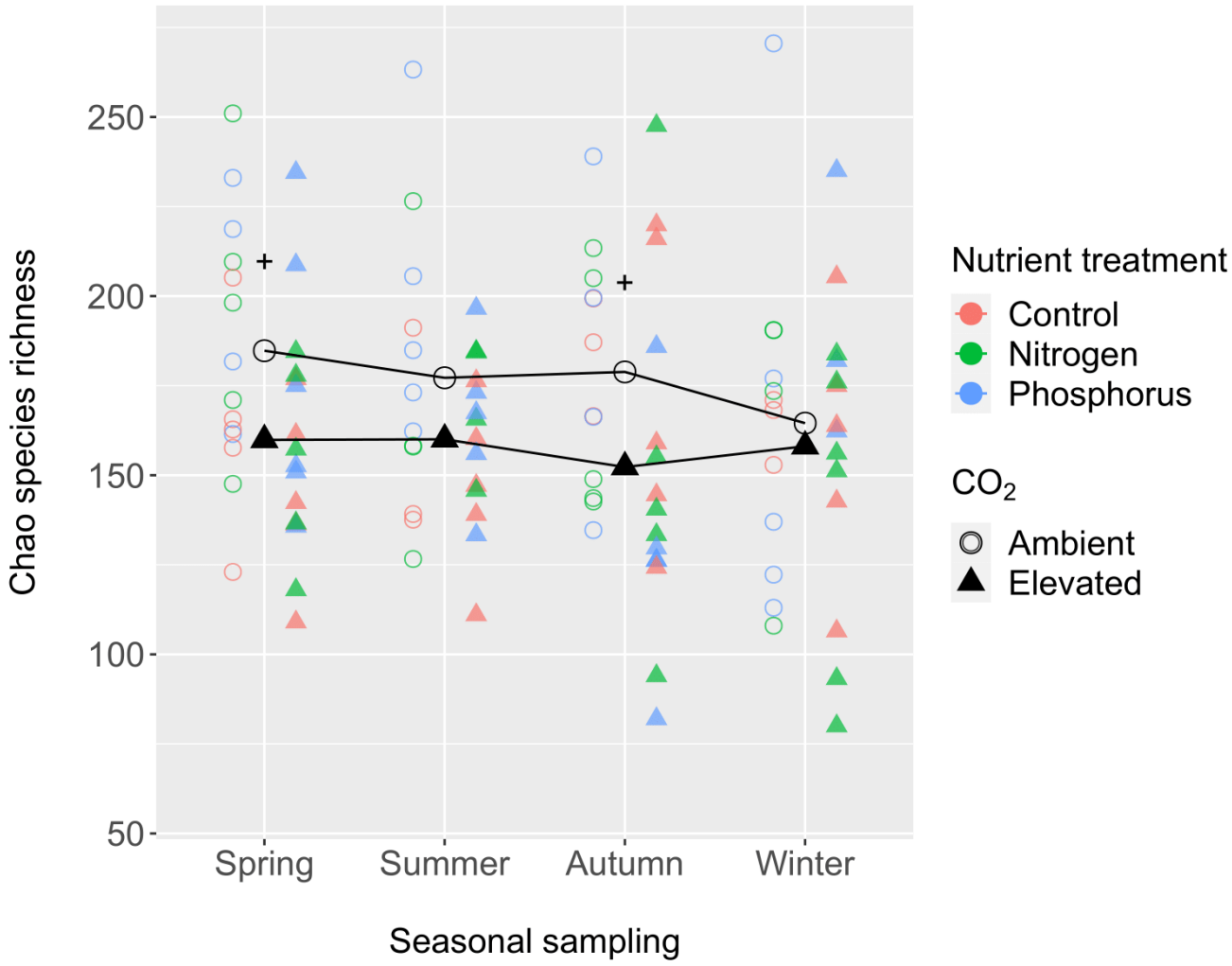


Figure 2. 10. The limestone grasslands ITS Chao species richness over a year. Mean ambient and elevated $CO_2$ results for each seasonal time point are indicated in black and joined with a black line to show trends over the year (though the straight lines are not intended to signify a linear change between time points). Nutrient treatments are colour coded: red = control nutrient treatment, green = nitrogen nutrient treatment, blue = phosphorus treatment. (*) indicate significant difference at time

point and number of stars indicate significance level (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, + $p < 0.08$.

2.4.1.9. Grassland alpha diversity comparison

Generally, bacteria species richness and biodiversity (slightly) was higher in the limestone compared to the acidic grassland [Bacteria alpha diversity at ambient $CO_2$: mean Shannon diversity (Acid = 5.65) (Limestone = 5.96), mean Chao species richness (Acid = 1593.59) (Limestone= 2363.91)]. However, fungal diversity and species richness was similar in both grasslands [Fungal alpha diversity at ambient $CO_2$: mean Shannon diversity (Acid = 3.75) (Limestone = 3.72), mean Chao species richness (Acid = 166.33) (Limestone = 166.23)]. Bacteria compared to fungi tended to have greater biodiversity and species richness in both grasslands.

### *2.4.2. Beta diversity*

2.4.2.1. Acidic grassland bacterial beta diversity

In the acidic grassland, $eCO_2$ produced bacterial communities that were dissimilar to communities in the ambient $CO_2$ treatment (PERMANOVA, df = 1, R2 = 0.046, F= 9. 48, $p < 0.001$) and the time of year produced communities dissimilar to one another (PERMANOVA, df = 3, R2 = 0.38, F = 27.98, $p < 0.001$).

Spring and summer bacterial communities, which did not differ to each other, differed from both autumn and winter communities, which differed to each other (see supplementary table for statistical contrasts S1.4). The spring summer and autumn winter bacterial communities clustered separately in the ordination plot (Fig. 2.11). Spring and summer communities cluster towards the south and south-west, autumn and winter communities cluster more towards the north and north east of the ordination plot.

Elevated $CO_2$ bacterial communities are dissimilar to ambient $CO_2$ bacterial communities in autumn (PERMANOVA, df = 1, R2, F= 4.95, p = 0.006). Autumn $eCO_2$ bacterial communities appear to cluster more closely with ambient spring and summer communities in the ordination space, as blue triangles appear to have a south-west trajectory away from blue circles.

Nutrient treatment (PERMANOVA, df = 2, R2 = 0.03, F = 3. 07, p = 0.0190), and the $eCO_2$ nutrient treatment interaction (PERMANOVA, df = 2, R2 = 0.07, F = 7.52, p < 0.001) produced dissimilar acidic grassland bacterial communities. The nutrient treatment $eCO_2$ interaction produced dissimilar communities in spring (PERMANOVA, df = 2, R2 = 0.18, F = 3.0835, p = 0.014) and winter (PERMANOVA, df = 2, R2 = 0.14, F = 2.15, p = 0.033) and in summer (PERMANOVA, df = 2, R2 = 0.145, F = 2.18, p = 0.077).

Bacterial communities in the acidic grassland, in the control nutrient treatment, were less seasonally dynamic with $eCO_2$. In the nitrogen treatment bacterial communities did not change with the time of year of $eCO_2$. The opposite effect was found in the phosphorus treatment where $eCO_2$ increased seasonally dynamic communities (see below).

In the ambient $CO_2$ (no nutrient addition) acidic grassland, bacterial communities in summer and autumn were dissimilar (df = 1, R2 = 0.53, p = 0.036) but were not dissimilar in the $eCO_2$ treatment (df = 1, R2 = 0.22, p = 1.000). Whereas, in the phosphorus ambient $CO_2$ treatment the communities were not seasonally different but with $eCO_2$, the bacterial communities in summer and autumn were dissimilar (df = 1, R2 = 0.62, p = 0.042).

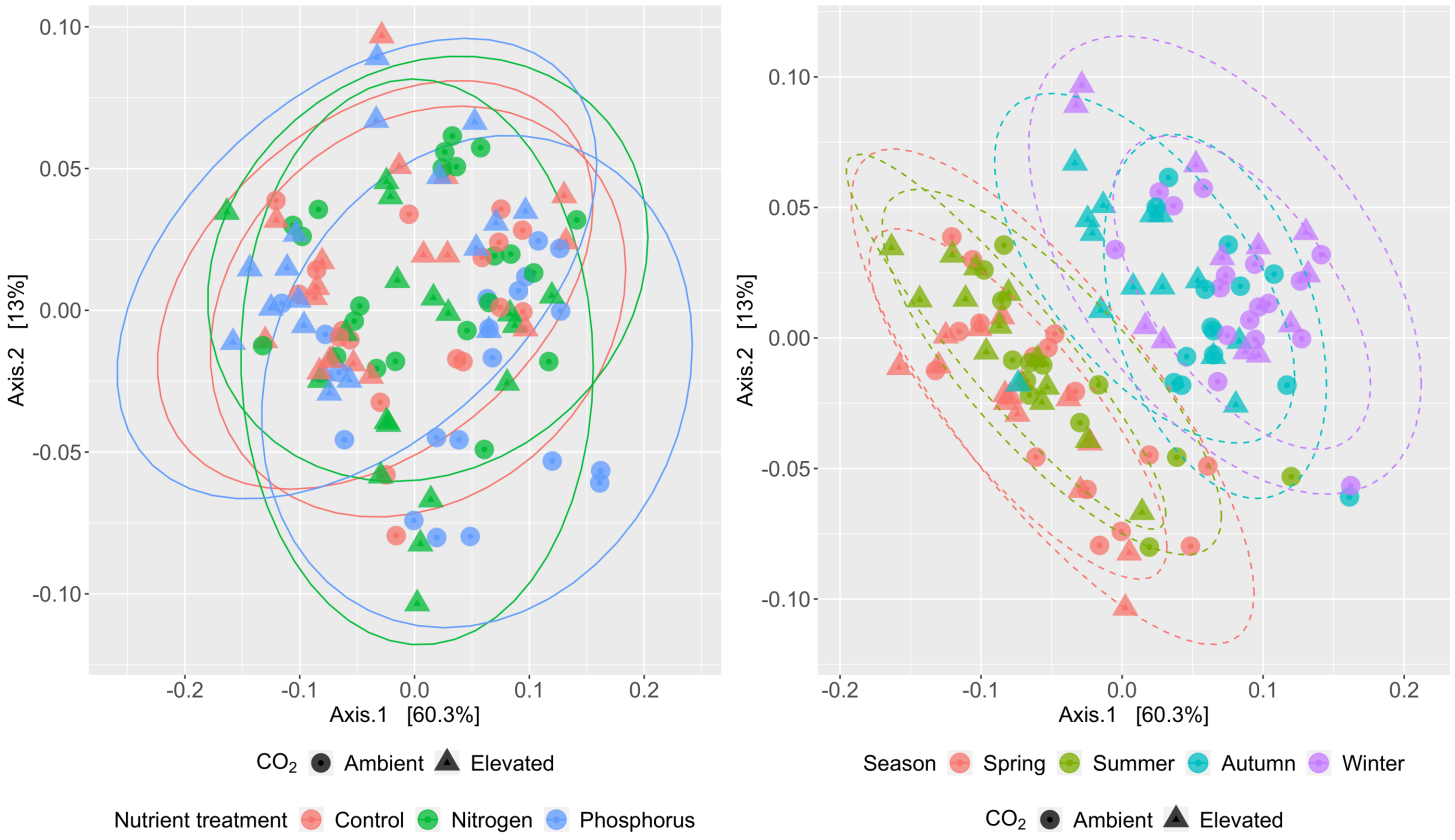


Figure 2. 11. The acidic grasslands 16S weighted UniFrac PCoA beta diversity over a year. Both plots are the same data, circle points are individual ambient $CO_2$ mesocosms and the triangles are elevated $CO_2$ mesocosms, displayed with different colour coding. The left plot has nutrient treatment colour coding: red = control nutrient treatment, green = nitrogen nutrient treatment, blue = phosphorus nutrient treatment. The right plot has season colour coding: red = spring, green = summer, blue = autumn and purple = winter. Ellipses indicate 95% confidence intervals and show broad clustering regions by group.

2.4.2.2. Acidic grassland fungal beta diversity

Elevated $CO_2$ fungal communities in the acidic grassland were not dissimilar to ambient $CO_2$ communities (PERMANOVA, df = 1, R2 = 0.01, F = 1.27, p = 0.2273) but did differ with the time of year (PERMANOVA, df = 3, R2 = 0.06, F = 2.35, p = 0.0035). The fungal community in autumn was

dissimilar to the community in summer (df = 1, R2 = 0.07, p = 0.18). The summer and autumn fungal communities did not clearly cluster away from each other in the ordination plot (Fig. 2.12).

The fungal communities in the acidic grassland differed with nutrient treatment (PERMANOVA, df = 2, R2 = 0.03, F = 2.03, p = 0.0259). Specifically, phosphorus treated communities were dissimilar to nitrogen amended communities (df = 1, R2 = 0.04, p = 0.075). However, this effect was not clearly observed in the clustering in ordination space (Fig. 2.12).

Elevated $CO_2$ and nutrient treatment interacted to produce dissimilar fungal communities (PERMANOVA, df = 1, R2 = 0.04, F = 2.26, p = 0.0144). Winter fungal communities, were affected by this interaction (PERMANOVA, df = 2, R2 = 0.11, F = 1.76, p = 0.054) but there was no evidence for this effect in other seasons.

Elevated $CO_2$ reduced the occurrence of seasonally different communities in the control nutrient treatment but had no effect in the nitrogen and phosphorus treatment. In the acidic grassland (no nutrient addition) at ambient $CO_2$, the autumn fungal community was dissimilar to the summer fungal community (df = 1, R2 = 0.21, p = 0.048) but this effect was not found in the $eCO_2$ treatment (df = 1, R2 = 0.15, p = 1.000).

The fungal acidic grassland communities had two distinct clusters in ordination space, but it was not clear what drove this.

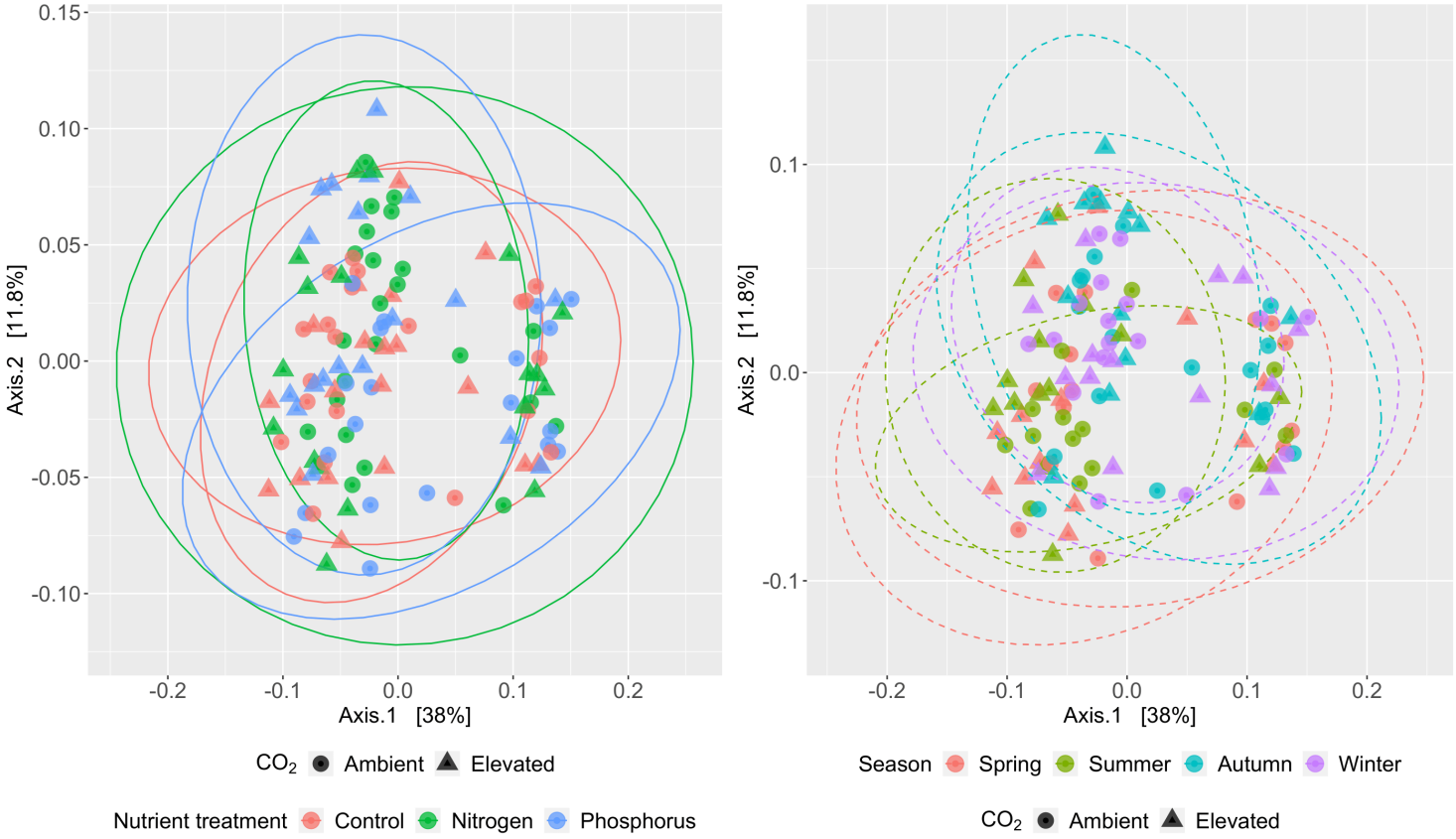


Figure 2. 12. The acidic grassland ITS weighted UniFrac beta diversity over a year. Both plots are the same data, circle points are individual ambient $CO_2$ mesocosms and the triangles are elevated $CO_2$ mesocosms, displayed with different colour coding. The left plot has nutrient treatment colour coding: red = control nutrient treatment, green = nitrogen nutrient treatment, blue = phosphorus nutrient treatment. The right plot has season colour coding: red = spring, green = summer, blue = autumn and purple = winter. Ellipses indicate 95% confidence intervals and show broad clustering regions by group.

2.4.2.3. Limestone grassland bacterial beta diversity

Bacterial communities in the limestone grassland under $eCO_2$ were dissimilar to ambient $CO_2$ bacterial communities (PERMANOVA, df = 1, R2 = 0.02, F = 3.08, p = 0.0246) and dissimilar depending on the time of year (PERMANOVA, df = 1, R2 = 0.28, F = 14.26, $p < 0.001$). The spring and summer bacterial communities, which were not dissimilar to each other, differed from both autumn and winter communities, which did not differ to each other (see supplementary table for statistical contrasts S1.5). Spring and summer communities cluster together in the south-west and

north-west ordination space and the autumn and winter communities cluster together towards the north-east ordination space (Figure 2.13).

Bacterial communities in the limestone grassland significantly with nutrient treatment (df = 2, R2 = 0.06, p < 0.001). Specifically, the bacterial community in the nitrogen amended plots differed significantly to the phosphorus amended plots (df = 1, R2 = 0.07, p = 0.009).

The bacterial communities, differed with nutrient treatment in the spring (PERMANOVA, df = 2, R2 = 0.16, F = 2.49, p = 0.030) and autumn (PERMANOVA, df = 2, R2 = 0.19, F = 2.50, p = 0.008), in winter (PERMANOVA, df = 2, R2 = 0.05, F = 1.7, p = 0.072) but not in summer (PERMANOVA, df = 2, R2 = 0.1, F = 1.24, p = 0.242).

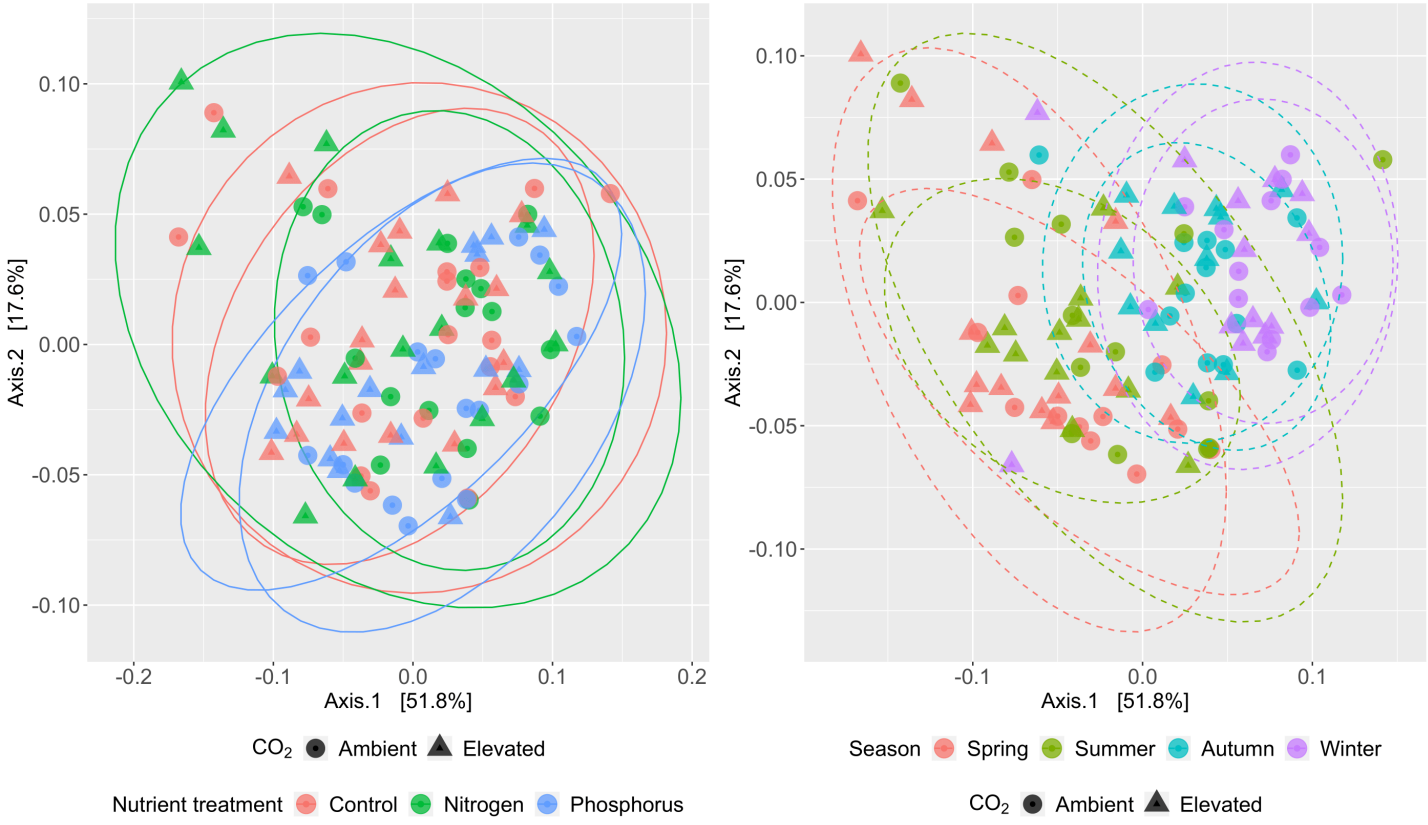

Figure 2. 13. The limestone grasslands 16S weighted UniFrac beta diversity over a year. Both plots are the same data, circle points are individual ambient $CO_2$ mesocosms and the triangles are elevated $CO_2$ mesocosms, displayed with different colour coding. The left plot has nutrient treatment colour coding: red = control nutrient treatment, green = nitrogen nutrient treatment, blue = phosphorus nutrient treatment. The right plot has season colour coding: red = spring, green = summer, blue = autumn and purple = winter. Ellipses indicate 95% confidence intervals and show broad clustering regions by group.

2.4.2.4. Limestone grassland fungal beta diversity

Fungal communities in the limestone grassland under $eCO_2$ did not differ from ambient $CO_2$ communities (PERMANOVA, df = 1, R2 = 0.01, F = 1.44, p = 0.1473) but fungal communities did vary with the time of year (PERMANOVA, df = 3, R2 = 0.05, F = 1.81, p = 0.0120). The summer fungal communities differed from the winter fungal communities (df = 1, R2 = 0.05, p = 0.072). This effect was not clearly shown by clustering in the ordination plot (Fig. 2.14).

Nutrient addition produced fungal communities that differed from one another in the limestone grassland (PERMANOVA, df = 2, R2 = 0.08, F = 4.43, $p < 0.001$). Specifically, the phosphorus treatment community was dissimilar to both the control (PERMANOVA, df = f, R2 = 0.05, $p < 0.01$) and the nitrogen treatment community (df = 1, R2 = 0.09, $p < 0.01$). The phosphorus treated limestone fungal community clustered towards the northern ordination area; whereas the nitrogen and control communities tended to extend towards a southern trajectory (Fig. 2.14).

The fungal limestone grassland community was significantly affected by nutrient treatment in spring (PERMANOVA, df = 2, R2 = 0.15, F = 2.49, p = 0.005) and in summer (PERMANOVA, df = 2, R2 = 0.18, F = 2.68, $p < 0.001$). Summer fungal communities were affected by the $eCO_2$ nutrient treatment interaction (PERMANOVA, df = 2, R2 = 0.11, F = 1.65, p = 0.042).

Similar to the acidic grassland fungal community, the ordination plots featured two main clusters with an east and west trajectory and it is unclear what drove this clustering. However, the eastern fungal

cluster community is more represented by elevated $CO_2$ fungal communities in the limestone grassland.

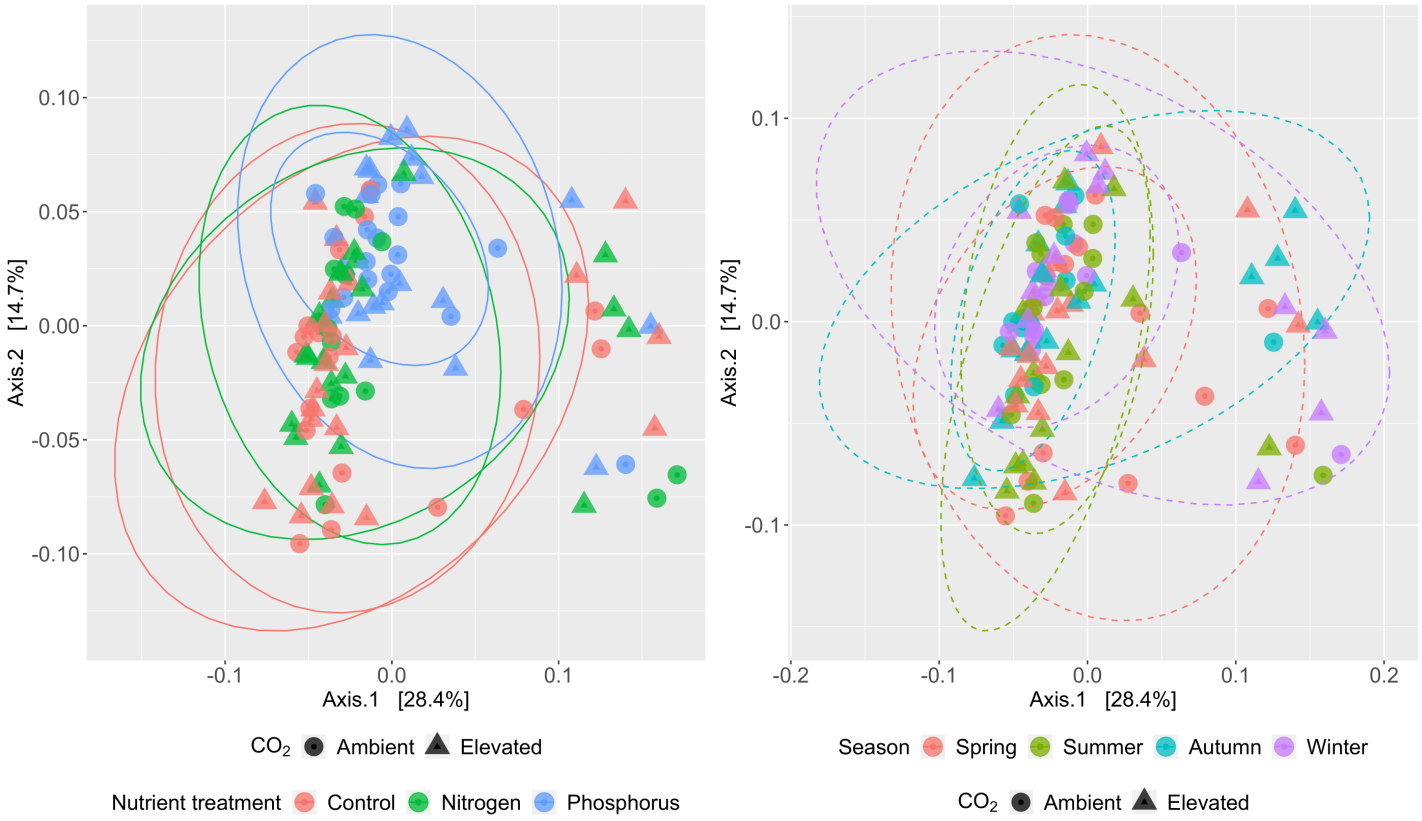


Figure 2. 14. The limestone grasslands ITS weighted UniFrac beta diversity over a year. Both plots are the same data, circle points are individual ambient $CO_2$ mesocosms and the triangles are elevated $CO_2$ mesocosms, displayed with different colour coding. The left plot has nutrient treatment colour coding: red = control nutrient treatment, green = nitrogen nutrient treatment, blue = phosphorus nutrient treatment. The right plot has season colour coding: red = spring, green = summer, blue = autumn and purple = winter. Ellipses indicate 95% confidence intervals and show broad clustering regions by group.

*2.4.3. Soil organic carbon and pH*

In the acidic grassland, $eCO_2$ increased organic carbon (LME, df =1 , 26.13, F = 4.74, p = 0.0463). The time of year (LME, df = 3, 80.98, F = 0.63, p = 0.613) or nutrient treatment (LME, df = 1, 25.94, F = 0.05, p = 0.963) did not affect organic carbon.

In the limestone grassland, organic carbon was unaffected by $eCO_2$ (LME, df = 1, 28.57, F = 0.36, p = 0.548) or time of year (LME, df = 1, 73.35, F = 0.86, p 0.421) but was affected by nutrient treatment (LME, df = 2, 59.38, F = 4.84, p = 0.012). The nitrogen treatment (t(65.42) = 2.99, p < 0.004), and phosphorus addition near (t(75.26) = -1.9, p = 0.06), reduced organic carbon.

The pH measurements for the acidic and limestone grasslands are only available for the first three seasonal samplings: spring, summer and autumn.

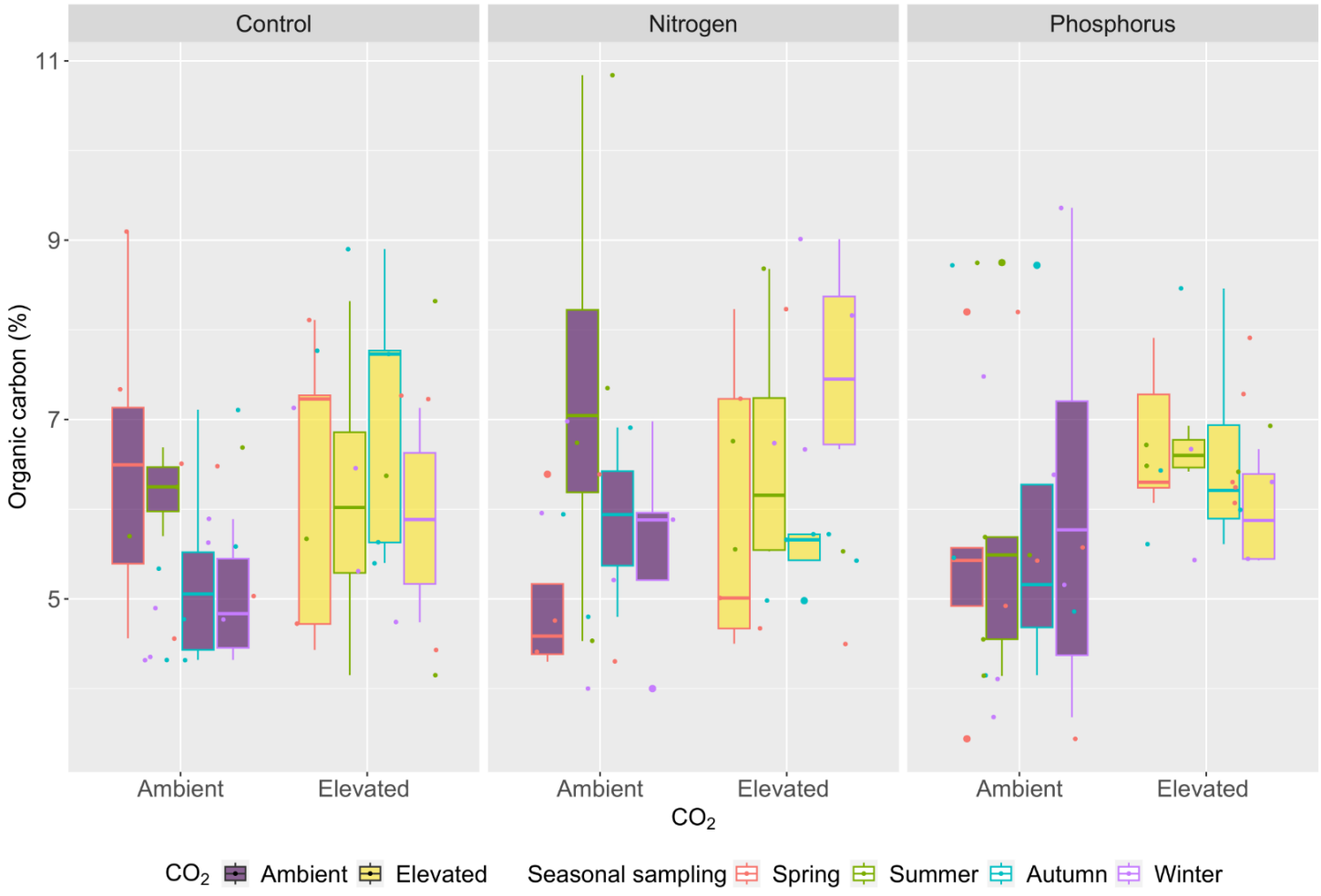

Figure 2. 15. The soil organic carbon content (%) of an acidic grassland over a year, with and without $eCO_2$ (A = ambient $CO_2$, E = elevated $CO_2$) and with control, nitrogen or phosphorus nutrient addition. Seasonal sampling times correspond to spring = T1 (red), summer T2 (green), autumn = T3 (blue) and winter T4 (purple). Small coloured dots illustrate replicate results from each time point.

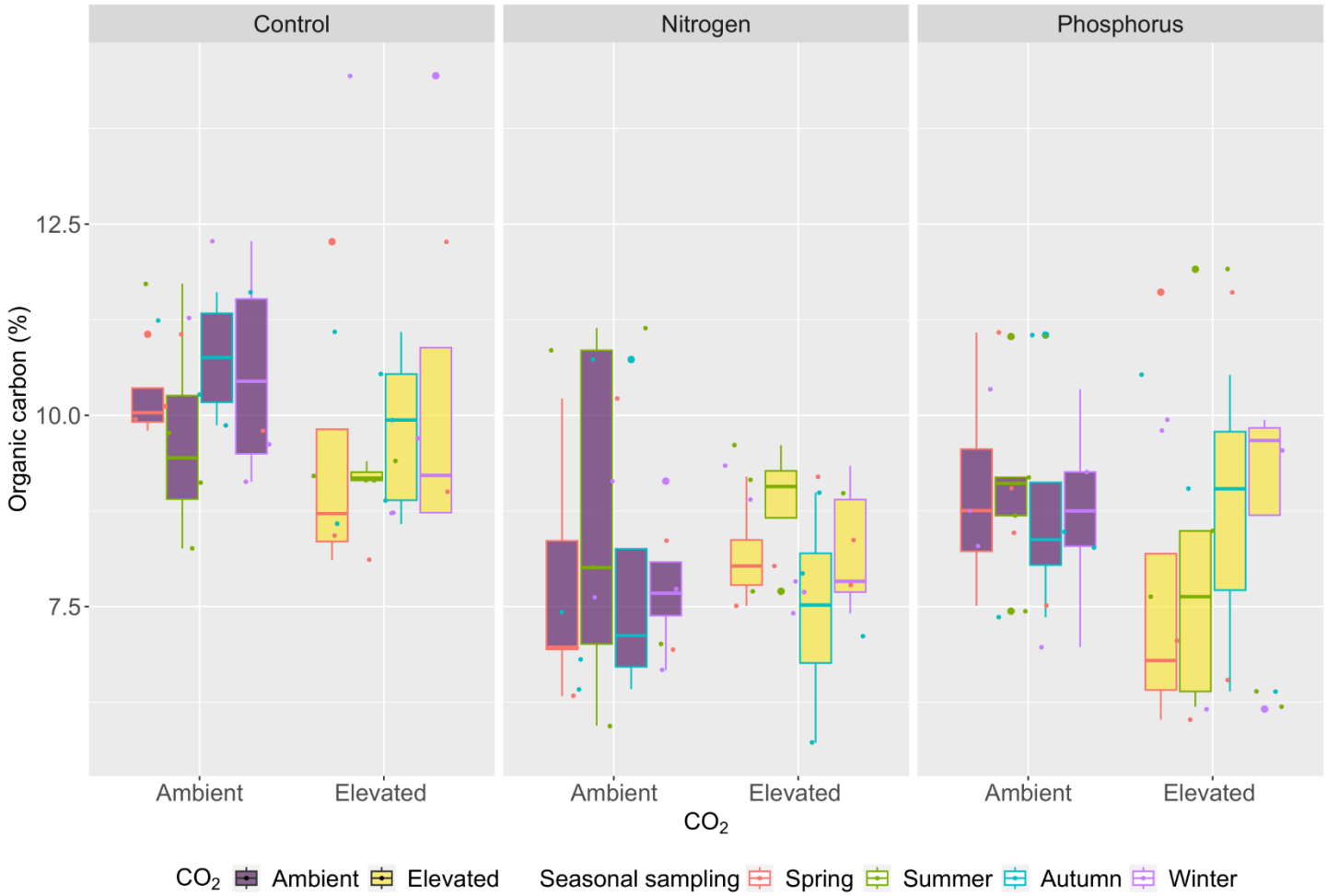


Figure 2. 16. The soil organic carbon content (%) of a limestone grassland over a year, with and without $eCO_2$ (A = ambient $CO_2$, E = elevated $CO_2$) and with control, nitrogen or phosphorus nutrient addition. Seasonal sampling times correspond to spring = T1 (red), summer T2 (green), autumn = T3 (blue) and winter T4 (purple). Small coloured dots illustrate replicate results from each time point.

The acidic grassland's soil pH was unaffected by $eCO_2$ (LME, df = 1, 32.78, F = 2.71, p = 0.09) and time of year (LME, df = 2, 34.77, F = 0.01, p 0.988) but was affected by nutrient addition (LME, df = 2, 12.79, F = 20.20, p <0.001). The nitrogen treatment reduced (t(13.96) = -3.64, p < 0.001) and

phosphorus treatment increased (t(13.17) = 2.42, p = 0.038) soil pH compared to the control nutrient treatment. The $eCO_2$ phosphorus treatment interaction decreased soil pH (t(12.71) = -1.92, p = 0.077).

Similar to the acidic grassland, the limestone grasslands soil pH was unaffected by $eCO_2$ (LME, df = 1, 29.773, F = 1.78, p = 0.1631). The limestone grasslands pH differed depending on the time of year (LME, df = 2, 49.22, F = 6.49, p 0.014), nutrient addition (LME, df = 2, 39.13, F = 22.31, p < 0.001) and the time of year nutrient treatment interaction (LME, df = 4, 50.09, F = 3.36, p = 0.018).

Soil pH was lower in summer (t(51. 46) = -3.41, p = 0.0018) and autumn (t(48.21) = - 4.16, p < 0.001) compared to spring. The limestone grasslands pH was overall reduced by nitrogen (t(71.61) = -7.46, p < 0.001) and phosphorus addition (t(72.96) = -2.82, p < 0.001).

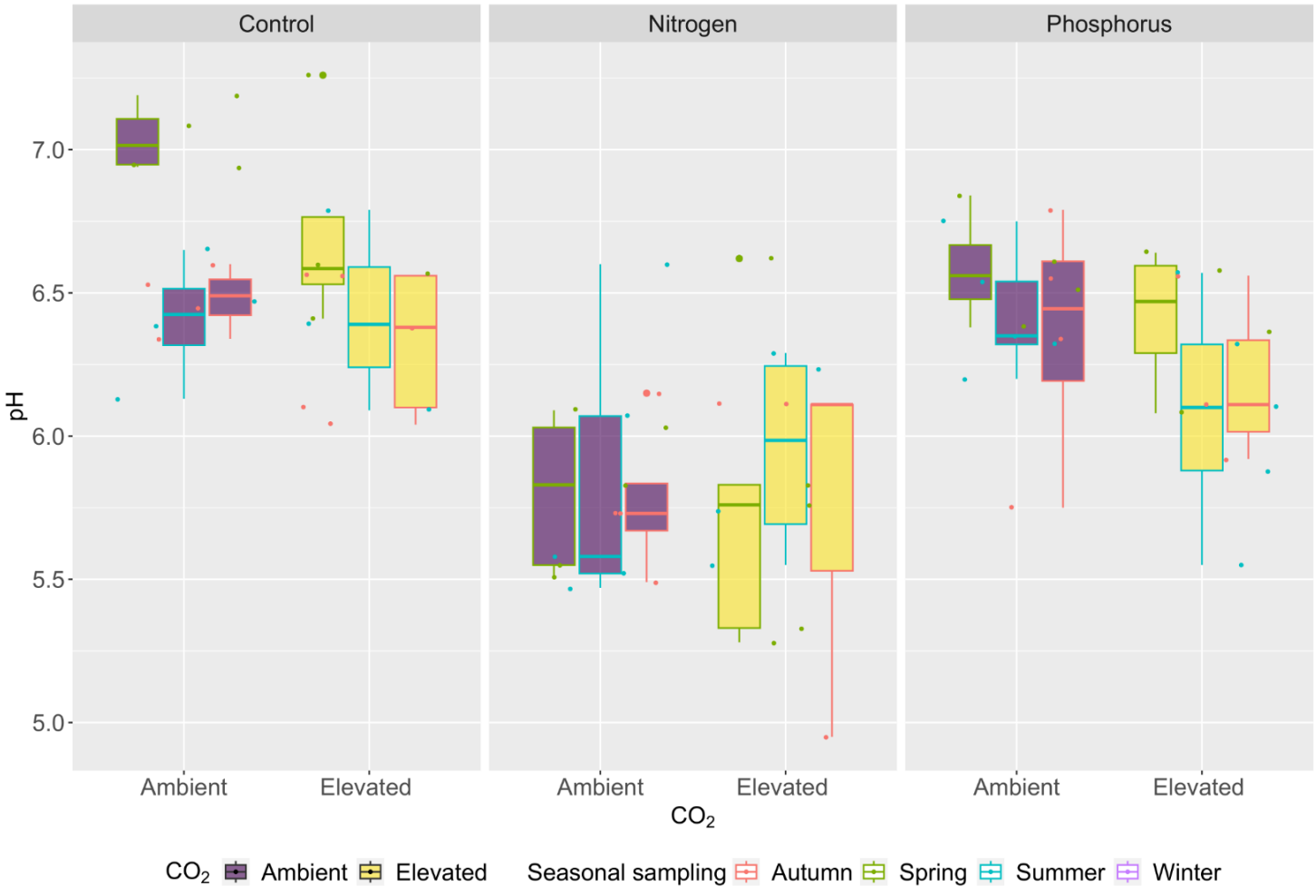

Figure 2. 17. The soil pH of an acidic grassland over a year, with and without $eCO_2$ (A = ambient $CO_2$, E = elevated $CO_2$) and with control, nitrogen or phosphorus nutrient addition. Seasonal sampling times correspond to spring = T1 (red), summer T2 (green), autumn = T3 (blue) and winter T4 (purple). Small coloured dots illustrate replicate results from each time point.

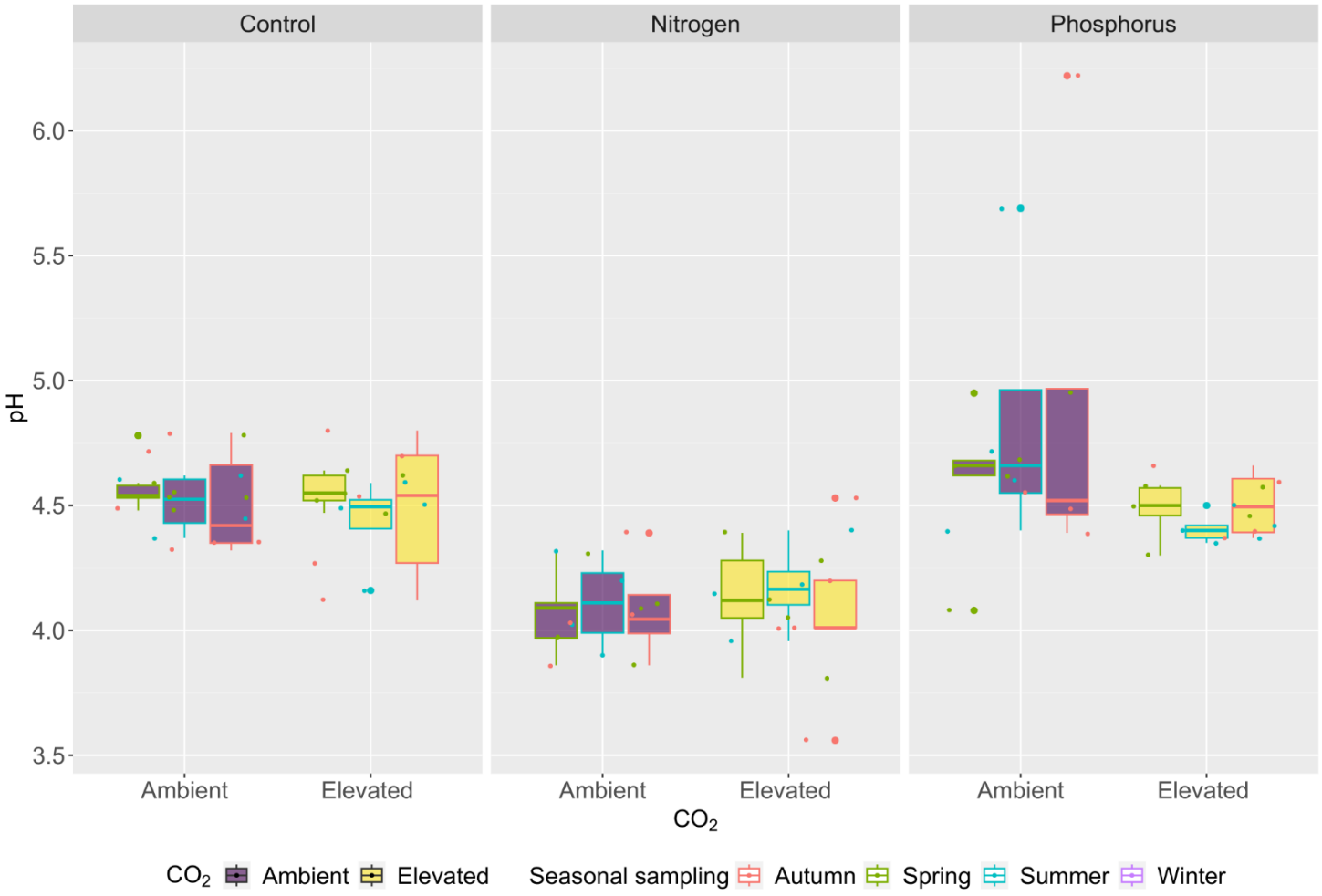


Figure 2. 18. The soil pH of a limestone grassland over a year, with and without $eCO_2$ (A = ambient $CO_2$, E = elevated $CO_2$) and with control, nitrogen or phosphorus nutrient addition. Seasonal sampling times correspond to spring = T1 (red), summer T2 (green), autumn = T3 (blue) and winter T4 (purple). Small coloured dots illustrate replicate results from each time point.

## 2.5. Discussion

### *2.5.1. Alpha diversity*

#### 2.5.1.1. Elevated $CO_2$ effects on alpha diversity

Overall, $eCO_2$ reduced the acidic grassland bacterial Shannon diversity, the acidic grassland spring bacterial Chao species richness and the limestone grassland bacterial and fungal Chao species richness. A reduction in alpha diversity can be analogous to a relative increase in dominance (Schwartz *et al.*, 2000), possibly due to a greater flow of plant carbon belowground with $eCO_2$ (Paterson *et al.*, 1997) and a relative increase in the abundance of species best adapted to that greater resource availability. Soil microorganisms dynamically respond to changes in resource availability (Fontaine *et al.*, 2007), and soil carbon composition, and the microbial community composition and function exist in a tight relationship (Ng *et al.*, 2014). As heterotrophic soil microorganisms are primarily carbon limited (Soong *et al.*, 2019), and the alpha diversity measurements in this experiment are relative, the alleviation of carbon limitation may have released constraints on certain species that now are able to dominate the community more, in turn reducing alpha diversity. This dominance effect is usually found in the rhizosphere, where diversity is reduced compared to bulk soil with the dominance of a few taxa (Philippot *et al.*, 2013). This effect could become more prevalent in bulk soil under $eCO_2$ as found in this experiment.

However, most $eCO_2$ experiments, including FACE experiments, do not report reductions in bacterial or fungal alpha diversity. Lipson *et al.*, (2014), found fungal diversity in terms of richness and evenness increased at the semiarid chaparral shrubland FACE in Southern California. In addition, the long-term $eCO_2$ BioCON experimental site, a 12-year $CO_2$ manipulation in temperate grassland in central Minnesota, USA, fungal richness was not found to change with $eCO_2$ but communities did reassemble (Tu *et al.*, 2015). No discernible effects of $eCO_2$ on bacterial biodiversity have also been reported (Lipson *et al.*, 2006; Hayden *et al.*, 2012). However, *Populus tremuloides* seedlings grown under five years of $eCO_2$ had increased bacterial biodiversity sampled from the roots (Janus *et al.*,

2005). The reductions in microbial alpha diversity in this study may contrast most reports in the literature, as investigating $eCO_2$ effects on phosphorus limited ecosystems is rare. For example, this mini-FACE system (Keane *et al.*, 2020) is one of two FACE systems globally that investigate the effects of $eCO_2$ on proven phosphorus limited environments, the other being the EucFACE Eucalyptus forest system in Australia where as far as I am aware no investigation into soil microbial diversity with $eCO_2$ has been done (EucFACE, no date). Phosphorus limitation is noted as an important constraint on $eCO_2$ ecosystem productivity in a recent meta-analysis (Jiang *et al.*, 2020). As increased ecosystem productivity is dependent on the microbial cycling of carbon and nutrients (Dijkstra *et al.*, 2013), constrains on ecosystem productivity are likely representative of microbial process as well. Therefore, phosphorus limited ecosystems are likely to respond uniquely to $eCO_2$.

In fact, the alleviation of phosphorus limitation (the phosphorus treatment) increased microbial alpha diversity in this experiment, indicating that phosphorus limitation may be driving microbial alpha diversity declines observed with $eCO_2$. In the limestone grassland, $eCO_2$ and phosphorus treatment interacted to increase bacterial Shannon diversity, and fungal Shannon diversity in the phosphorus treatment was higher compared to the nitrogen treatment (control treatment was in between). In addition, the limestone grassland bacterial species richness was reduced by $eCO_2$ in the control nutrient treatment but close-to-increased (near-sig) in the phosphorus. However, increased microbial alpha diversity with $eCO_2$ was only found in the limestone grassland not the acidic. Although both grasslands are phosphorus limited, the limestone grassland has a more neutral pH compared to the acidic grassland (mean soil pH; acidic grassland = 4.39, limestone grassland = 6.25). Soil pH is commonly reported to have strong controls over soil microbial communities over regional scales (Hartman *et al.*, 2008; Baker *et al.*, 2009; Jesus *et al.*, 2009; Jones *et al.*, 2009; Lauber *et al.*, 2009). Therefore, it seems that in phosphorus limited grasslands, $eCO_2$ reduces alpha diversity; but this trend can be reversed by the alleviation of phosphorus limitation but only in more neutral pH soils. The nitrogen treatment in the limestone grassland strongly reduced soil pH and was associated with reduced alpha diversity. This further suggests that the alpha diversity response to nutrient and $eCO_2$ are partly controlled by soil pH.

2.5.1.2. Time of year effects on alpha diversity

Bacteria alpha-diversity changed depending on the time of year. There was a trend for increased bacterial alpha diversity in autumn and winter compared to spring and summer in all nutrient and $CO_2$ treatments. In both grasslands, autumn and winter increased bacterial Shannon diversity compared to spring and summer. Bacterial Chao species richness followed the same trend in the acidic grassland and was higher in winter compared to spring and summer in the limestone grassland. As this experiment contained one time series of samplings from spring to winter, it is unclear whether this trend was increasing through time and year on year, or if this was a seasonal pattern, where bacterial alpha diversity is reduced by spring and summer each year and then gradually increases as the year progresses. But the similarity in reduced alpha diversity in spring and summer vs autumn and winter, and the overall trend of a reduction with $eCO_2$, could indicate that an increase in rhizosphere carbon is driving both reductions. In grasslands, spring and summer is when the greatest root biomass and exudation rates are reported (Garcia-Pausas *et al.*, 2011; Shen *et al.*, 2020). Increased belowground plant activity in spring and summer could therefore be driving the reductions in alpha diversity compared to autumn and winter, and is more likely to be a seasonal cycle rather than a year-on-year increase. As samples were taken over one year this cannot be fully evaluated. None-the-less, the similarity in the $eCO_2$ response also driving reductions in alpha diversity in this study do indicate a rhizosphere driven response.

2.5.1.3. Possible effects of community size on alpha diversity

It is unfortunate that the quantitative spike used in this experiment was unsuccessful. A measure of the microbial community size though time and for different treatments in this study would have provided information on whether greater biodiversity coincided with greater or reduced community size and whether reduced biodiversity coincided with greater of reduced community size. High-throughput sequencing can provide information on how the abundance of taxa within a sample relate to the abundance of other taxa within the same sample. However, it has limitations as it is unable to assess

total microbial DNA across samples, which affects result interpretation (Smets *et al.*, 2016). For example, one (treatment or time-point) sample may appear to have a high relative abundance of a certain taxa, and another sample a relatively low abundance (sample with greater biodiversity) but the sample with the lower relative abundance may have a considerably larger number of microbial cells within the soil and a much greater cellular abundance of the taxa in question. In this experiment specifically, dramatic changes in biodiversity are hard to interpret without a measure of community size as large relative increases in one taxa cannot be distinguished from a relative decrease in another taxa. Therefore, the proportional nature of high-throughput sequencing alone makes it difficult to assess how the total amounts of microbes are changing across samples that could have very large differences in community size (cell numbers) (Smets *et al.*, 2016).

Another method that could provide information on the community size would be the use of qPCR. During the qPCR reaction, the amount of DNA present in the sample is amplified exponentially. The increase in fluorescence intensity is monitored in real-time by the qPCR machine. The cycle at which the fluorescence signal exceeds a predetermined threshold (Ct value) is recorded for each sample. The Ct value is inversely proportional to the amount of target DNA in the sample, with lower Ct values indicating higher abundance. Statistical analysis can be performed to compare microbial population sizes between different samples or treatments (Yu *et al.*, 2005). IF this experiment was to be repeated it could benefit from the additional use of qPCR to assess the 16S and ITS populations between different time and treatments.

### *2.5.2. Beta diversity*

#### 2.5.2.1. How $eCO_2$ effects the soil microbial communities

Elevated $CO_2$ produced different bacterial communities in both grasslands and interacted with nutrient treatment to produce different acidic grassland fungal communities. Microbial community structure, in particular bacterial community structure, is sensitive to $eCO_2$. Previous research conducted in grasslands has found that microbial community change is both resistant to $eCO_2$ (Ebersberger *et al.*,

2004; Guenet *et al.*, 2012) and sensitive to change (Castro *et al.*, 2010; He *et al.*, 2012; Yu *et al.*, 2018). Experiments have also been done on $eCO_2$ grassland studies, where $^{13}C$ labelled tracers were used to understand how additional plant carbon is incorporated into the soil microbial communities. A $^{13}CO_2$ pulse labelling experiment was conducted in the long-term Giessen FACE experiment (GiFACE) in Germany on a temperate grassland (Denef *et al.*, 2007). They found little of the $^{13}C$ tracer incorporated into the PLFAs after three hours, but there was greater incorporation into the fungal compared to the bacterial PLFAs after 10 hours. After eleven months, the $^{13}C$ enrichment of fungal PLFAs was largely still present but had decreased, while the bacterial PLFAs were greater enriched in the $^{13}C$ compared to a few hours after the pulse labelling. They concluded that additional $eCO_2$ is rapidly processed by arbuscular mycorrhizal fungal species (relative enrichment in the arbuscular mycorrhizal fungal biomarker PLFA 16:1ω5) and only much later by the bacterial communities. Furthermore, they attributed this to either a fungal-mediated translocation of rhizosphere carbon into the bacterial biomass or bacterial preference for dead root or fungal necromas carbon over the direct use of rhizosphere carbon (Denef *et al.*, 2007). This study can provide information on how the fungal and bacterial communities may respond individually to $eCO_2$. However, that long-term FACE experiment reported that the bacteria and archaea (Brenzinger *et al.*, 2017), and the fungi arbuscular mycorrhizal communities (Maček *et al.*, 2019) were unaffected by $eCO_2$. Although, more subtle increases and decreases of specific AM fungal taxa were observed with $eCO_2$ (Maček *et al.*, 2019). Nevertheless, that grassland was characterised by nitrogen limitation rather than phosphorus limitation (Denef *et al.*, 2007) and observed increased fungal (AM) richness with $eCO_2$, which suggests that different grassland responses to $eCO_2$ compared with this thesis's experiment may be taking place. Furthermore, the unique nature of the seasonal time series in this study provides insight into why microbial communities changed with $eCO_2$.

2.5.2.2. How the time of year shapes the microbial communities

The bacterial community, in both grasslands, differed in the $eCO_2$ treatment compared to the ambient $CO_2$ treatment and differed depending on the time of year. Both bacterial communities had strong

spring/summer *vs* autumn/winter communities with the two groups significantly different to each other. Spring and summer bacterial communities clustered together in both ordination spaces and away from the autumn winter cluster. However, this effect was more pronounced in the acidic grassland. The different spring summer vs autumn winter bacterial communities could be driven by the availability and quality of carbon resources partitioned belowground (Garcia-Pausas *et al.*, 2011; Shen *et al.*, 2020), which is suggested to also be responsible for the seasonal reductions in alpha diversity. Seasonally different microbial communities are reported in grassland ecosystems (Bardgett *et al.*, 1999; Habekost *et al.*, 2008; Slaughter, Weintraub and McCulley, 2015) and a change in carbon source and availability is commonly reported to partly account for differences.

2.5.2.3. How $eCO_2$ modifies the seasonal microbial communities

There is evidence that $eCO_2$ in the acidic grassland altered the seasonal dynamics of the microbial communities, during the year of this sampling. In the acidic grassland, the $eCO_2$ autumn bacterial samples (blue triangles) tended to cluster more towards the ambient $CO_2$ summer samples (Fig. 2.11). In autumn, the $eCO_2$ bacterial community differed from the ambient $CO_2$ bacterial community, but did not differ from the ambient $CO_2$ summer community. This indicates $eCO_2$ caused the summer bacterial community to extend into autumn, when the communities would usually differ from each other (ambient $CO_2$ treatment). There is some evidence this effect was present in the fungal acidic grassland community, but only for the control nutrient treatment. Under ambient $CO_2$ fungal communities differed in autumn compared to summer. Whereas, under $eCO_2$ there was no difference between any of the seasonal communities. This could be the result of a greater amount of rhizosphere carbon availability or/and extended exudation season (Phillips, Finzi and Bernhardt, 2011; Phillips *et al.*, 2012; Usyskin-Tonne *et al.*, 2021), driven by greater atmospheric $CO_2$.

It is unclear why this effect was not found in the limestone grassland, particularly in the bacterial community, as it also has the spring summer *vs* autumn winter community clustering (Fig. 2.13) and the bacterial community changed depending on the time of year. In addition, $eCO_2$ bacterial

communities differed compared to ambient $CO_2$ communities, indicating sensitivity to increased carbon availability. However, when analysing each time point in isolation there was no $eCO_2$ effect on beta diversity, which suggests a less sensitive response to $eCO_2$ compared to the acidic grassland. The limestone bacterial community overall changed depending on the time of year, but this effect was produced by nutrient treatment, as within each nutrient treatment $eCO_2$ did not produce different bacterial communities. The limestone grassland bacterial community was less different between seasons in the year of sampling, compared to the acidic grassland. The limestone bacterial community (seasonal perspective within each nutrient treatment) was different in the spring and following winter only in the nitrogen treatment, but did not seasonally change in the control or phosphorus treatment. Therefore, the bacterial limestone microbial community appeared less seasonally dynamic and less responsive to change by $eCO_2$ compared to the acidic grassland community. More seasonally dynamic belowground communities or key taxa/ life histories within those communities may be more sensitive to $eCO_2$, as they may be shaped to a greater extend by carbon quality and availability.

In both grasslands, $eCO_2$ did not produce different fungal communities. However, nutrient addition, in both grasslands, did produce fungal communities that differed to each other. Fungi may be more sensitive to nutrient addition due to above ground biodiversity changes associated with nutrient addition. Previous work on the mini-FACE experiment found that nutrient treatment rather than $eCO_2$ changed the plant functional types in the grasslands (Taylor *et al.*, in press). An increase in legumes was found in the phosphorus treatment and an increase in sedges was found in the nitrogen treatment (Taylor et al., in press). In a seasonal microbial community investigation on artificially controlled above ground biodiversity experiment (Jena experiment), the proportion of fungi was decreased in the presence of legumes (Habekost *et al.*, 2008). In grasslands studies across China, plant diversity had a stronger linkage with soil fungal diversity than with bacterial diversity (C. Wang *et al.*, 2022). Fungi, rather than bacteria, may be more influenced by nutrient addition rather than $eCO_2$ because of the functional roles that fungi occupy in relation to plants and their greater sensitivity to plant functional changes. For example, fungi functional guilds are arbuscular mycorrhizal fungi (AMF), ectomycorrhizal fungi (ECMF), saprotrophic fungi and pathotrophic fungi (Nguyen *et al.*, 2016; Yang

*et al.*, 2017). Therefore, a change in plant functional types with nutrient addition, rather than $eCO_2$, may influence the functional niches that exist for fungi to occupy, driving stronger community changes seen in this experiment. Microbial diversity research is currently shifting towards investigating microbial functions and traits and their interactions with other species rather than just taxonomy because of this (Frac *et al.*, 2018).

### *2.5.3. Different organic carbon grassland responses to $eCO_2$*

The limestone and acidic grassland exhibit opposing organic carbon storage trends with $eCO_2$. Elevated $CO_2$ caused and increase in organic carbon ($p = 0.0463$) in the acidic grassland but a trend for reduction in the limestone grassland, which was further reduced by both phosphorus and nitrogen treatment. The opposing directions of carbon storage in the soils may represent opposing priming of organic matter directions. In the acid grassland under $eCO_2$ negative priming may be occurring where an increase in carbon storage (reduced decomposition) is found, and in the limestone grassland positive priming may be occurring (increased decomposition) which results in a trend for carbon storage loss (Kuzyakov, Friedel and Stahr, 2000; Kuzyakov *et al.*, 2019). Under $eCO_2$ in both grasslands, an increase in phosphorus and nitrogen cycling enzymes has been found (Keane *et al.*, 2020), suggesting that additional carbon, from $eCO_2$, is invested by the plants into alleviating nutrient limitation. However, plants seem to only benefit from this increased carbon availability in the limestone grassland because previous research on this experiment found that in the limestone grassland, aboveground productivity increased (16%) and the microbial biomass phosphorus remained unchanged; whereas in the acidic grassland, aboveground productivity and phosphorus uptake declined (11% and 20%, respectively), but P immobilization into microbial biomass increased (36%) (Keane *et al.*, 2023). Therefore, these two phosphorus limited grasslands may represent a trade-off that exists under $eCO_2$. It appears that under $eCO_2$, plants are relatively less carbon limited but relatively more nutrient limited, to overcome this nutrient limitation they invest greater amounts of carbon belowground to overcome this limitation. In the limestone grassland, this investment pays off and plants increase phosphorus uptake and above ground biomass. In the acidic grassland, this

investment does not pay off and the microbial community benefits from the greater carbon and nutrient availability. In the acidic grassland (as discussed above), there is a stronger microbial diversity response to $eCO_2$ and it is suggested that the declines in diversity are driven by greater dominance of certain species caused by greater carbon availability. The priming response discussed here also indicates that the acidic grasslands' microbial community has greater access to carbon and nutrients, compared to the limestone grasslands', and that this greater resource availability shapes the microbial community more so.

The acidic grassland microbial community in this study, was comparatively more sensitive to $eCO_2$ while the limestone grassland was more sensitive to nutrient treatment. This effect, although also observed in beta diversity responses, was particularly apparent in the organic carbon content of the soil. This was surprising as previous research has shown that the limestone grassland is comparably more carbon limited (Keane *et al.*, 2020), so a greater sensitivity to carbon was expected. Both grasslands are phosphorus limited but the limestone grassland has a Ca-P weatherable bedrock; whereas, the acidic grassland's phosphorus, because of the low soil pH, will be bound to iron and aluminium secondary minerals for which access is severely restricted for plants and microbes (Keane *et al.*, 2023). Recent research has found that nitrogen addition, in the long-term like in this study, can alleviate phosphorus limitation by increasing microbial phosphatase activity to mineralise organic P, or increasing acid exudates to mobilize mineral Ca-P (Chen, van Groenigen, *et al.*, 2020; Keane *et al.*, 2020; R. Wang *et al.*, 2022). Therefore, the more abundant supply of weatherable inorganic phosphorus (Ca-P) in the limestone grassland may explain why it appears more sensitive to nutrient addition, as the microbial community is able to use both nitrogen and phosphorus to overcome phosphorus limitation. The loss of organic carbon in the phosphorus treatment, particularly with the combination of phosphorus and $eCO_2$ treatment, may also indicate rhizosphere priming. However, instead of relatively greater phosphorus limitation driving this priming, when the limestone grassland is supplied with phosphorus there is a relatively greater stress of nitrogen limitation that then drives the priming of organic matter.

Plants need nutrients in stoichiometric ratios, the relative increase in one nutrient may increases the relative limitation of another (Dijkstra *et al.*, 2013). Therefore, the alleviation of phosphorus (and carbon with $eCO_2$) limitation in the limestone grassland may increase the relative nitrogen limitation of the grassland also driving soil organic carbon loss by stimulating the microbial decomposition of soil organic matter. Whereas, in the acidic grassland nutrient addition does not affect soil organic carbon as plants do not have as much access to the nutrients due to microbial immobilisation (Keane et al., 2023).

## 2.6. Conclusions

This study conducted in two phosphorus-limited grasslands over the course of a year revealed alterations in bacterial communities under elevated $CO_2$ (eCO2), while fungal communities appeared relatively less unaffected. Elevated $CO_2$ led to a reduction in bacterial biodiversity and spring bacterial species richness in the acidic grassland. In the limestone grassland, $eCO_2$ resulted in reduced bacterial and fungal species richness. The observed decrease in alpha diversity is attributed to the alleviation of carbon limitation and a relative increase in the dominance of specific species. There was evidence that more seasonally dynamic bacterial communities are more sensitive to $eCO_2$ as they are shaped to a greater extend by the availability of rhizosphere carbon. In the acidic grassland, $eCO_2$ appeared to extend the bacterial and fungal communities into autumn, disrupting the usual community change at that time. Fungal communities, whilst appearing more resistant to $eCO_2$, were more sensitive to nutrient manipulation, possibly due to nutrient manipulation driving greater aboveground plant functional change and the close relationship fungi have with plants. Despite both grasslands being phosphorus-limited, their contrasting responses to eCO2, with increased organic carbon in the acidic grassland and a trend towards reduction in the limestone grassland (further reduction with nutrient addition), suggest a potential trade-off where either plants or the microbial communities benefit from elevated $CO_2$.

# Chapter 3. Quantification of storage effects in analysis of upland soil samples

## 3.1. Abstract

Analytical samples collected during fieldwork or generated in large experiments often needs storing due to the technical and physical constraints of processing such samples immediately. This is an unavoidable part of research for many scientists and there is limited consistent guidance on how to appropriately store soil samples to minimise the effects on measured soil properties. Soil from an upland *Calluna vulgaris* moorland in The Peak District National Park, UK, was subjected to four different soil storage preservation treatments: air-drying, freeze-drying, refrigeration (4 °C) and freezing (-18 °C). Commonly measured soil properties were analysed on the un-stored soil immediately and the stored soil from the four storage treatments, after one and three months of storage. Statistical similarity was assessed, where the relative change in measured soil properties compared to the un-stored soil was considered similar when it was within 10% of the un-stored soil. The analytical measured properties assessed were: extractable total nitrogen, nitrate, ammonium & phosphorus; microbial biomass, dissolved inorganic and organic carbon, total organic carbon and $\delta^{13}$ C of organic carbon; and activity of five soil enzymes (BG, NAG, POX, PER, PHO). Statistical similarity was only found for $\delta^{13}$ C of organic carbon, although, some storage treatments met a less stringent 20% similarity limit. Generally, freezing and refrigeration out performed air-drying and freeze-drying at maintaining soil properties. Large changes in measured soil properties were found in the freeze-drying and air-drying treatments, particularly for enzymes: POX, PER, PHO; and dissolved organic & inorganic carbon, microbial biomass, extractable nitrate & ammonium. These findings indicate that soil storage should be avoided when possible, with refrigeration -and to a lesser degree freezing- performing the best at preserving measured soil properties. However, the variability in measured soil properties after storage raises questions about the utility of storage in soil science, suggesting the need to consider alternative approaches for research. Researchers should declare soil storage in their studies to enhance scientific rigor.

## 3.2. Introduction

Ecological research often requires sample collection in remote locations, meaning that ecological samples, including soils, cannot be immediately analysed. The situation is exacerbated where the researcher may be away in the field for an extended period. In addition, laboratory or greenhouse experiments can produce large volumes of samples during intense harvests, again potentially delaying analysis. Ideally, samples would be processed and analysed immediately, however this is not always feasible for remote work or if the quantity of samples collected are greater than the instrumental and/or human capacity for processing. So, researchers face a decision about the most appropriate method and time to store samples to limit their degradation prior to analysis.

Fresh soil samples are preferred for microbiological studies (Anderson, 1987), but when this is not possible, freeze-drying, refrigeration, freezing and air-drying may be used depending on the property of interest and the intended storage time. However, the storage recommendations for soil properties vary considerably. Many studies have been conducted testing soil sample storage, for properties ranging from microbial biomass to phospholipid fatty acid profiles (Petersen and Klug, 1994; Bandick and Dick, 1999; Sessitsch *et al.*, 2002; Pesaro *et al.*, 2003, 2004; Goberna *et al.*, 2005; DeForest, 2009; Liu, Yao and Huang, 2009; Kim *et al.*, 2016): the consensus is that soils respond uniquely to storage depending on storage method, duration, soil type and the soil property of interest.

Lowering the temperature or removing water from the soil reduces the microbial activity, preserving some of the soil's conditions at the time of sampling (Zelles *et al.*, 1991). When soils are refrigerated a small amount of microbial activity is expected to continue and resources are depleted more slowly (Coxson and Parkinson, 1987). Freezing soil is expected to slow microbial processes further but cause osmotic stress similar to drought, due to salts concentrating in the liquid phase during ice crystal formation (Calcott and Gray, 2006). When comparing the microbial biomass of stored soil (4°C and -20°C), using the chloroform fumigation method, mixed results have been found. Stenberg et al., (1998b) found the effects on the microbial biomass of 12 different soils were generally smaller when

the soil was stored frozen at −20°C compared to refrigerated at +2°C. Whereas, Ross *et al.*, (1980) found when testing microbial biomass via three different methods, no storage temperature (25°C, 4°C and -20°C) was satisfactory at preserving field conditions, but mostly 4°C was adequate for short periods of less than 28 days.

Generally, cold storage (Pesaro *et al.*, 2004; Lee *et al.*, 2007; Liu, Yao and Huang, 2009) is preferred over drying treatments, but there is evidence drying is more appropriate for enzyme analysis (Gianfreda and Bollag, 1996; Bandick and Dick, 1999). However, drying of soil is found to increase nutrient availability and pH of the soil (Erich and Hoskins, 2011). In particular, air-drying is known to the increase the nitrogen (N) pool of stored soil (Jones and Willett, 2006; Inselsbacher *et al.*, 2011; Ros, Temminghoff and Hoffland, 2011; Gütlein, Dannenmann and Kiese, 2016). Nitrogen (N) is dynamic in the soil and therefore it is expected that storage allows transformations to take place (Li *et al.*, 2012a). The N pool of soil is similarly affected by sieving, which is routinely used during soil sample preparation (Li *et al.*, 2012b), and the N content of soil has been found to increase immediately after sampling when soil was only refrigerated (Bailey *et al.*, 2021). Soinne, Räty and Hartikainen (2010) found that air-drying, although not altering the total amount of extractable phosphorus (P), altered the distribution of P in soil pools. But air-drying is commonly used as a storage method in university and commercial laboratories (Hoskins, 1995; Mallarino, 2003) and is convenient for extended fieldwork as it can be achieved with no equipment.

Wallenius *et al* (2010) found when assaying for soil enzyme activity how soils respond to freezing or air-drying was dependent on enzyme assayed for and soil type. This complicates predicting how to store soil for enzyme investigation, as often multiple enzymes are tested at once, as more than one enzyme is involved in the steps of soil organic matter decomposition. Researchers are unlikely to use different soil storage practices for different enzyme analysis, as this is likely to increase the chance of biasing results. Furthermore, there is often conflicting information on how long samples can be stored. Rhymes *et al.*, (2021) highlighted many studies offer contradicting advice for soil storage temperature

and duration, with some recommending immediate analysis and others recommending storing soil at 4°C for 8 weeks (Černohlávková *et al.*, 2009) or -18°C for up to 13 months (Stenberg *et al.*, 1998).

To date however, no storage methods paper has included the use of soil from boreal or arctic regions; instead, they mainly focus on mineral and agricultural soils (Lee *et al.*, 2007; Peoples and Koide, 2012; Inselsbacher, 2014; Bailey *et al.*, 2021). The type of soil is often noted as an important factor in determining how soil will respond to storage (Wallenius *et al.*, 2010), so this makes it difficult for researchers wanting to make informed decision on how best to store soil not from mineral or agricultural environments. In addition, storage papers often focus and make recommendations based on one soil property, so when designing studies that involve a number of analysis the decision about how to store soil becomes complicated. One storage recommendation for one soil property may not be suitable for another, but researchers want soil storage to be consistent within the study. Therefore, when faced with deciding how to store soil samples, the preferred solution is to either base choices on pre-existing studies with similar soil type and properties, or if there is no available literature, to conduct an appropriate storage study.

Work in upland, boreal and arctic environments often involves acidic soils of high organic carbon content (Djukic *et al.*, 2010; Buckeridge *et al.*, 2013; Prietzel and Christophel, 2014; Koller and Phoenix, 2017; Siles *et al.*, 2017) but information on the impacts of storage on these types of soil is lacking. With this in mind, this study aims to test how this soil type responds to storage prior to testing for a range properties that are often undertaken on soils. For this study, access to freshly collected soil, which can be immediately analysed, is important so as to not involve pre-storage before the initiation of the study. Therefore, local upland organic soil from The Peak District National Park was used since it is (a) accessible from the University of Sheffield laboratories, and (b) has organic carbon content, pH, nutrient availability and vegetation similar to many arctic, boreal and other upland landscapes. A number of commonly used soil properties were tested in this study to investigate how each soil property may be individually affected by storage.

The storage treatments were, refrigerated 4°C (R), frozen -18°C (F), air-dried (AD), and freeze-dried (FD). Air-dried storage was chosen because it should be possible in almost all situations, while refrigerated and frozen storage treatments were selected to represent methods that could be available for long fieldwork seasons in remote areas where only basic lab (or domestic) facilities are available. Freeze-drying was included as it preserves the soil microbial communities for DNA sequencing (Weißbecker, Buscot and Wubet, 2017), so should strongly inhibit microbial activity. Although, freeze-drying is not often available on remote fieldwork, soil could be frozen and shipped to the nearest research institution for freeze-drying. Once freeze-dried, samples could be stored at room temperature and transported over international borders without the need for cold storage.

The soil properties analysed in this study were chosen as they are used in environmental research to investigate carbon cycling in soil. Dissolved organic carbon was analysed, as it the most important source of carbon for soil microorganisms (Marschner and Bredow, 2002) representing a labile freely available carbon pool in the soil which turns over quickly (Neff and Asner, 2001). Soil organic carbon is the organic fraction of soil carbon and therefore is the pool where carbon is sequestered in the soil. Soil organic carbon is a source of energy for all soil fauna and flora, and their activity controls the release of soil organic carbon as $CO_2$ to the atmosphere (Whalen, 2009). Soil nutrient availability influences carbon dynamics strongly as nutrient availability in the soil strongly controls soil organic matter decomposition (Schmidt *et al.*, 2011; Stuart Chapin, Matson and Vitousek, 2012). Microorganisms produce extracellular enzymes to degrade organic matter into soluble compounds that can be assimilated (Schmidt *et al.*, 2011), so their abundance in the soil can be a measure of the availability of nutrients in the soil and the activity of the microbial community (Dick, 2015). Microbial biomass carbon is the amount of carbon stored within the cells of the microbial community and can indicate the relative size of the community. Together these soil indicators provide information on the microbially mediated carbon dynamics of soil and can help to disentangle the mechanisms behind carbon sequestration and loss.

It was hypothesised that a soil storage treatment and duration of storage is appropriate for a property when the stored and un-stored soil have a change in property within 10% maximum (the similarity limit). A 10% similarity was considered appropriate because that is the maximum level of difference preferred when testing two different methods of analysis (ISO, 2004) for environmental research. If two methods of environmental analysis are considered equivalent at 10% similarity, then stored and un-stored soil should be considered equivalent within the same margin for environmental research.

## 3.3. Materials and Methods

### *3.3.1. Study site*

Soil was collected (~ 4 kg) from the organic horizon (this included the main rooting zone) of *Calluna vulgaris* (heather) dominated moorland, High Bradfield, South Yorkshire, UK 53°25'54''N, 001°35'00''W, on the 15$^{th}$ January 2021. A shovel was used to remove the litter layer from a plot (~2 x 2 m) and the dark organic layer of soil of around 20 cm depth was removed. Afterwards, the litter layer was returned onto the plot to minimise disturbance. The site is 393 m above sea level, has mid-summer (Jul-August) temperatures of 20°C to 11°C, winter (Dec-Feb) low temperatures of -1°C to 6°C, and has an annual precipitation average of 807 mm $y^{-1}$ (National Oceanic and Atmospheric Administration, 2019). The soils are non-calcareous and siliceous with a thin peat layer, typical of acidic moorland.

### *3.3.2. Experimental design*

The experimental had three sampling time points (Fig. 3.1). The first time point used freshly harvested un-stored soil and had twelve analytical replicates to ensure an accurate quantification of each soil measurement before soil storage took place. This created the baseline quantification of each response variable for the experiment and was used to compare how storage treatment and duration affected the soil. The two subsequent samplings took place after the soil was stored for one and three

months in one of the four different storage treatments. Each time point for each storage treatment had six replicates per analysis type (Fig. 3.1.).

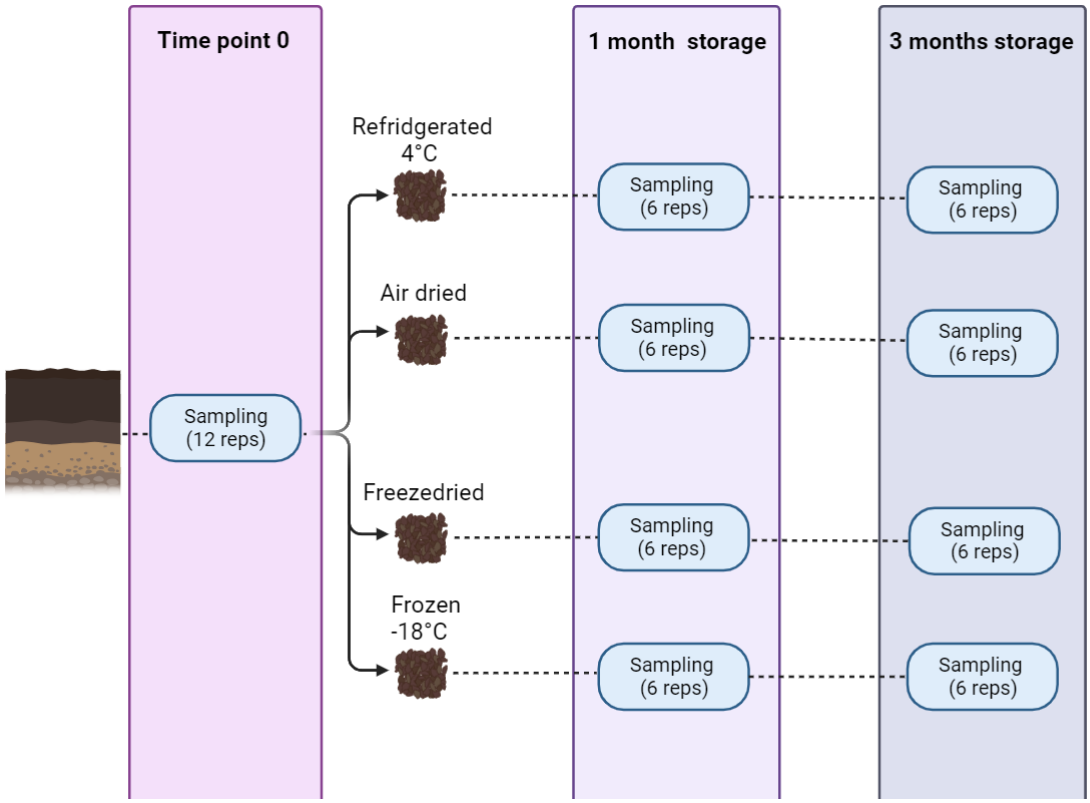


Figure 3. 1. Schematic flow diagram illustrating the processing of soil through different soil storage treatments streams, replication number and soil storage duration.

Freshly harvested soil was sieved (4mm) and mixed by hand twice to ensure a well-mixed soil composite free from large stones and root material. Immediately, the soil was divided into 1/5, one was used for the first analysis (Time point 0) and the remaining four were used to apply the storage treatments to the soil. Soil was homogenised so that replication was considered analytical replication, to increase the accuracy of measurements made on the stored and un-stored soil, and to minimise variation introduced by multiple bags of soil being stored (i.e. chance that one bag might not be closed as well as another, or temperatures variation in different areas in the fridge or freezer). Homogenisation allowed the interpretation of variation introduced by analysis (i.e. the size of

standard deviation for different soil property measurements on the stored and un-stored soil). The decision to homogenise the soil used in the study was a trade-off where homogenisation would reduce the actual replication reducing statistical power and comparability to real studies. However if the soil was not homogenised, due to the sample size constraints and the ability of one person to conduct the lab work in a time sensitive manor, analytical replication in the study would not have been able to be conducted. Therefore, results could have been strongly affected by variation introduced by analysis and soil property measurement accuracy could have been reduced. None-the-less it is important to consider that statistical implications of homogenisation on the results and that actual replication is reduced in this experiment.

The soil in each storage treatment was divided into two aliquots (one for 1 month storage and one for three months storage). The 1 month and 3 month soil was kept in separate (double bagged) bags. This was done so that when the 1 month analysis took place the bag of soil would not have to be deforested and re-frozen (or opened in moist room temperature air) and re-turned to storage. This reduces a possible disturbance effect of the soil stored for the three months analysis.

For all storage treatments, apart from the air-drying, the division into the 1 month and 3 month aliquots was done prior to the application of the storage treatment. For the air-drying treatment, soil was divided into two aliquots after air-drying. Soil in all aliquots were double bagged in plastic bags and kept in together in the centre of the shelves in the fridges, freezers, freeze-dryer, or cupboard. At the 1 month analysis one bag was randomly selected for sampling.

For the air-drying treatment, soil was thinly spread on to A4 tin trays to dry in a well-ventilated room for 5 days. Soil was shaken every day to bring fresh soil to the surface. Freeze-dried soil was frozen for 48 hours at -18°C, and then freeze-dried. The freezedryer (Edwards Modulyo, Crawley Sussex, England) was run for 72 hours at 0.14 mbar at an ice condenser temperature of -64°C. Air-dried and freeze-dried soil was then stored in a cupboard in the laboratory (~20 °C) out of sunlight on the same shelf. Refrigerated and frozen soil was stored in a fridge (4$^{o}$C) and freezer (-18$^{o}$C).

On the same day that the storage treatments were applied to the soil, the measurements of the un-stored baseline soil undertaken. Measurements taken from the baseline soil (time point 0), and at 1 and 3 months analysis, took one week to conduct. The order that response variables were measured in that week was kept consistent throughout the whole experiment. So, at the next analysis time all response variables were measured in the same way on the same days in sequence to keep storage duration the same for each property.

During the analysis for the un-stored soil (time point 0), soil that was not being used for quantification was stored in a 6°C refrigerator to mimic field conditions as closely as possible. The soil was harvested at spring time, so 6°C conforms with the average temperature range for this period of time (National Oceanic and Atmospheric Administration, 2019).

During the 1 month and 3 month analysis, soil was kept in storage treatment conditions until analysis was completed. Air-dried and freeze-dried soil was kept in the same cupboard in the laboratory and refrigerated soil was kept on the same shelf in the refrigerator. The frozen soil was not kept in the freezer as the soil had to be defrosted first so that accurate weights of soil could be weighed out for analysis, so the soil was defrosted and stored at 4°C during this time.

### *3.3.3. Analytical methods*

#### 3.3.3.1. Enzymes

Soil extracellular enzyme activity was assessed for four enzymes involved in the decomposition of soil organic matter (SOM ) and covering C, N and P cycling (Table 3.1). Enzyme activity was measured spectrophotometrically using the microplate assay technique (Jackson, Tyler and Millar, (2013). B-glucosidase, b-N acetylglucosaminidase and acid phosphatase, were assayed using PNP linked substrates and absorbance was measured spectrophotometrically 405nm. DOPA substrate (L-3, 4-dihydroxyphenylalanin) was used to measure the activity of lignin peroxidase and lignin phenol

oxidase; the activity was measured at 450nm after 0mins, 1.5h, and 18.5hr (total incubation time of 20hrs).

The soil slurry concentration, substrate concentration and incubation time for PNP linked substrate enzymes was optimized (German *et al.*, 2011) using baseline (time zero) soil before the assays. The soil and buffer slurry concentration was optimized to 0.375 g $mL^{-1}$, standard incubation times were maintained and the substrate concentrations used were B-glucosidase 40mM (1.5 hr incubation time), b-N acetylglucosaminidase 8mM (3.5hr incubation time), acid phosphatase 50mM (0.5 hr incubation time). Enzyme activity was expressed as substrate nmol $hr^{-1}$ $g^{-1}$ dry soil.

Table 3. 1. The role of extracellular soil enzymes and their significance in soil organic matter decomposition and turnover (Ch *et al.*, 2017; Luo, Meng and Gu, 2017; Wang *et al.*, 2020)

| **Enzyme name** | **Reaction** | **Source** | **End product** | **Soil function indicated** | **Factors influencing enzyme activity** |
|---|---|---|---|---|---|
| **B-1,4-glucosidase (BG)** | Cellobiose hydrolysis | Bacteria, archaea, fungi, plants, animals | Glucose | C-cycling | Temperature, pH, water, $O_2$, location of organic matter, fungicides, mineral elements |
| **Acid phosphatase (PHO)** | Hydrolysis of esters and anhydrides of phosphoric acid | Plants, fungi, bacteria | Phosphate ($PO_4$) | P-cycling | Organic matter content, pH, management practices, pollution, plant species |
| **B-N-acetylglucosaminidase (NAG)** | Hydrolysis of chitin oligomers | Bacteria, archaea, fungi, plants, animals | N-acetly-B-D-glucosaminide | N- and C-cycling | Availability of N, soil depth, pH, ect. |
| **Phenol oxidase (POX)** | Lignin hydrolysis | Plants, fungi, bacteria | C compounds (humic substances) | C-cycling | Low substrate specificity, Oxygen as final electron acceptor, pH, precipitation, temperature, SOM content, N enrichment, management practices |

### 3.3.3.2. Soil Organic Carbon $\delta^{13}C$

Soil was dried at 105ºC for 48 hours and then ground to a fine powder. Soil organic carbon $\delta^{13}C$ was determined by acid digesting 50mg of soil in 1.5 ml Startedt tubes with 700 µL of 6M HCl in a method adapted from Hedges and Stern (1984). Acid was added in small increments to remove the inorganic carbon from the soil until effervescence stopped. The samples were left to react for 24 hrs, and then dried at 105ºC for 24hrs in a fume cupboard in a Techne Dri-Block (DB200/3). Between 1-2 mg of acid stripped dry soil was loaded into tin boats and run on an isotope-ratio mass spectrometer (ANCA GSL 20-20 Mass Spectrometer, Sercon PDZ Europa) for carbon content and $\delta^{13}C$ signature (Harris, Horwáth and van Kessel, 2001). Due to a balance malfunction, the carbon content of the soil was not accurate and was removed from this study. As the $\delta^{13}C$ is based on ratios of carbon, it was not affected by the balance malfunction.

### 3.3.3.3. Extractable Phosphorus

Soil P was extracted by shaking 1 g of fresh soil with 25ml 2.5% acetic acid for 2 hours, and then filtering through Whatman 42 filter paper. Phosphorus was then measured using an ascorbic acid microplate method adapted from Kuo S (1996). The extract was neutralised with NaOH, and reacted with a mixed reagent containing sulfuric acid, ammonium molybydate tetrahydrate, potassium antimony tartrate hydrate, and ascorbic acid, to produce an intense blue colour. The absorbance was measured after 10 minutes at 880nm. Phosphate standards were produced from potassium dihydrogen phosphate in miliQ water, which was diluted in 2.5% acetic acid to form a calibration curve. P mg $Kg^{-1}$ dry soil was calculated by dividing the P in well (mg $L^{-1}$) * dilution factor * Extraction volume (L) by soil dry weight (Kg).

3.3.3.4. Dissolved Organic Carbon

Dissolved organic carbon was extracted by shaking 5 g of soil and 35 ml MilliQ for 10 minutes, and then filtering through Whatman no. 1 filter paper (Harrison and Bardgett, 2004). The carbon content of the extracts was determined by running triplicate analytical replicates on a Total Organic Carbon analyser (Sievers 5310C Portable TOC Analyser, University of Sheffield Geography Department) with a dilution factor of 1:5.

3.3.3.5. Extractable Total Nitrogen, Nitrate, and Ammonium

Nitrogen was extracted from the soil in a 1:5 ratio with 1M potassium chloride, and then filtered through Whatman no. 1 filter paper (Allen, 1989; Li *et al.*, 2012b). Extractable nitrate and ammonium was measured on an auto-analyser (Skalar SAN++ System Continuous Flow Analyser, University of Sheffield Geography Department). Nitrate was reduced to $NO_2^-$ by a copper-cadmium redactor column, reacted with sulphanilamide to form a diazo compound, and then coupled with N-1-naphthylethylene diamine dihydrochloride to form a purple azo dye.

For total nitrogen, 15ml aliquots of potassium chloride extracts, were digested with potassium persulfate and sodium hydroxide, in an autoclave at 121°C at 20 p.s.i. for 30 minutes (Langner, C.L., 1982; Kalff, J., 1984). The persulfate under alkali conditions oxidises the organic nitrogen and ammonium to nitrate. The digested samples were run on the Skalar SAN++ System Continuous Flow Analyser and measured for nitrate following the method outlined above.

3.3.3.6. Microbial Biomass Carbon

Microbial biomass carbon was measured by splitting each soil sample in two, and fumigating one sample with chloroform under a vacuum for 24 hours (Vance, Brookes and Jenkinson, 1987). The fumigation of soil with chloroform under a vacuum causes microbial cell lysis. Carbon was extracted from both samples by shaking 5 g of soil with 25 ml of potassium sulfate for 30 minutes, and then

filtering through Whatman no. 1 filter paper. The carbon content of both samples was measured on a Sievers 5310C Portable TOC Analyser. Microbial carbon biomass was calculated by subtracting the carbon in the fumigated sample by the carbon in the non-fumigated sample. Microbial carbon biomass was expressed as mg C $kg^{-1}$ dry soil. Data was corrected with the standard kEC value of 0.35 (Joergensen and Mueller, 1996).

3.3.3.7. Soil moisture

Soil moisture content was assessed by mass loss by drying soil for 72 hrs at 105°C using triplicate replicates. This was measured to express resulted per dry weight of soil and was also measured on the un-stored soil (time zero).

### *3.3.4. Statistical Analysis*

Statistical analysis was carried out in R Version 4.1.2 (R Core Team, 2021). Data for each variable was standardised, so data from different treatments, time points, and response variables could be compared. Data was standardised by calculating the relative change from the baseline (time zero) measurements by the following equation:

$$relative\ change = \frac{measured\ varaible\ for\ each\ treatment}{measured\ variable\ for\ the\ baseline}$$

The Levene's and Anderson-Darling tests were used to assess the Normality and homoscedasticity of the data. All variables were natural log transformed apart from $\delta^{13}C$. Mixed effects models were conducted with the *nlme* package (Pinheiro *et al.*, 2013) to test the effects of the fixed factors storage time and storage treatment, the random effect factor replicate, and their interaction on the relative change from the baseline. As most of the data (all but $\delta^{13}C$) had heterogeneous error variance, the models were set up to allow heteroscedasticity per group for the effect levels of Storage treatment

and/or Storage time. The variance function *varIdent()* was combined using the *varComb()* function in the *weights*= argument. This allowed for different variances for different factors, which was selected by comparing various variant structures and selecting the structure which best fit of the data by AIC (Akaike information criteria). For some variables (Phosphatase activity and the $\delta^{13}C$ soil organic carbon), the interaction of storage treatment and time was removed from the models, as it was non-significant. This improved the models statistical power.

Similarity between the baseline (time zero) and storage treatment at each time point was assessed, rather than testing for difference. This was done because interpreting a lack of statistically significant difference as evidence of no or negligible effect of storage treatment is inaccurate. A lack of statistical significance from a t-test ($P \geq 0.05$) could be assumed to provide evidence for similarity of the means, as a statistical difference between the means is not found and the means are assumed to be close together. But, large *p*-values can be obtained from large standard deviations and small samples sizes. Therefore, noisy experiments with small sample sizes could show the similarity of two storage methods, hence similarity cannot be accurately assessed on a non-significant t-test result (Rita and Ekholm, 2007).

3.3.4.1. Justification for the Similarity test

Power analysis has been suggested as a method to overcome this problem (Osler, 2002). This approach suggests that large *p*-values can be accompanied by the large power of a t-test, to conclude similarity between two means. However, this approach only provides a partial solution as it still bases the testing on the null hypothesis 'there is no difference', which implies the aim of the test is to gain support for the hypothesis 'there is a difference' (Rita and Ekholm, 2007). When testing for similarity, the alternative hypothesis would be 'the means of the two methods are *close enough* to each other'. Therefore, power analysis cannot resolve the problem of showing similarity of two means as the hypothesis testing is incorrect. Schuirmann (1987), conducted a detailed critique of power analysis and similarity testing. They found that the similarity approach (two one-sided tests procedure) is

uniformly superior to testing of no difference supplemented with power analysis, as long as the significance levels of the one-sided tests are set to no greater than 0.05.

Testing for similarity is an approach that is used commonly in the pharmaceutical industry when comparing the treatment of two drugs to evaluate if they can be used interchangeably for treatment (bioequivalence). This approach is less common in environmental studies as it requires the setting of similarity levels, to determine how 'close enough' is. The choice of similarity level is dependent on the intended application. In the pharmaceutical industry the similarity level is set by the authorities. Pharmaceutical industries have favoured a 20% difference in the adsorption of two drug products (CHMP, 2010), to determine similarity. Whereas, a comparison of colony counting methods used in water microbiology favours a 10% similarity level (ISO, 2004).

Previous environmental studies that have used this statistical approach favour the use of a 20% similarity level (Wallenius *et al.*, 2010; Jennifer M. Rhymes *et al.*, 2021). One reason for this, outlined by Wallenius *et al* (2010), is that experiments testing storage treatments on different types of soil are more interested in a uniform relative change from the baseline across soils tested, rather than the magnitude of response. Therefore, as most enzyme studies (the response variable they assessed storage treatment for) are based on comparing different soils, they chose the less conservative 20% level. As the experiment in this chapter uses one type of soil, a 10% similarity level was used. However, a 20% similarity level is additionally illustrated to provide a guide for other researchers who may use this study to inform their own soil storage practices.

3.3.4.2. Similarity test

In this chapter, similarity between the baseline measurements and storage treatments for each time point was assessed following the approach outlined in detail by Rita and Ekholm (2007) with the hypothesis being 'change in quantity from the baseline is at a maximum 10%' against the null 'change in quantity from the baseline exceeds 10%' using 0.05 level of significance.

The *emmeans* function from the *emmeans* package (Lenth, 2022) was used to calculate 95% predicted fitted upper and lower confidence intervals from the mixed effects models. Similarity between the baseline and storage treatments was determined when the upper and lower limit of the predicted fitted confidence intervals fit within 10% positive and negative variance from the baseline. For log transformed data, when the log transformed relative change fits within -0.2231 and 0.1823, it is accepted that the similarity limits are met and the null hypothesis is rejected. For untransformed data, when the relative change from the baseline fits within 0.8 and 1.2, it is accepted the similarity limits are met and the null hypothesis is rejected. Where relative change from the baseline was not within the similarity limits, the storage treatment is not similar to the baseline (time zero).

## 3.4. Results

### *3.4.1. Enzymes assays*

#### 3.4.1.1. Beta glucosidase (BG)

No soil storage treatment assessed, for a duration of one and three months, had a similar beta-glucosidase activity to the un-stored soil, as the 95% confidence intervals of the stored soil did not fall within the 10% similarity boundary (Fig 3.2. no error bar span is within the dashed boundary).

Air-drying and freeze-drying soil tended to increase the mean BG enzyme activity, whereas freezing and refrigerating soil tended to decrease mean BG enzyme activity (note, these differences are not statistically tested because this work tests for similarity). The storage treatment and duration with the apparent smallest mean change in LOG RC (log relative change compared to the t=0 unstored soil) BG enzyme activity from the baseline was the air-drying treatment after 3 months of storage (0.72 nmol $h^{-1}$ $g^{-1}$ dry soil), followed by the refrigeration treatment after 1 month of storage (- 0.77 nmol $h^{-1}$ $g^{-1}$dry soil). BG enzyme activity tended to change the least between 1 and 3 months of storage under freezing conditions (mean LOG RC 0.08 nmol $h^{-1}$ $g^{-1}$ dry soil). Freeze-drying soil and storage for 1 month caused the apparent greatest mean change in BG enzyme activity compared to the baseline (2.4 log nmol $h^{-1}$ $g^{-1}$ dry soil). Between 1 and 3 months of soil storage, all storage treatments had tended

towards decreased mean BG enzyme activity, apart from the freezing treatment that had a slight increase in activity (Fig 3.2).

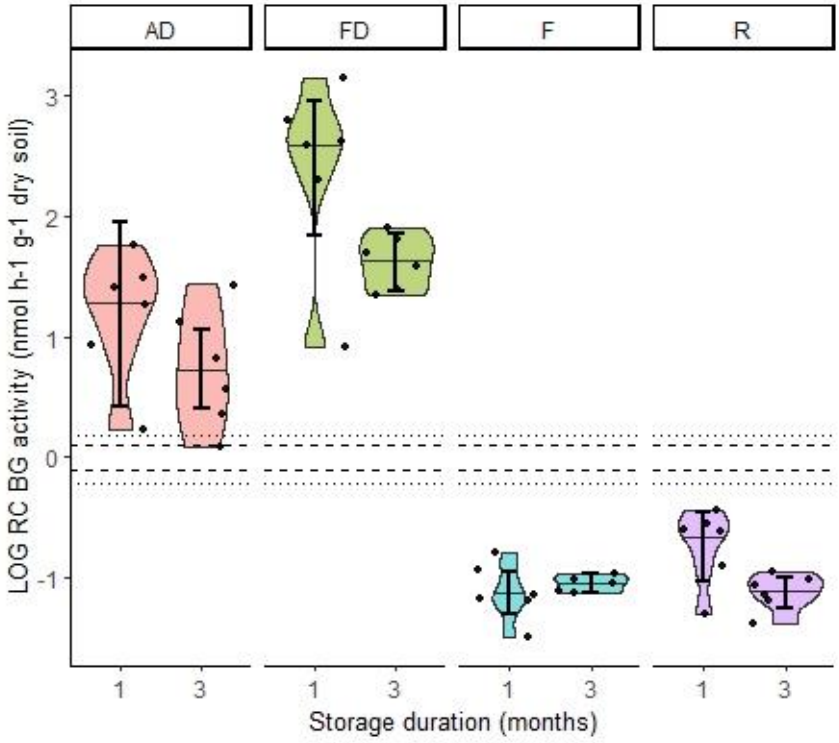


Figure 3. 2. The natural log of the relative change compared to baseline (t=0 un-stored) soil in soil beta-glucosidase enzyme activity after 1 and 3 months of soil storage, compared to un-stored soil. Storage treatments are air dried (AD), freeze-dried (FD), frozen at -18 °C (F) and, refrigerated 4 °C (R). The inner dashed line represents the 10% variation from the baseline boundary, referred to as the similarity limit. The outer dotted line illustrates 20% variation from the baseline. Error bars are the upper and lower 95% confidence intervals extracted from the mixed model. Horizontal lines within the violin plots represent the mean relative change of six replicates.

3.4.1.2. Acid Phosphatase (PHO)

No soil storage treatment, for a duration of one and three months, had a similar acid phosphatase activity to the un-stored soil, as the 95% confidence intervals extracted from the model do not fall within the 10% similarity boundary (Fig 3.3. no error bar span fall between the inner dashed lines)

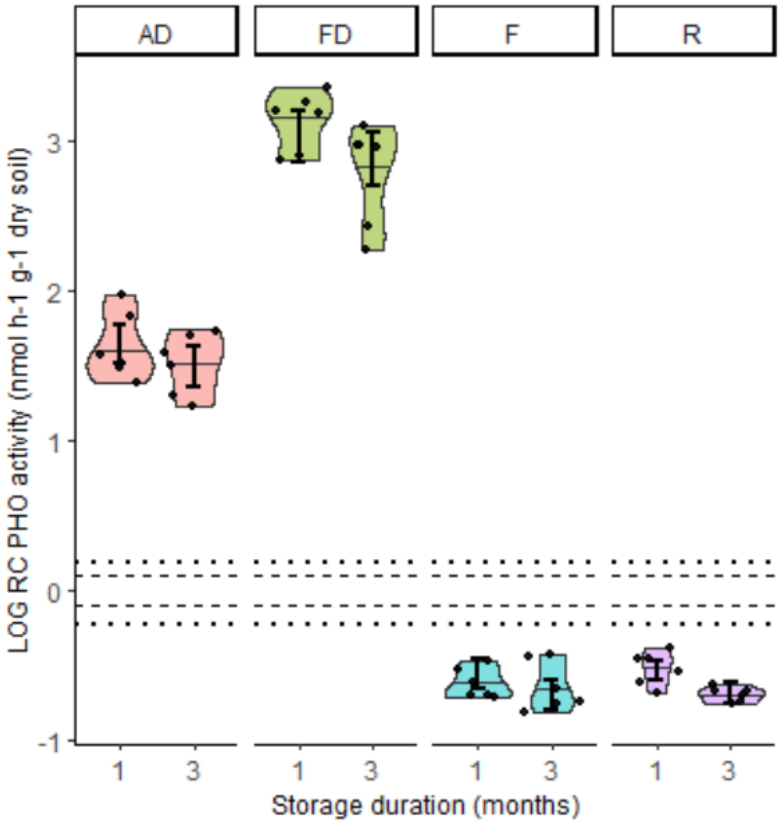


Figure 3. 3. The natural log of the relative change compared to baseline (un-stored) soil in soil acid phosphatase enzyme activity after 1 and 3 months of soil storage, compared to un-stored soil. Storage treatments are air dried (AD), freeze-dried (FD), frozen at -18 °C (F) and, refrigerated 4 °C (R). The inner dashed line represents the 10% variation from the baseline boundary, referred to as the similarity limit. The outer dotted line illustrates 20% variation from the baseline. Error bars are the upper and lower 95% confidence intervals extracted from the mixed model. Horizontal lines within the violin plots represent the mean relative change of six replicates.

The drying treatments (AD and FD) tended to increase, whereas the cooling treatments (R and F) tended to decreased, the PHO activity of the soil. The treatment that appeared to produce the smallest mean LOG RC in PHO activity, compared to the un-stored soil, was refrigerating soil for one month (- 0.52 nmol $h^{-1}$ $g^{-1}$ dry soil). Freeze-drying soil tended to cause the greatest mean LOG RC in PHO activity (3.17 nmol $h^{-1}$ $g^{-1}$ dry soil). PHO activity under freezing conditions appeared to change the least between 1 and 3 months. For all treatments, storage past 1 month tended to reduce mean LOG RC in PHO activity (Fig 3.3).

3.4.1.3. B-N-acetyleglucosaminidase (NAG)

No soil storage treatment, for a duration of one and three months, had a similar B-N-acetylglucosaminidase activity to the un-stored soil, as the 95% confidence intervals did not fall within the 10% similarity boundary (Fig 3.4. no error bar span fits within the inner dashed line.)

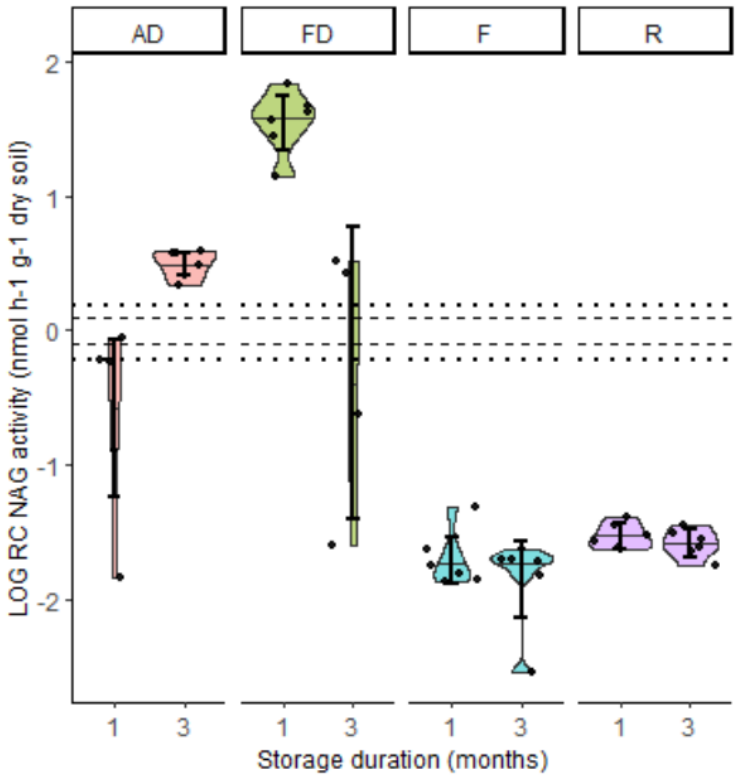


Figure 3. 4. The natural log of the relative change compared to baseline (t=0 un-stored) soil in B-N-acetyleglucosaminidase enzyme activity, compared to soil baseline measurements after 1 and 3 months of soil storage. Storage treatments are air dried (AD), freeze-dried (FD), frozen at -18 °C (F) and, refrigerated 4 °C (R). The inner dashed line represents the 10% variation from the baseline boundary, referred to as the similarity limit. The outer dotted line illustrates 20% variation from the baseline. Error bars are the upper and lower 95% confidence intervals extracted from the mixed model. Horizontal lines within the violin plots represent the mean relative change of six replicates.

The NAG activity of air-dried soil tended to have larger variation after one month of storage (SE = 0.3) compared to three months of storage (SE = 0.04). Freeze-dried soil stored for three months had

larger variation (SE = 0.41) in NAG activity than freeze-dried stored for one month (SE = 0.09). Cooling (refrigeration and freezing) treatments tended to reduce the activity of NAG but this reduced mean LOG RC activity remained similar at one and three months of storage (F1 = - 1.7, F3 = - 1.85, R1 = -1.5, R3 = -1.59 nmol $h^{-1}$ $g^{-1}$ dry soil).

3.4.1.4. Lignin peroxidase (PER)

No storage treatment assessed, for a duration of one and three months, had a similar lignin peroxidase activity to un-stored soil, as the 95% confidence intervals extracted from the model did not fall within the 10% similarity limit (Fig 3.5. no error bar span is within the dashed similarity boundary).

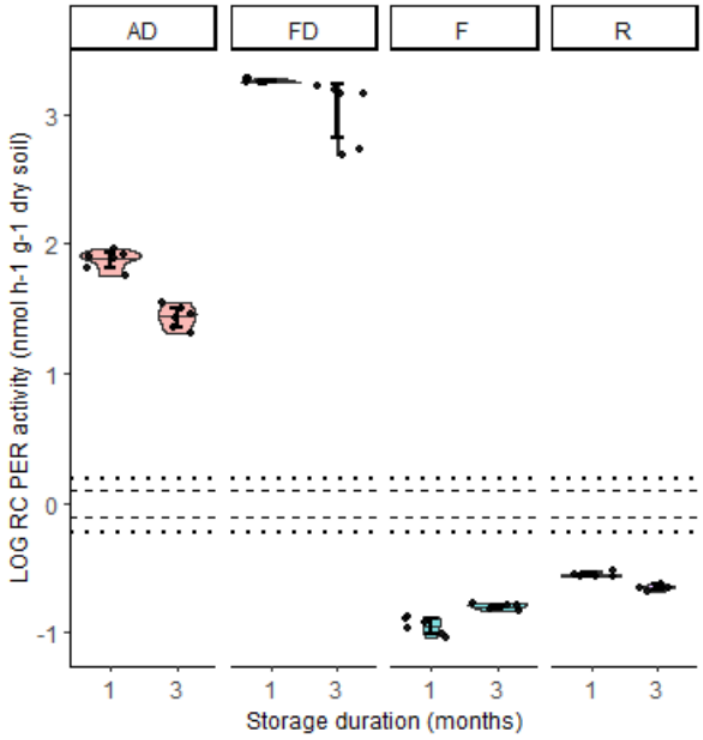


Figure 3. 5. The natural log of the relative change compared to baseline (t=0 un-stored) soil in lignin peroxidase enzyme activity after 1 and 3 months of soil storage, compared to un-stored soil. Storage treatments are air dried (AD), freeze-dried (FD), frozen at -18 °C (F) and, refrigerated 4 °C (R). The inner dashed line represents the 10% variation from the baseline boundary, referred to as the similarity limit. The outer dotted line illustrates 20% variation from the baseline. Error bars are the upper and lower 95% confidence intervals extracted from the mixed model. Horizontal lines within the violin plots represent the mean relative change of six replicates.

Refrigerated soil appeared to have the closest mean LOG RC in PER activity compared to the un-stored soil. Refrigerated soil after one and three months had mean LOG RC in PER activity of – 0.56 and – 0.65 nmol $h^{-1}$ $g^{-1}$ dry soil. Soil in the drying treatments tended to increase in mean LOG RC in PER activity. Whereas, soil in the cooling storage treatments tended to reduce in mean LOG RC in PER activity. Between one and three months of storage the mean LOG RC in PER activity of the soils tended to decrease, apart from in the frozen soil which slightly increased by 11 nmol $h^{-1}$ $g^{-1}$ dry soil.

3.4.1.5. Lignin phenol oxidase (POX)

No treatment assessed, for a duration of one and three months, had a similar lignin phenol oxidase activity to the un-stored soil, as the 95% confidence intervals did not fall within the 10% similarity boundary (Fig 3.6. no error bar span is within the dashed 10% boundary).

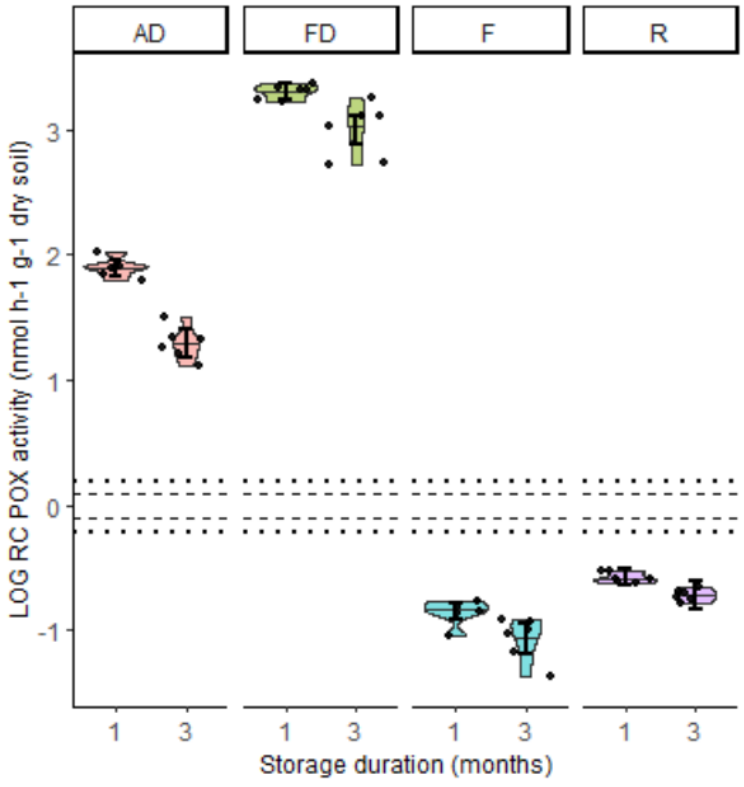


Figure 3. 6. The natural log of the relative change compared to baseline (t=0 un-stored) soil in lignin phenol oxidase enzyme activity measurements after 1 and 3 months of soil storage, compared to un-stored soil. Storage treatments are air dried (AD), freeze-dried (FD), frozen at -18 °C (F) and,

refrigerated 4 °C (R). The inner dashed line represents the 10% variation from the baseline boundary, referred to as the similarity limit. The outer dotted line illustrates 20% variation from the baseline. Error bars are the upper and lower 95% confidence intervals extracted from the mixed model. Horizontal lines within the violin plots represent the mean relative change of six replicates.

Refrigerated soil tended to have the closest mean LOG RC in POX activity compared to the un-stored soil. The mean LOG RC in POX activity after one and three months of storage was – 0.58 and – 0.72 nmol $h^{-1}$ $g^{-1}$ dry soil. Drying treatments tended to increase, and cooling treatments tended to reduce, the LOG RC in POX activity. The soil storage treatment that tended to have the greatest mean change in LOG POX activity was the freeze-drying treatment. Between one and three months, the LOG RC in POX activity in the air-dried soil treatment appeared to reduce by a relative change of 0.6 nmol $h^{-1}$ $g^{-1}$ dry soil.

### *3.4.2. Delta $^{13}C$*

All treatments tested for up to three months had a similar $\delta^{13}C$ signature of soil organic carbon as the un-stored soil (Fig 3.7: all error bars fall within the similarity inner dashed boundary).

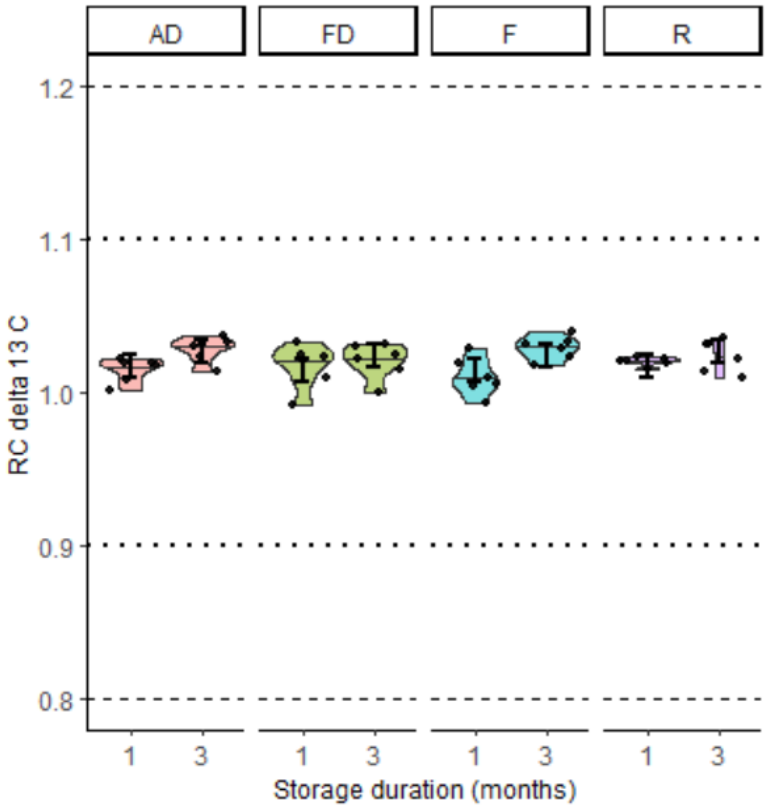


Figure 3. 7. The relative change (RC) in the $\delta^{13}C$ signature of soil organic carbon after 1 and 3 months of soil storage, compared to un-stored soil. Storage treatments are air dried (AD), freeze-dried (FD), frozen at -18 °C (F) and, refrigerated 4 °C (R). The inner dashed line represents the 10% variation from the baseline boundary, referred to as the similarity limit. The outer dotted line illustrates 20% variation from the baseline. Error bars are the upper and lower 95% confidence intervals extracted from the mixed model. Horizontal lines within the violin plots represent the mean relative change of six replicates.

Between one and three months of soil storage the mean $\delta^{13}C$ signature of soil tended to slightly increase. Frozen and air-dried soil had an apparent mean RC (relative change compared to t=0 unstored soil) increase of 0.2, freeze-dried soil had an apparent mean RC increase of 0.1 and refrigerated soil had no increase.

*3.4.3. Dissolved organic carbon (DOC)*

No soil storage treatment, for a duration of one and three months, had a similar dissolved organic carbon content to the un-stored soil, as the 95% confidence intervals did not fall within the 10% similarity boundary (Fig 3.9. no error bar span is within the dashed boundary).

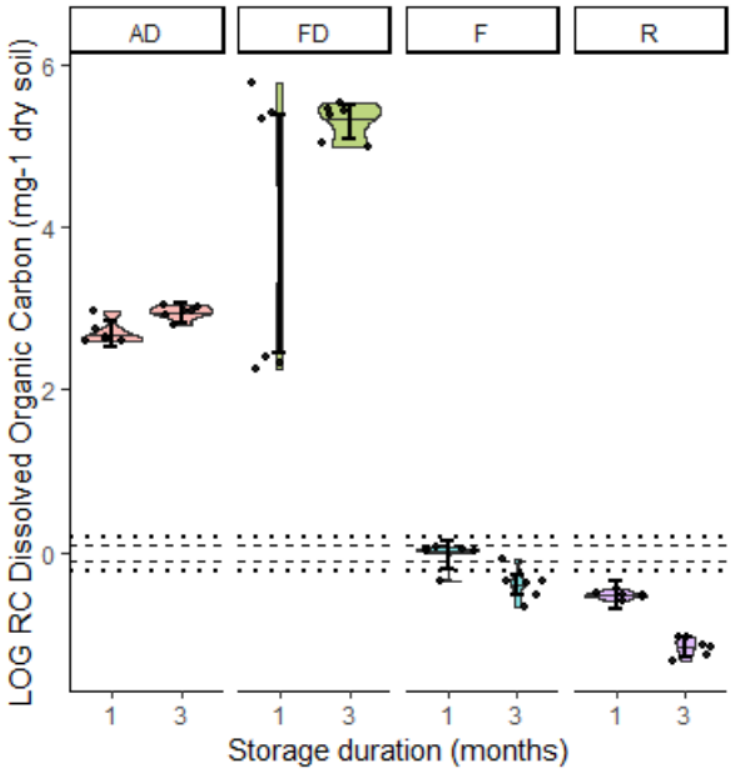


Figure 3. 8. The natural log of the relative change in soil dissolved organic carbon after 1 and 3 months of soil storage, compared to un-stored soil. Storage treatments are air dried (AD), freeze-dried (FD), frozen at -18 °C (F) and, refrigerated 4 °C (R). The inner dashed line represents the 10% variation from the baseline boundary, referred to as the similarity limit. The outer dotted line illustrates 20% variation from the baseline. Error bars are the upper and lower 95% confidence intervals extracted from the mixed model. Horizontal lines within the violin plots represent the mean relative change of six replicates.

Freezing soil for one month tended to change the dissolved organic carbon content of the soil the least. The 95% confidence intervals for freezing at one month although not meeting the 10% limit set in the experiment falls within a more lenient 20% similarity boundary (outer dotted boundary).

Freezing soil for one month, and three months, appear to reduce the mean LOG RC in DOC by -0.037 and -0.039 $mg^{-1}$ dry soil. Drying treatments tended to increase, and cooling treatments decrease, the mean LOG RC in DOC compared to un-stored soil. In the cooling treatments, storage duration tended to reduce the mean LOG RC in DOC compared to un-stored soil. In the drying treatments, mean LOG RC in DOC appeared to increase with storage duration. Air-drying appeared to increase the mean LOG RC in DOC from 2.69 to 2.94 $mg^{-1}$ dry soil and freeze-drying from 3.91 to 5.29 $mg^{-1}$ dry soil, between one and three months of soil storage.

### *3.4.4. Dissolved inorganic carbon (DIC)*

No storage assessed, for a duration of one and three months, had a similar inorganic dissolved carbon content to the un-stored soil, as the 95% confidence intervals did not fall within the 10% similarity boundary (Fig 3.10. no error bar span is within the dashed inner boundary).

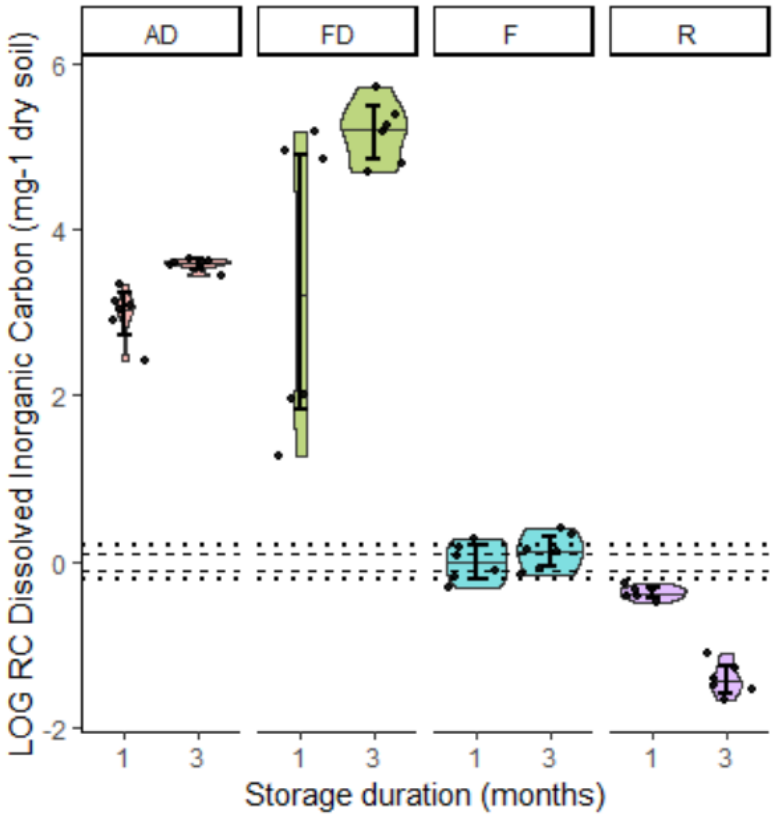


Figure 3. 9. The natural log of the relative change (LOG RC) in soil dissolved inorganic carbon after 1 and 3 months of soil storage, compared to un-stored soil. Storage treatments are air dried (AD), freeze-dried (FD), frozen at -18 °C (F) and, refrigerated 4 °C (R). The inner dashed line represents the 10% variation from the baseline boundary, referred to as the similarity limit. The outer dotted line illustrates 20% variation from the baseline. Error bars are the upper and lower 95% confidence intervals extracted from the mixed model. Horizontal lines within the violin plots represent the mean relative change of six replicates.

Similar to DOC, the frozen soil appeared to have the most similar mean LOG RC in DIC compared to the un-stored soil. Although not fitting within the 10% similarity limit set in this experiment, the 95% confidence intervals fit within a more lenient 20% limit.

### *3.4.5. Microbial Biomass Carbon (MCB)*

No storage treatment assessed, for a duration of one and three months, had a similar microbial biomass carbon to the un-stored soil, as the 95% confidence intervals extracted from the model did

not fit within the 10% similarity limit (Fig. 3.11. no error bar span lies within the inner dashed boundary).

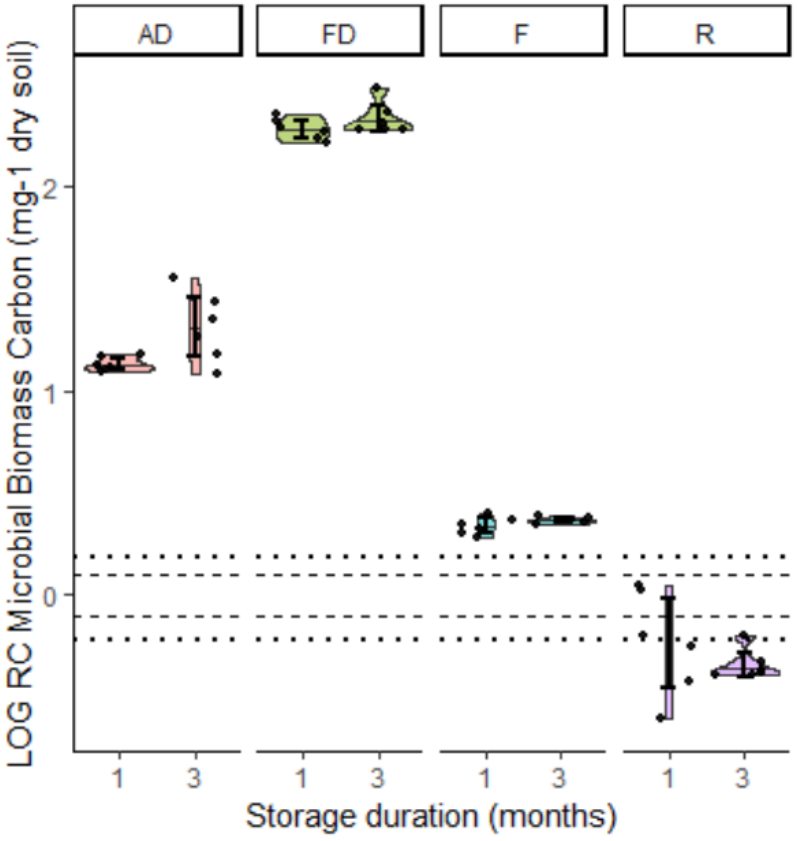


Figure 3. 10. The natural log of the relative change (LOG RC) in soil microbial biomass carbon after 1 and 3 months of soil storage, compared to un-stored soil. Storage treatments are air dried (AD), freeze-dried (FD), frozen at -18 °C (F) and, refrigerated 4 °C (R). The inner dashed line represents the 10% variation from the baseline boundary, referred to as the similarity limit. The outer dotted line illustrates 20% variation from the baseline. Error bars are the upper and lower 95% confidence intervals extracted from the mixed model. Horizontal lines within the violin plots represent the mean relative change of six replicates.

Freeze-dried and air-dried soil appear to have a large increase in mean LOG RC in MCB after one and three months of storage. Whereas, freezing soil caused an apparent small increase and refrigerating soil a reduction, in the mean LOG RC in MCB. Frozen soil had close microbial biomass carbon at one

and three months of storage, having a mean LOG RC in MCB of 0.336 and 0.365 $mg^{-1}$ dry soil respectively.

### *3.4.6. Extractable Phosphorus (P)*

No storage treatment assessed, for a duration of one and three months, had a similar extractable phosphorus content compared to the un-stored soil, as the 95% confidence intervals extracted from the model did not fit within the 10% similarity limit (Fig. 3.12. no error bar span lies within the inner dashed boundary).

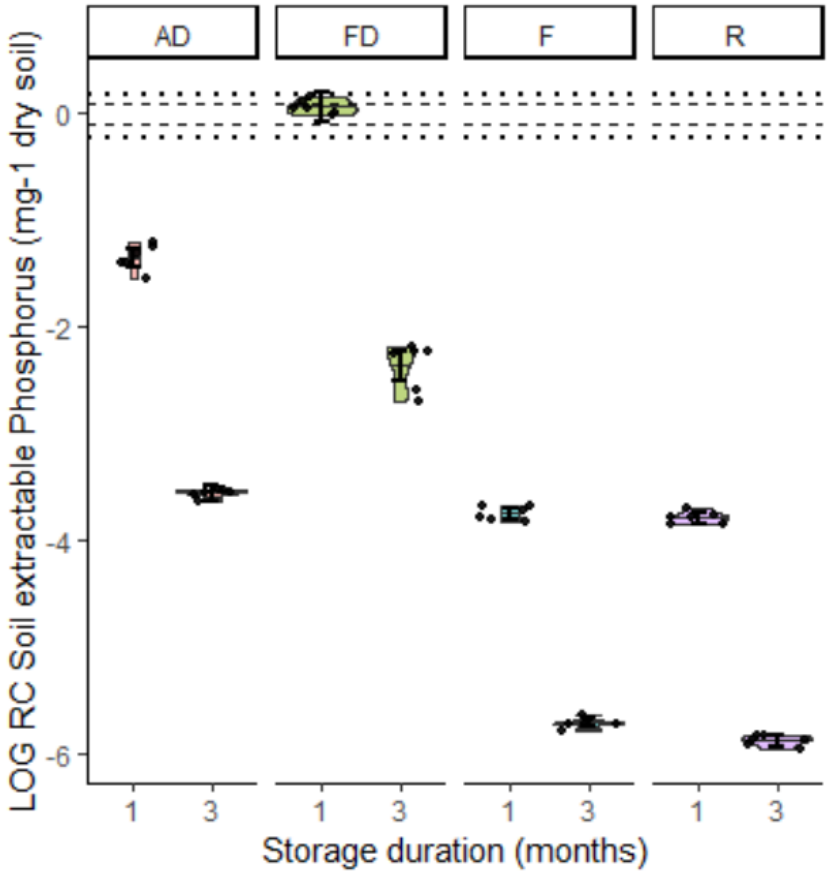


Figure 3. 11. The natural log of the relative change (LOG RC) in soil extractable phosphorus after 1 and 3 months of soil storage, compared to un-stored soil. Storage treatments are air dried (AD), freeze-dried (FD), frozen at -18 °C (F) and, refrigerated 4 °C (R). The inner dashed line represents the 10% variation from the baseline boundary, referred to as the similarity limit. The outer dotted line illustrates 20% variation from the baseline. Error bars are the upper and lower 95% confidence

intervals extracted from the mixed model. Horizontal lines within the violin plots represent the mean relative change of six replicates.

Freeze drying soil for one month has the closest LOG RC in P (log relative change in phosphorus) content compared to un-stored soil. This is shown in figure 8, as the error bar span most closely overlaps the 10% similarity boundary. All soil storage treatments, apart from freeze-drying for one month, caused an apparent large mean LOG RC in P concentration. P appeared to strongly reduce with time in all treatments. Soil LOG RC in P in the drying treatments appeared to reduce less than in the cooling treatments with time. Air-dried and freeze-dried soil stored for three months appears to have a close or higher LOG RC in P than soil stored by refrigeration and freezing for one month (AD3 = -3.56, FD3 = -2.36, F1 = -3.75, R1 = -3.79 $mg^{-1}$ dry soil).

### *3.4.7. Extractable Nitrate ($NO_3$)*

No soil storage treatment assessed, for a duration of one and three months, had a similar extractable nitrate to the un-stored soil, as the 95% confidence intervals did not fall within the 10% similarity boundary (Fig 3.13. no error bar span is within the dashed boundary).

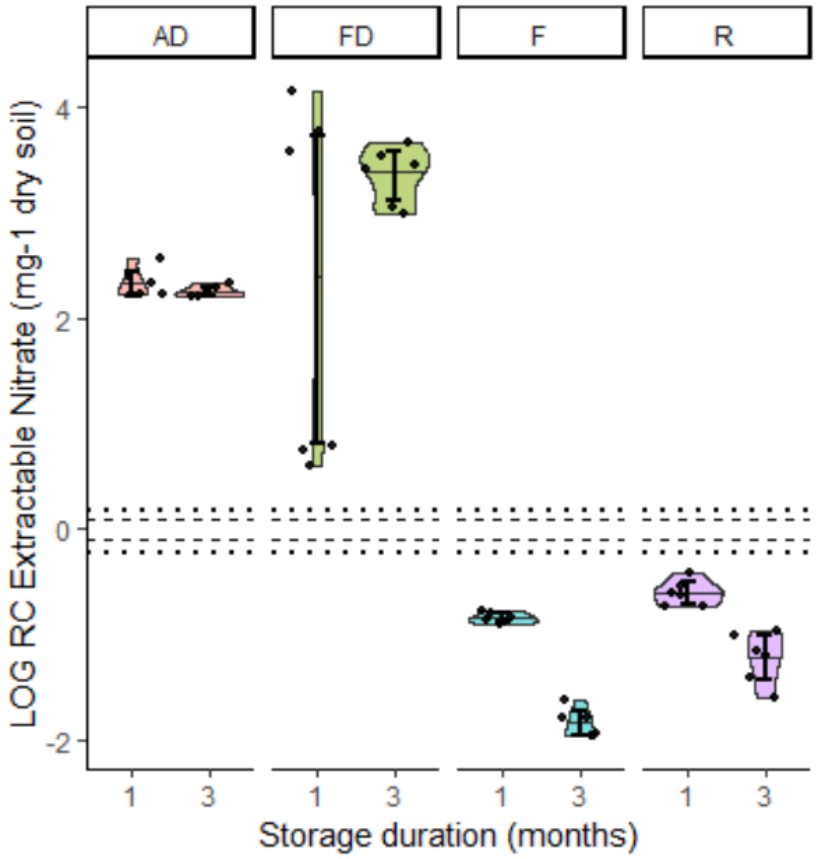


Figure 3. 12. The natural log of the relative change (LOG RC) in soil extractable nitrate after 1 and 3 months of soil storage, compared to un-stored soil. Storage treatments are air dried (AD), freeze-dried (FD), frozen at -18 °C (F) and, refrigerated 4 °C (R). The inner dashed line represents the 10% variation from the baseline boundary, referred to as the similarity limit. The outer dotted line illustrates 20% variation from the baseline. Error bars are the upper and lower 95% confidence intervals extracted from the mixed model. Horizontal lines within the violin plots represent the mean relative change of six replicates.

Drying treatments tended to increase, whilst cooling treatments tended to decrease, the mean LOG RC in $NO_3$. Freeze-dried soil stored for one month had a large variation in mean LOG RC in $NO_3$ (SE = 0.7). Refrigerating soil for one month had the most similar mean LOG RC in $NO_3$ to the baseline (-0.6 $mg^{-1}$ dry soil). Air-drying soil maintained a similar mean LOG RC in $NO_3$ concentration between one and three months (AD1 = 2.27 $mg^{-1}$ dry soil AD3 = 2.25 $mg^{-1}$ dry soil).

### *3.4.8. Extractable Ammonium ($NH_4$)*

No soil storage treatment assessed, for a duration of one and three months, had a similar extractable ammonium content to the un-stored soil, as the 95% confidence intervals did not fall within the 10% similarity boundary (Fig 3.14. no error bar span is within the dashed boundary).

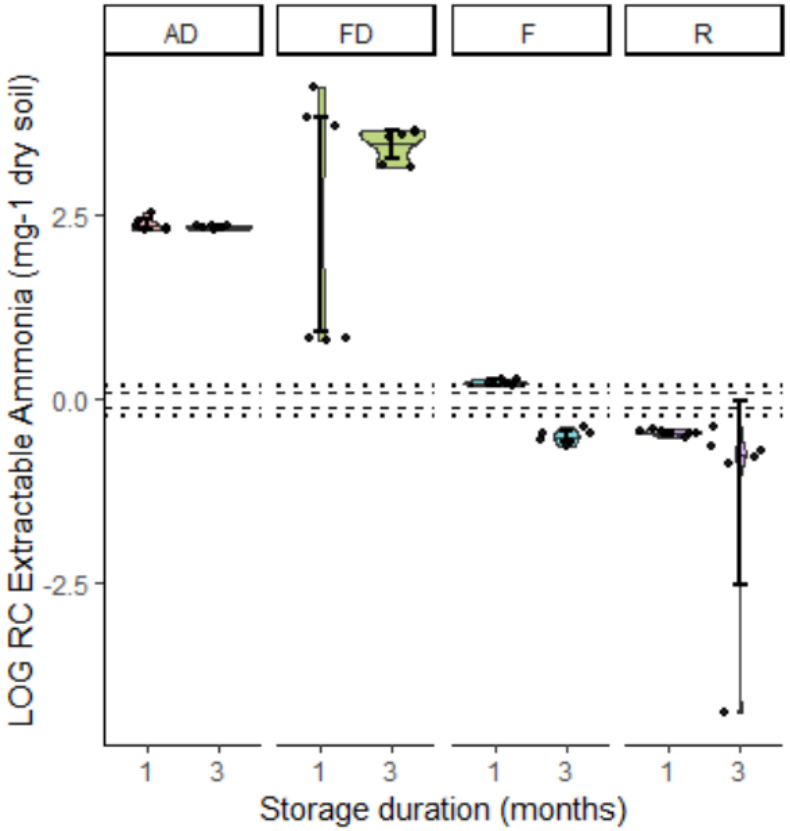


Figure 3. 13. The natural log of the relative change (LOG RC) in soil extractable ammonium after 1 and 3 months of soil storage, compared to un-stored soil. Storage treatments are air dried (AD), freeze-dried (FD), frozen at -18 °C (F) and, refrigerated 4 °C (R). The inner dashed line represents the 10% variation from the baseline boundary, referred to as the similarity limit. The outer dotted line illustrates 20% variation from the baseline. Error bars are the upper and lower 95% confidence intervals extracted from the mixed model. Horizontal lines within the violin plots represent the mean relative change of six replicates.

Freezing soil for one month appeared to have the closest ammonium concentration compared to the un-stored soil (mean LOG RC 0.220 $mg^{-1}$ dry soil). Freeze-drying soil for one month tended to increase $NH_4$ (mean LOG RC 2.37 $mg^{-1}$ dry soil) and it was highly variable (SE = 0.69). Air-dried soil

had the closest extractable $NH_4$ between one and three months storage (mean log RC: AD1 = 2.36 $mg^{-1}$ dry soil, AD3 = 2.31 $mg^{-1}$ dry soil). Storage of soil between one and three months tended to reduce the mean LOG RC in $NH_4$.

### *3.4.9. Total Extractable Nitrogen (TN)*

No treatment is appropriate, as no 95% confidence intervals extracted for the models fit within the inner dashed similarity limit as shown in Figure 3.15.

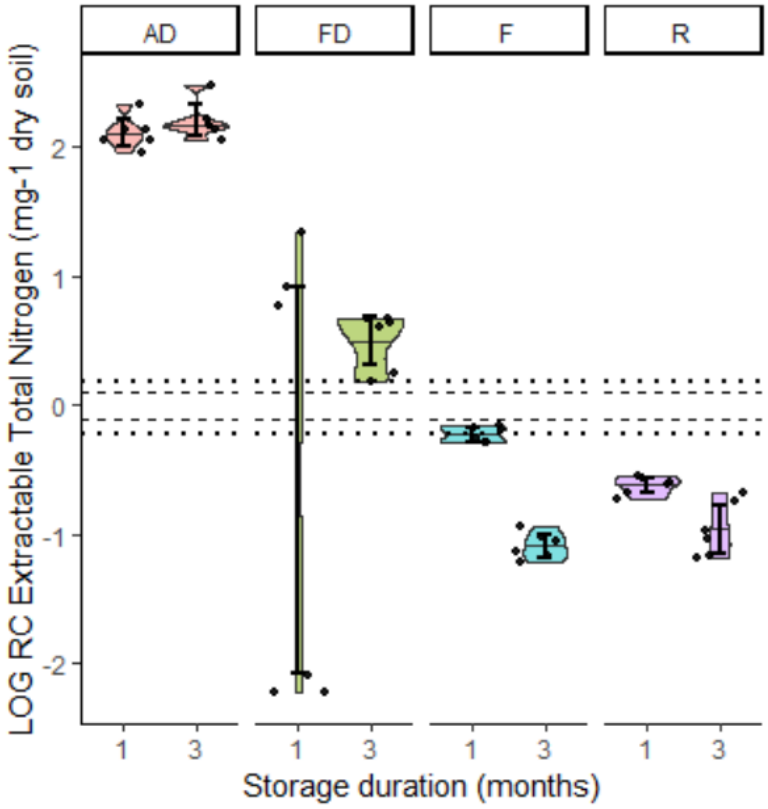


Figure 3. 14. The natural log of the relative change (LOG RC) in total soil extractable nitrogen after 1 and 3 months of soil storage, compared to un-stored soil. Storage treatments are air dried (AD), freeze-dried (FD), frozen at -18 °C (F) and, refrigerated 4 °C (R). The inner dashed line represents the 10% variation from the baseline boundary, referred to as the similarity limit. The outer dotted line illustrates 20% variation from the baseline. Error bars are the upper and lower 95% confidence intervals extracted from the mixed model. Horizontal lines within the violin plots represent the mean relative change of six replicates.

Freeze dried soil stored for one month both increased and decreased LOG RC in TN (total nitrogen concentration) compared to the baseline, as the variation between the replicates was so large (mean

log RC = -0.585, SE = 0.7). Frozen soil stored for one month had the closest LOG RC in TN compared to the baseline (mean LOG RC = 0.0026). Cooling treatments tended to reduce the TN of soil over time. Air-dried soil had a large increase in TN and it was similar at one and three months of storage (mean LOG RC: AD1 = 2.12, AD3 = 2.204)

### *3.4.10. Baseline soil properties*

Table 3. 2. Table of mean un-stored baseline soil properties (n =12). From the top down: Enzymes BG, PHO, NAG, PER, POX, Soil moisture, Organic carbon, delta $^{13}C$ of organic carbon, extractable phosphorus, dissolved organic carbon, dissolved carbon, dissolved inorganic carbon, microbial biomass carbon, extractable nitrate, extractable ammonium, extractable total nitrogen.

| Soil properties | units | Mean | Median | Standard deviation | Standard error |
|---|---|---|---|---|---|
| **BG** | nmol $hr^{-1}$ $g^{-1}$dry soil | 1275.03 | 1284.39 | 82.57 | 23.84 |
| **PHO** | nmol $hr^{-1}$ $g^{-1}$dry soil | 20907.91 | 21034.60 | 1631.97 | 471.11 |
| **NAG** | nmol $hr^{-1}$ $g^{-1}$dry soil | 803.41 | 810.16 | 121.66 | 35.12 |
| **PER** | nmol $hr^{-1}$ $g^{-1}$dry soil | 49.29 | 49.49 | 7.21 | 2.08 |
| **Soil moisture** | % dry weight | 69.67 | 69.84 | 0.76 | 0.22 |
| **OC** | % | 12.10 | 13.08 | 1.94 | 0.56 |
| **$\delta^{13}C$** | ‰ | -28.15 | -28.15 | 0.21 | 0.06 |
| **P** | mg $kg^{-1}$ dry soil | 61.13 | 61.17 | 0.91 | 0.26 |
| **DOC** | mg $kg^{-1}$ dry soil | 608.32 | 580.11 | 53.79 | 15.52 |
| **DC** | mg $kg^{-1}$ dry soil | 656.11 | 627.82 | 51.36 | 14.83 |
| **DIC** | mg $kg^{-1}$ dry soil | 47.79 | 49.48 | 6.38 | 1.84 |
| **MBC** | mg $kg^{-1}$ dry soil | 17.91 | 17.71 | 2.64 | 0.76 |
| **$NO_3$** | mg $kg^{-1}$ dry soil | 2.94 | 2.81 | 0.53 | 0.15 |
| **$NH_4$** | mg $kg^{-1}$ dry soil | 80.99 | 80.88 | 1.58 | 0.46 |
| **TN** | mg $kg^{-1}$ dry soil | 188.49 | 189.44 | 6.55 | 1.89 |

## 3.5. Discussion

Most storage methods for this soil were found to not maintain statistically similar soil properties to the un-stored soil, over one and three month storage. This raises questions about the appropriateness of soil sample storage and suggests selection of storage method (where there is no option other than to store soils), may be a case of choosing the options that causes the least change in the characteristic being measured, while also recognising that there will be some change. The one exception was $\delta^{13}C$ that remained statistically similar to the baseline for both 1 and 3 months.

Although, in a few cases either the upper or the lower 95% confidence intervals (illustrated as error bars on the box plots) overlapped within the 10% similarity boundary, for statistical similarity to be confirmed both the upper and lower 95% confidence intervals must fall within the 10% similarity boundary. In some cases, analytical replicates fell within the 10% similarity boundary but the 95% confidence intervals and/or not all of analytical replicates did. Sometimes, the variation in tests results from replicates in the same treatment and storage duration was too large to meet the 10% similarity limit (95% confidence intervals were too wide to fit within the 10 % similarity limit), even when the majority of replicates fell within the 10% similarity limit. This may be a limitation of the approach, as it does not consider the variation in analytical replicates when setting the similarity limit. In future, researchers conducting similar studies may want to observe the variation in analytical replicates of the un-stored soil when setting similarity limits, and opt for a more lenient similarity limit if unstored soil replicate variation is large. However, in this experiment if the 20% similarity limit was used, no change in the main findings would occur, as results that failed to meet the 10% limit also failed to meet the 20% limit.

### *3.5.1. Type of soil storage*

How the soil in this study responded to the choice of soil storage treatment can be broadly split into drying treatments (freeze drying & air-drying) and cold storage treatments (refrigeration & freezing).

Although nearly all measured soil properties after one and three months storage did not maintain 10% similarity in test results to the un-stored soil, cold storage treatments tended to perform better at preserving measured soil properties nearer to the un-stored soil. There is however, a few notable exceptions to this generalisation. For example, freeze-drying for one month preserved the soil extractable phosphorus content better than any other storage treatment. In addition, a case could be made for air-drying being the best storage treatment for the NAG and BG enzyme assays. Nevertheless, the cold storage (freezing and refrigerating) appeared superior overall at preserving the measured soil properties closest to the un-stored soil at t=0.

3.5.1.1. Drying storage treatments

How soils respond to drying can be dependent upon the physical and chemical components of the soil (Kemper, Rosenau and Dexter, 1987). In this study, air-drying and freeze-drying of this soil tended to increase the relative change of most soil measured soil properties. There is some evidence that drying soil, although usually increasing, can decrease the aggregate stability. In particular, the concentration of clay-sized particles, 2:1 alumino-silicates, Fe- and Al-oxides, soil organic matter content, soil water status and exchangeable polyvalent cations such as $Ca^{2+}$ (Kemper and Rosenau, 1984; Kemper, Rosenau and Dexter, 1987; Six *et al.*, 2000, 2001; Denef *et al.*, 2002; Kaiser, Kleber and Berhe, 2015) all contribute to aggregate stability under drying conditions. Clayey soil tending to form more stable aggregates as they dry (Kemper, Rosenau and Dexter, 1987). The physical and chemical characteristics of the soil used in this experiment (Table 3.2.) resulted in dried soil that tended to show an increase in measured soil properties like dissolved organic carbon and enzyme activities compared to un-stored soil.

Rather than illustrating a true increase in soil properties like enzyme activity and dissolved carbon after drying, drying may have disrupted the soil matrix and broken down the physical protection of organic matter within aggregates. Most analysis of soil properties in this experiment, and more generally in soil science, include an extraction phase where the soil is mixed with a solution (usually

acid or salt based) to dissolve ions, polymers, proteins or cells into the solution to later be analytically quantified by chemical reaction. Disruption of soil aggregates prior to the extraction phase could increase the dissolution rate of the soil compared to soil that did not undergo this disruption. Therefore, increasing the amount of ions, polymers, proteins or cells dissolved into the extraction solution, when the *amount within* the soil has not changed but rather the *amount dissolved* into the extraction solution has increased.

In this study, the soil was sieved and well mixed by hand twice to homogenise the soil and alleviate the chance that natural spatial heterogeneity existing in the soil would introduce variation to the analysis. This preparation of the soil, however, will not have broken down aggregates fully as smaller aggregates will be more resistant to break-down. Therefore, when the soil underwent the drying storage pre-treatments, this may have broken-down the aggregates further, increasing the pool of nutrients available to extracting solutions.

Along with drying potentially increasing the exposure of soil surfaces to solution, from the breakdown of aggregates, drying could theoretically increase soil measured properties as seen in this study by disrupting and changing organic bonds, and the death and lysis of soil microbial organisms. Drying is shown to increase surface acidity by increasing the concentration of solutes, affecting the solubility of nutrients (Erich and Hoskins, 2011). Air-drying has been found to increase extractable organic matter (Bartlett and James, 1980; Lundquist *et al.*, 1999), which was also found in this study. In addition, extractable nitrate has been found to increase from close to zero, to after 27 days, increase in wet soil to 120 mg N $m^{-2}$ and to 690 mg N $m^{-2}$ in dry soil (Venterink et al., 2002).

3.5.1.2. Cold storage treatments

The cold storage of soil (refrigeration and freezing) generally altered soil properties less than drying, but still tended to reduce the relative change in measured soil properties past the 10% similarity limit. Therefore, freezing and the refrigeration of this soil for one and three months was found to not be an appropriate soil storage treatment as they did not preserve soil properties to within 10% of the un-

stored soil values. Refrigerating soil is advised as a preferred soil storage method, with recommendations of 2-4°C for up to three months (ISO 10381-6, 2009). Refrigeration slows down microbial processes, so resources are depleted more slowly but microbial activity continues (Coxson and Parkinson, 1987). Freezing soils is only recommended for the storage of soils that undergo annual freezing (Verchot, 1999), due to the effects ice has on microbial cells and soil structure (Stenberg *et al.*, 1998). The soil used in this study during winter will experience freezing and near freezing temperatures, and the SOM seems to be more resilient to freezing conditions, compared to drying treatments.

### *3.5.2. Storage duration*

Soil did not respond uniformly to soil storage duration across the experiment. For instance, for soil extractable phosphorus, longer storage duration strongly reduced extractable phosphorus in all types of storage treatment. However, for dissolved inorganic carbon for instance, storage duration strongly increased the mean dissolved inorganic carbon in some treatments like air-drying and freeze-drying but reduced it in other treatments like freezing. Overall, there was a slight trend for cold storage treatments to have a more reduced relative change in measured soil properties at three months of storage compared to one month, which might reflects a depletion in microbially available resources over time. A small amount of microbial activity may have remained in the stored soils, and greater immobilisation of these resources into the microbial biomass may have reduced their concentration in the bulk soil.

In this study, a similar mean and variation around the mean between one and three months of storage was rare. Aside from the stored soil ideally having similar soil properties to the un-stored soil, it would be best if soil properties also remain consistent between one and three months of storage (in addition to staying within the bounds of "similarity"): this would suggest a stable soil storage condition. Three examples that did seem to have similar means and variation around the means at one and three months are the microbial biomass carbon of frozen soil, the extractable ammonium content

of air-dried soil and all treatments for the $\delta^{13}C$ of organic carbon. However, only the $\delta^{13}C$ of carbon was statistically similar to the un-stored soil.

### *2.5.3. Soil measured properties*

One surprising result from the study was that the microbial biomass carbon of air-dried and freeze-dried soil was much larger than the un-stored soil. This was unexpected as drying puts a physiological stress on microorganisms in the soil (Fierer and Schimel, 2002; Blackwell *et al.*, 2010), often resulting in cell lysis and microbial death. After the rewetting of dried soil, a pulse of nutrients is commonly reported and attributed to microbial cell lysis (Gordon, Haygarth and Bardgett, 2008; Borken and Matzner, 2009; Butterly *et al.*, 2009; Bünemann *et al.*, 2013). The apparent increase in microbial biomass carbon in the air-dried and freeze-dried soil could be from a rapid increase in microbial growth, as a result of the nutrient pulse from microbial cell lysis after drying. However, the incredibly low moisture content of freeze-dried soil at one month (0-1% moisture content– supplementary material S2.1), which had the largest microbial biomass, makes this highly unlikely. Some microorganisms are adapted to very low moisture, in particular microbes adapted to severe drought conditions (Hosseyni Moghaddam *et al.*, 2021). But it is unlikely that the community present in this soil has these adaptations. Therefore, a few possible explanations for the increase in microbial biomass found are provided below. Firstly, the increase in microbial biomass in the freeze-drying treatment could be from an artificial increase caused by a combination of physical and structural changes to the soil organic matter with chloroform fumigation under a vacuum, which increased the extractable $K_2SO_4$ carbon. However, there appears to be no mention of this risk associated with this technique recorded in the literature. Secondly, freeze-drying could kill off the microbial community in the soil without causing cell lysis, possibly through cell dehydration, whilst also increasing the surface area of the soil through the break-down of aggregates. This potentially could explain a much larger microbial biomass carbon result from fumigation and extraction, as dead cells could be liaised by the technique. However, there is also no mention of this in the literature and both explanations are purely speculative. Thirdly, in the literature there is evidence that chloroform fumigation is inhibited in water

logged soils (Lee *et al.*, 2016), so the increased microbial biomass found in freeze-dried and air-dried soil could be the result of increased efficiency of chloroform fumigation.

Another unusual result from this experiment is the preservation of extractable phosphorus content of the soil only found in the freeze-drying storage treatment after one month. Between one and three months of storage, for all storage treatments, there was a strong decrease in the soil extractable phosphorus concentration. A small amount of microbial activity could have depleted the phosphorus availability in the stored soil over time. Freeze-dried soil after one month had similar phosphorus availability compared to un-stored soil, but it did not meet the 10% similarity limit. However, it did meet a less stringent 20% similarity limit. The small change in phosphorus availability, in the freeze-dried soil after one month of storage compared to the un-stored soil, could reflect that freeze-drying reduced the microbial activity most out of all of the storage treatments. Freeze-drying soil combines severe stresses that injure and kill microorganisms through membrane fracture and rupturing of microbial cells (Mazur, 1970; Calcott and MacLeod, 1975; Blagodatskiy *et al.*, 1987). Water and the protein hydrate shell are removed from the soil, preventing the diffusion of molecules and microbial activity (Kurkal *et al.*, 2005; Ball, 2008). Freeze-drying the soil in this study is likely to have killed and injured the microbial community as soil moisture was successfully removed to near 100% removal. Whereas air-drying only removed water content to ~ 95% at one month of storage, so the phosphorus could have been depleted more quickly from a larger microbial activity. There are two caveats to this understanding of the experiment. Firstly, the soil microbial biomass carbon was much higher in freeze-dried and air-dried soil. Secondly, this pattern of strong nutrient reduction with time is not reflected in other nutrients and enzyme activities tested.

The trend for higher extractable forms of nitrogen found in this study under drying treatments could also be explained by the similar logic of a break-down in the physical structure of the soil matrix under drying, which allows for a greater extraction of nitrogen into KCl extraction. The lack of a greater extraction of phosphorus into acetic acid extraction solution could be explained by the unique properties of this soil and that the extraction solution was a weak acid rather than a salt solution. The

soil used in this study is acidic (~pH 4), so the pool referred to as plant available phosphorus, which is the weak acetic acid extractable pool, may have been fully dissolved in the soil solution and available to microorganisms prior to extraction and considered more of an dissolved phosphorus pool. Therefore, the break-down in the soil matrix would not have increased the availability of phosphorus in the drying treatments. The availability of soil phosphorus across treatments could reflect the combined size and activity of the microorganism community, where a potentially more active/larger community found in the refrigeration and freezer storage were able to immobilise the phosphorus at a faster rate. However, in this study phosphorus immobilisation was not measured.

Freezing compared to refrigeration is expected to reduce the activity of microbes to a greater extent, due to osmolality and physical liquid water limitation inducing stress (Öquist *et al.*, 2009; Ewert and Deming, 2014). This is not reflected in this data, as frozen and refrigerated soil have close phosphorus availability at one month and again at three months. It is possible that during the defrosting of the frozen soil, before analysis, the additional available phosphorus could have been immobilised to a similar level as in the refrigerated soil. To test this in future studies smaller aliquots of frozen soil should be stored and soil properties extracted directly from the frozen soil without the need for defrosting first.

Air-dried and freeze-dried soil had a higher soil blank absorbance when assessing for NAG activity. The higher soil blank absorbance in the drying treatments was sometimes higher than the absorbance in the sample wells resulting in negative NAG soil enzyme activity for those replicates. This contributes to the large variation found in the air-drying at one month and freeze-drying at three months of storage (Fig. 3). Wallenius *et al* (2010), found when assessing how freezing and air-drying soil affected soil enzyme activity, using MUF linked enzyme substrates, that fluorescence of the soil blanks was increased. They suggested, that drying increases the water repellence of samples and that similar drying processes take place during freezing due to sublimation (Diehl, Ellerbrock and Schaumann, 2009). Therefore, the solubility of dried soil is reduced and this alters the concentration of light absorbing solutes in the buffer. They found this effect was more pronounced in compost and

in forest soil where humic organic matter was highest, similar to the high organic soil used in this study. Interestingly, in this study, the effect was not consistent for all replicates in the air-dried and freeze-dried soil. Soil microorganisms and their secreted extracellular enzymes occupy the soil matrix in a patchy distribution (Mummey and Rillig, 2008; Raynaud and Nunan, 2014) where they cluster in mechanically resistant aggregates (<250 µm) (Tisdale and Oades, 1982; Vos *et al.*, 2013). The breakdown of aggregate stability in the drying treatments is likely to increase spatial variability of the soil, increasing variability in soil properties. Consistent with this, freeze-dried and air-dried soil had much larger variability than frozen or refrigerated soil for most enzyme analyses, along with freeze-dried soil for DOC, DIC, $NO_3^-$, $NH_4^+$, TN.

Freeze-dried and sometimes air-dried soil KCl extracts once digested in the autoclave had visible precipitation. The high variability around the mean in the extractable total nitrogen content of freeze-dried, and to a lesser degree air-dried soil, may have been caused by this precipitation. As discussed earlier, the physical and chemical changes that soil undergoes under drying conditions could alter the way the soil reacts to common soil solution-based extractions. Therefore, freeze-drying and air-drying soil is not recommended for total nitrogen KCl digestion, as it is likely to lead to an underestimation of the total nitrogen concentration of the soil.

## 3.6. Conclusions

In conclusion, this study shows that the storage of soil should be avoided if possible for all soil properties apart from the organic carbon $\delta\ ^{13}C$ signature of organic carbon. Soil can respond unpredictably to storage treatment and soil storage reduces the accuracy and integrity of conclusions. If the storage of soil is unavoidable, then changes that arise may be less of an issue for comparison studies evaluating the change in a single soil with, for instance, a field experimental treatment. This is because comparison studies are more focussed on the magnitude of change between treatments and not absolute values of soil measured properties, but the effects of storage should be highlighted as a potential limitation. If studies are interested in using different types of soils or soils from different

geographical regions, the storage of soil should be avoided if at all possible, particularly drying treatments, as different types of soil could respond uniquely to storage treatment preventing the accurate detection of changes in measured soil properties. Out of all of the storage methods tested refrigeration could be considered the best and so is recommended for cases where short-term (less than one month) storage must take place. In addition, high organic soils accustomed to seasonal freezing temperatures, like the soil used in this study, may be resistant to short-term freezing to preserve soil properties. None-the-less, freezing or refrigeration still did not preserve soil properties to statistically similar amounts found in the un-stored soil. Furthermore, the unexpected large variation in measured soil properties after storage seen in this study begs the question whether storage should be avoided completely and whether soil science should move towards standard practice of field extractions and the preservation of extractants, as there is evidence that extracts are more stable under storage compared to soil ( Rhymes *et al.*, 2021). If at all possible, soil storage should be avoided.

# Chapter 4. Consequences of plant-to-soil carbon cut-off following an extreme climatic event simulation

## 4.1. Abstract

Anthropogenic climate change is increasing the occurrence of extreme events. Extreme events have a range of impacts on ecosystems but a common result can be widespread plant mortality. Ecosystems that are considered substantial carbon sinks, such as arctic, heathland and boreal ecosystems, could be sensitive to a loss in plant cover and their sequestration ability could be weakened or reversed. Ecosystems can take up to 2-4 years to recover from extreme event damage. Previous research has indicated that damaged sites have reduced ecosystem respiration and soil decomposition rates. This indicates a reduction in microbial and plant root activity, driven by a reduction in plant carbon input and/or less carbon loss from soils. It is unclear whether this will lead to a net increase in carbon sequestration or greater loss of previously sequestered carbon to the atmosphere. Therefore, an understanding of how sensitive ecosystems respond to the abrupt cut-off of fresh plant carbon, and how soil microbes modify carbon cycling in response is needed. An extreme event proxy experiment (the clipping of aboveground plant material) was set-up on trenched matched paired plots on *C.vulgaris* dominated heathland. Carbon cycling (soil respiration, dissolved organic carbon, soil organic carbon, microbial biomass, soil temp, soil moisture) was investigated following clipping through a time-series over a year and carbon and nutrient cycling (as above and including: five enzymes involved in nitrogen, phosphorus and carbon cycling, extractable phosphorus, extractable nitrate, ammonium and total nitrogen) was investigated the following year during an intensive sampling. Soil respiration was reduced by the EE proxy over the time-series, after 238 days the microbial biomass and dissolved organic carbon was higher in EE proxy plots compared to control plots. Soil organic carbon, although not affected by EE proxy, was found to be highly dynamic and vary by around 50% during the experiment. Soil moisture was affected by the EE proxy but the

direction of change depended on the day. A year after clipping, soil respiration remained reduced compared to controls. Whereas, extractable ammonium and soil temperature was higher compared to controls. This was likely resulted from reduced plant uptake and reduced plant shading increasing solar irradiance. The results of the experiment were complicated by a site-level drought that damaged control plots but some inferences into how plant carbon and root activity shapes the microbial cycling of carbon can be inferred.

## 4.2. Introduction

Increasing frequency and intensity of extreme events (EE) are both a consequence of, and part of, climate change (IPPC, 2018). This is of considerable concern since they can have disproportionate impacts on ecosystem structure and functioning, as they take organisms past lethal thresholds (Miriti *et al.*, 2007; Bokhorst *et al.*, 2009; Smith, 2011; Bjerke *et al.*, 2014, 2017; IPPC, 2018). In upland, boreal and arctic regions a number of different types of EE are currently threatening ecosystems, where they can cause widespread plant mortality (Bokhorst *et al.*, 2015; Bjerke *et al.*, 2017; Treharne *et al.*, 2019; Myers-Smith *et al.*, 2020).

Extreme events are usually identified by their large impact on ecosystems compared to their relatively short duration. Different definitions exist and include a climatologically rare event that alters ecosystem structure or function outside the bounds of normal variability (Gutschick and BassiriRad, 2003), or events driven by interacting factors, which separately may not, but together, cross ecological thresholds to trigger an extreme response (Smith, 2011). Extreme events include severe drought, periods of heavy rainfall (flooding), heat waves, extreme winter warming, fire and extreme pest and pathogen outbreaks (IPPC, 2007). Although the ecological impacts of particular events are highly variable, and their 'extremeness' is largely context dependent (Smith, 2011), one mark of their 'extremeness' can be widespread plant mortality leaving landscapes markedly changed (Bigler *et al.*, 2006; Gitlin *et al.*, 2006; Miriti *et al.*, 2007; Thibault and Brown, 2008; Niu *et al.*, 2014; Orsenigo *et al.*, 2014; Treharne *et al.*, 2019; Coppi, Lazzaro and Selvi, 2022). Widespread plant mortality is likely

to drastically alter how ecosystems function, and some ecosystems never fully recover to pre-disturbance levels (Maxwell *et al.*, 2019).

### *4.2.1. Plants influence soil carbon cycling*

Plant and soil community relationships are tightly coupled by mutual responses, where a change in environmental conditions such as temperature or moisture can cause plants to alter their inputs to the soil via litter and root exudates, which provide carbon and nutrients to the soil, and through microbial processes regulate nutrient availability to plants (Bardgett *et al.*, 2013). Root exudates are mostly low molecular weight organic compounds such as sugars, organic acids and amino acids. In neutral or alkali soils they can acidify the rhizosphere which along with ammonium supply can enhance the mobilisation of soluble calcium (Ca) phosphates increasing phosphorus supply to plants (Marschner, 2011). Plant root respiration provides ATP and the reducing equivalents for the uptake of nutrients and production of plant biomass (Atkin, Edwards and Loveys, 2000). Plant fixed carbon (i.e., plant respiration, and respiration associated with rhizosphere exudates and decomposition of fresh litter) has been found to dominate carbon released to the atmosphere via ecosystem respiration (Grogan *et al.*, 2001). Around 33±60% of soil respiration is attributed to roots (Bowden *et al.*, 1993; Pregitzer *et al.*, 1998) and around half of photosynthetically fixed carbon is released back to the atmosphere via root respiration (Lambers, Atkin and Millenaar, 2002).Therefore, EE that cause high levels of plant mortality will directly reduce ecosystem respiration by reducing the contribution of root exudates to respiration, as well as reducing plant respiration, to respired $CO_2$.

The transfer of carbon by plants is an important process in determining plant interactions with the soil environment (Canarini *et al.*, 2019). Plants represent a major pathway of carbon transfer to soils, linking plant carbon fixation in the canopy to soil carbon losses, influencing soil respiration strongly (Mencuccini and Hölttä, 2010), the structure of the soil microbial communities (Eisenhauer *et al.*, 2017), and contributing towards reduced or enhanced soil organic carbon (Kuzyakov, 2010).

Plants can select soil microorganism communities (Berg and Smalla, 2009; Huang *et al.*, 2014), to provide benefits to plant health such as growth promotion, nutrient uptake, stress tolerance and resistance to pathogens (Trivedi *et al.*, 2020). Therefore, plants can suppress or stimulate the microbial decomposition of SOM (soil organic matter) (Fontaine, Mariotti and Abbadie, 2003; Blagodatskaya *et al.*, 2007; Dijkstra *et al.*, 2013). Plants ability to influence the soil microorganism community can scale-up to affect ecosystem and even global carbon cycling processes. Microorganism activity influences the uptake and release of $CO_2$ at the ecosystem level (Bardgett, Freeman and Ostle, 2008). As microorganisms play a central role in decomposition of litter and soil organic carbon, they play a direct role in global carbon cycling and climate change (Treseder *et al.*, 2012; Xu *et al.*, 2014). Therefore, while microorganism activity could be disrupted should plant photosynthate carbon flow stop or decline following EE, we do not know much about how microorganisms will respond to that disruption in carbon flow, or what the consequences will be for soil carbon cycling will alter.

### *4.2.2. How increased plant carbon supply affects carbon cycling*

Research about the impacts of changes in plant carbon supply to below ground has mainly focussed on *increased* supply by plants (Phillips, 2007; Phillips, Finzi and Bernhardt, 2011; Fransson, 2012; Kuzyakov *et al.*, 2019). This has been studied by increasing the atmospheric $CO_2$ concentration around plants mainly by two approaches: either by using controlled chambers (i.e. leaf chambers or climate chambers) where the $CO_2$ level is raised and compared to ambient plant growth, or by using multiple plants grown *in situ* with natural $CO_2$ springs, solar domes, OTC (open top chambers) or FACE (free air carbon dioxide enrichment) to raise the atmospheric level of plant communities and the soil environment (Kuzyakov *et al.*, 2019). FACE experiments tend to replicate future atmospheric $CO_2$ the best as they create the least disturbance (heating, wind speed and turbulence, air humidity, soil moisture etc.) with the soil being part of the unaltered ecosystem (Kuzyakov *et al.*, 2019). Results from FACE experiments have shown increased $CO_2$ leads to increased fine root production (Drake *et al.*, 2011; Phillips *et al.*, 2012) and an increase in carbon allocation by rhizodeposition (Paterson *et*

*al.*, 1997). This creates a larger flux of carbon transferred to the soil leading to greater substrate availability for microorganism (Los, 2013), so they can increase in biomass but then also have a greater demand for nitrogen and phosphorus, and so increasing microbial N and P immobilisation. Increased carbon supply creates an environment where competition between plants and soil microorganisms is greater (Haase *et al.*, 2007; Keane *et al.*, 2023) and plant growth is no longer limited by atmospheric $CO_2$ but by soil nitrogen and phosphorus availability in the soil (Garten, Iversen and Norby, 2011).

Lots of our understanding about the role that photosynthate carbon plays in ecosystem carbon cycling also comes from addition experiments, where carbon supply is increased by direct glucose and nutrient additions to the soil. Increased plant labile carbon supply belowground is reported to contribute to both soil carbon losses and gains (see below). Therefore, predicting how ecosystems may respond to plant carbon cut-off, as may happen with plant mortality from an extreme event, is complex. Disentangling the mechanisms behind soil responses to carbon cut-off will also be challenging.

### *4.2.3. Priming of soil organic matter*

Some research shows that plant carbon additions stimulate the decomposition of SOM through decreased metabolic efficiency (microbial carbon use efficiency) or the positive priming of organic matter (Fontaine *et al.*, 2007; Manzoni *et al.*, 2012). Fontaine *et al* (2007) investigated the stability of deep old carbon in soil in an incubation experiment where fresh plant-derived carbon added to the subsoil stimulated the mineralisation of older previously slow cycling stable soil carbon. This is an example of positive priming, where the input of fresh organic matter to the soil increased the decomposition of soil organic matter and soil respiration. Zhou *et al* (2021) investigated how multiple labile inputs of $^{14}C$ glucose, 20% of microbial biomass every two months and 4% of microbial biomass every ten days, effected $CO_2$ production and soil organic matter priming over a 200 day incubation. They found the addition of labile inputs reduced the carbon incorporated into the

microbial biomass, resulting in a net loss of soil organic matter via positive priming. They attributed this positive priming to a shift in the microbial community whereby an increase in a K-strategist fungi arose.

As well as evidence of positive priming, where a loss in soil carbon is found due to increased soil organic matter mineralisation with the addition of new substrate. Negative priming can result when new substrate additions act to retard soil organic matter mineralisation or increase microbial necromas over time (Liang *et al.*, 2011), increasing soil organic carbon (Qiao *et al.*, 2014). Blagodatskaya and Kuzyakov, (2008) suggested the controls on whether negative or positive priming of soil takes place in response to fresh carbon inputs could be related to the amount of carbon additions relative to the microbial biomass. When the level of carbon additions were two to five times greater than the microbial biomass they found evidence of negative priming, where the microbial community switched carbon supply from the soil organic matter to the fresh carbon inputs. The microbes preferred to utilise the fresh carbon inputs as they were easily available and highly accessible (preferential substrate utilisation) (Kuzyakov, 2002; Kuzyakov and Bol, 2006).

### *4.2.4. How plant carbon cut-off may effect carbon cycling*

The cessation of plant carbon to the soil could theoretically contribute to either greater soil carbon sequestration or greater loss to the atmosphere. Recently fixed plant carbon dominates soil respiration (Parker *et al.*, 2020), so the cut-off of plant carbon input to the soil, would reduce carbon inputs (reducing soil sequestration) and/or reduce carbon loss to the atmosphere via respiration, and/or reduce any previous negative priming that was stimulated by the plant inputs (increasing soil sequestration). Another way soil microorganism could respond to the cut-off of fresh plant carbon inputs, which would contribute to reduced soil carbon sequestration, is to switch carbon substrates. In the absence of plant labile carbon, soil microorganisms will use more recalcitrant SOM for their carbon substrate needs (Cheng, 1999; Kramer and Gleixner, 2008; De Graaff *et al.*, 2010). Although an overall reduction in plant photosynthates carbon is expected after an EE,  pulse of labile carbon is

initially expected following extreme event damage, as high plant mortality releases labile carbon to the soil from decomposing roots (Bowden *et al.*, 1993). However, the later reduction in photosynthates carbon would lead to the greater respiration of 'old' soil carbon, rather than recently fixed plant carbon (Parker *et al.*, 2020). SOM is less energetically favourable compared to photosynthate carbon as it contains more complex carbon sources which require more enzymatic steps to break-down (Agren and Bosatta, 1987), so the rate of SOM microbial mineralisation may decline. None-the-less, without plant delivery of photosynthate carbon to soils, and the possible loss of 'old' carbon through soil respiration, the amount of carbon stored in soils could also decline following EE. Evidence from extreme winter warming damaged sites in the Arctic suggest reduced soil organic carbon decomposition following plant stress and mortality (Treharne *et al.*, 2019). In that experiment, labile litter decomposition (buried litter bags) was reduced under extreme winter warming damaged plots indicating reduced microbial activity. Reductions in gross primary productivity and net ecosystem exchange following extreme event damage have also been reported (Parmentier *et al.*, 2018; Treharne *et al.*, 2019). These results from extreme event damaged ecosystems could indicate less overall decomposition of soil organic matter and a reduction in ecosystem photosynthesis. But the soil organic matter which is decomposed and respired could contain a larger amount of 'previously sequestered' carbon reducing further the carbon sink capacity of an ecosystem following damage, in addition to the reduction in carbon sequestration due to reduced photosynthesis.

Plant mortality will reduce competition with soil microorganisms, potentially providing a greater nutrient pool to the microbes. In Arctic ecosystems for example, plant competition for nutrients reduces amino acid and ammonium to almost undetectable levels (Weintraub and Schimel, 2005), and is thought to be responsible for summer low extracellular enzyme concentrations, which would usually drive greater decomposition in warmer temperatures (Buckeridge *et al.*, 2013). Furthermore, canopy removal could increase soil temperature by reducing shading and increase water availability (due to reduced transpiration), both of which could increase microbial activity and SOM decomposition rates. Dan *et al* (2016) found when investigating the decomposition rates of soil organic matter from an elevation gradient and incubating the soils at different temperatures and

moisture concentrations; soil moisture, temperature and elevation all affected decomposition. In addition, Illeris, Michelsen and Jonasson (2003) in a subarctic tundra manipulation experiment found that water additions rather than nutrient additions increased microbial biomass by 24% and root respiration by 47%. This highlights that the controls on soil organic matter decomposition are not just carbon input dependent but are controlled by a variety of soil, weather, location and climate factors. These factors all combine to create unique environments for soil microorganisms to decompose soil organic matter.

The structure of soil microbial communities will likely change in response to new environmental selection pressures (reduced rhizodeposition, increases in soil moisture and temperature) caused by damage following EE, so the functional decompositional properties of the microbial community may also change. Girdling experiments, where the transfer of plant carbon but not transpiration is stopped, provides evidence that plant labile carbon can supress or stimulate nitrogen mineralisation rates and shape the microbial community structure (Kaiser *et al.*, 2011; Chen *et al.*, 2012; Parker *et al.*, 2020). In girdled plots, if plants have a preference for organic nitrogen over inorganic nitrogen, nitrate and ammonium, there can be a suppression of microbial nitrogen mineralisation, as plants outcompete microorganisms for organic nitrogen sources which would otherwise be used by microorganisms for mineralisation (Dannenmann *et al.*, 2009). Plants once girdled can switch from using nitrogen for growth to increasing nitrogen uptake and storage, where an increase in nitrogen accumulation in roots is found following girdling (Dannenmann *et al.*, 2009). In this study the free-living microbial biomass was reduced following girdling along with a reduction in soil dissolved organic carbon, but interestingly the most abundant symbiotic mycorrhizal partner of the plants *C. geophilum's* abundance or colonisation of fine roots was not reduced. Zeller *et al* (2008) found nitrogen mineralisation increased following girdling, they suggested that another mechanism limiting microbial activity was stopped via girdling such as a cessation of mineralisation inhibition factor by the plants (Erickson *et al.*, 2000). They also found the difference in mineralisation rates between girdled and non-girdled plots increased over the season, suggesting that root decay may have started proving microorganisms with another source of carbon. In addition, girdling experiments show that in the absence of

photosynthate carbon, a more saprotrophic fungi community can dominate, with a higher utilisation capacity to degrade complex carbon substrates (Koranda *et al.*, 2013). This could lead to greater decomposition of recalcitrant soil organic carbon.

### *4.2.5. Addressing a knowledge need*

For accurate climate models to exist an understanding of how abrupt plant carbon cut off influences microbial carbon cycling is needed, especially, as climate models often bias their quantifications by missing details of microbial mechanisms and do not always represent EE well (Conant *et al.*, 2011; Schmidt *et al.*, 2011; Treseder *et al.*, 2012; Todd-Brown *et al.*, 2013; Zha and Qianla, 2020). This has been highlighted as a weakness in recent years and an international soil carbon network was set-up to identify gaps in knowledge (Harden *et al.*, 2018). One way models do not capture microbial processes fully is due to an incomplete understanding on the changes to soil organic carbon, both its spatiotemporal distribution across ecosystems and the understanding of processes leading to carbon sequestration to, or respiration from, soils (Naylor *et al.*, 2020). In the past, soil decomposition was modelled only using first order decay rates, with no consideration for microbial processes. More recently, an emerging field in climate modelling incorporates microbial ecology into existing process-based models, to try to better model microbial processes that were previously ignored (Schimel and Weintraub, 2003; Allison, Wallenstein and Bradford, 2010; German *et al.*, 2012; Zha and Zhuang, 2018). These microbe-based models report better reproduction of field and observation studies (Moorhead and Sinsabaugh, 2006; Lawrence, Neff and Schimel, 2009; Todd-Brown *et al.*, 2012; Treseder *et al.*, 2012; Wieder, Bonan and Allison, 2013). However, some microbial processes such as community structure are still rarely considered in large-scale ecosystem models, causing notable uncertainty on model projections as microbial community dynamics are directly involved in soil processes such as priming, which can lead to soil carbon loss under climate change (Bouskill *et al.*, 2012; Graham *et al.*, 2014, 2016; Kaiser *et al.*, 2014; Sulman *et al.*, 2014; Wieder *et al.*, 2015). This is of particular concern for regions with soils containing large stores of carbon, potentially representing a substantial feedback to climate. There should also be concern in regions undergoing unprecedented

climate warming, where a greater number of EE are expected (IPCC, 2018). Both of these factors are at play in high latitude ecosystems.

### *4.2.6. Aims and objectives*

Therefore, the aims of this work was to simulate the loss of photosynthesis from death of the plant canopy arising from an extreme event to investigate how soil carbon cycling function and microbial community structure change following extreme event damage. To address the aims, a simulation experiment was established on upland organic rich moorland soil in the UK. The site was covered by ericaceous shrubs, predominantly *Calluna vulgaris* (heather), which has also been the subject of above-ground extreme event research in boreal regions (Treharne *et al.*, 2019, 2020a). Original plans for a boreal or sub-arctic site could not be implemented because of Covid travel restrictions. Clipping of all shoots was used as a proxy for extreme climatic event damage, which causes the sudden removal of the canopy and severe vegetation mortality. We use the term 'proxy' rather than simulation here in acknowledgement that a specific EE was not simulated, but rather a common outcome (cut-off of plant carbon to soil) was simulated. This experiment is therefore relevant to extreme climatic event damage that cause mortality or loss of plant canopies, such as that arising frost drought, extreme winter warming events, fire and extreme herbivore/pest outbreaks.

Soil temperature and moisture, were monitored because the treatment removed plant transpiration and shading, and along with soil dissolved organic carbon, soil respiration, microbial biomass and soil organic carbon for the duration of the experiment (12 months). At 12 months after the clipping treatment (proxy for EE damage), during the following growing season, an additional intensive sampling was conducted to investigate the legacy effects of the extreme event damage. Soil enzyme activities were measured to investigate the activity of the microbiome, in particular to investigate the production of enzymes used to break-down carbon sources and phosphorus and nitrogen organic matter sources. Plant available phosphorus, nitrate, ammonium and total nitrogen were measured to investigate nutrient availability in EE damaged sites.

It was hypothesised that: 1) The EE proxy will cause an initial pulse of labile carbon availability, so the microbial community will initially increase soil respiration and biomass. EE proxy damaged plots over winter will become more carbon limited than control plots and the microbial community will decrease soil respiration and biomass. 2) After winter decomposition processes, EE proxy damaged plots will be more carbon limited but less nutrient limited (no plant uptake of nutrients so no competition); the microbial community will have reduced biomass and reduce soil respiration, and will use more 'old' recalcitrant SOM for their carbon substrate needs compared to control plots. 3) EE proxy damage will increase soil temperature (due to increased exposure of the soil to the sun), and increase soil moisture by removing plant transpiration.

The additional occurrence of drought during the experiment unexpectedly damaged plots; therefore, drought control plots of visually healthy *C. vulgaris* were included for the final intensive sampling.

## 4.3. Materials and Methods

### *4.3.1. Study Site*

This study was performed in the grounds of The University of Sheffield Bradfield Environmental Laboratory, High Bradfield, South Yorkshire, UK 53°25'54''N, 001°35'00''W. The site lies 393 m above sea level on top of a hill. The geology is Millstone grit and the underlying bedrock is Crawshaw Sandstone (British Geological Survey, 2014). The soil is non-calcareous and siliceous with a thin highly organic layer. In the experimental plots, the evergreen dwarf shrub *C. vulgaris* dominates (refer to figure 4.1 for a photograph of the vegetation cover) to the extent that other vascular plant species occur only occasionally (*Vaccinium myrtillus* and *Festuca ovina).* Peak summer temperature (Jul) range from 20°C - 11°C and winter lows (Dec-Feb) range from -1°C - 6°C, and the site receives an annual average of 807 mm $y^{-1}$ precipitation (National Oceanic and Atmospheric Administration, 2019).

Figure 4. 1. Photograph of matched plots, flowering c. Vulgaris, and the gas chamber of a LiCOR sampling soil respiration rates

### *4.3.2. Experimental design*

A matched-plot design with eight replications of paired clipped (EE proxy), and control (non-clipped plots) were used to compare how the abrupt cut-off plant carbon supply (clipped shoots compared to control) affects soil C-cycling (refer to figure 2.2. for schematic of experimental design). Matched plots were randomly selected from a 13 m x 26 m *C. vulgaris* dominated study area ensuring similar

biomass amount (judged by eye) for matched plots. For each matched pair, the control and treatment plots were randomly assigned to the two plots. The plots were 50 x 50 cm and plots in the matched pair were side by side (creating a rectangle shape 50 x 100 cm for the pair). All plots, including unclipped plots, were trenched at the boarders with a sharp spade (~50 cm depth) to stop the lateral flow of carbon via roots from outside of the plots. All stems within clipped plots were cut approximately 5 cm above the ground level and removed from the plot (this ensured all leaves were removed). Plots were re-trenched approximately every 4 months to sever any new root growth. Retrenching was applied after sampling so as not to cause a disturbance effect. Within plots soil PVC respiration collars (10.4 cm diameter, 4.3 cm height) were inserted 1cm into the ground 3 days before first sampling (T=1, see below), to ensure a good seal for $CO_2$ flux and to allow $CO_2$ pulses from installation to settle (LiCor, 1997). Regrowth of vegetation was clipped monthly.

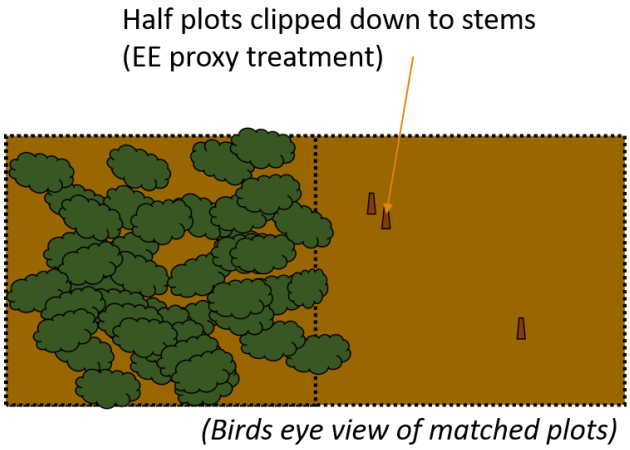


Figure 4. 2. Schematic drawing from a bird's eye perspective of the experimental set-up of one pair of matched plots

For brevity, in this study plots are referred to as EE proxy (clipped vegetation with trenching), and control plots (trenched plots with no clipping of vegetation), and drought control plots (additional control plots added due to unanticipated site-level drought damage). At the start of the experiment there were eight paired matched plots (one clipped, one with above ground vegetation remaining, in

each pair) trenched around the boarders. Due to the combination of a site level drought and the physical disturbance of trenching, the most damaged four matched plots were discarded from the experiment (visually inspected dead vegetation with no recovery from winter) just before the last time point (379 days). Four new undisturbed control plots with no trenching (visually healthy heather) were introduced into the experiment to provide additional controls.

### *4.3.3. Field sampling and laboratory analysis*

The plots were set up on 14th July 2020, the first sampling was conducted on the 15th July (day 1), and consecutive sampling was conducted on the 28th July (day 13), 21st August (day 37), 21st October (day 98), 4th January (day 173), and the following year on 10th March (day 238) and 15th July (day 379). After treatment application, for all 7 sampling points, soil respiration, dissolved organic carbon content, total organic carbon content, soil moisture content, soil temperature and microbial biomass were measured. Day 379 was an intensive sampling time-point, where soil enzyme activities, plant available nitrate, ammonium, phosphorus, and microbial nitrogen were additionally measured. Between the last two sampling days (day 239-379) a heatwave interacted with the trenching to stress and damage control plots. The damage was visually identified as control plots that had high shoot mortality. The five matched pairs, both clipped and control plots that were the most damaged were discarded from the experiment. Five drought-control plots were randomly selected from heather nearby that visually appeared healthy. These plots were not trenched prior to measurements and only included in the final intensive sampling (day 379). As the damage from the heatwave was only visible after day 238, the original plot selection was included up until then.

For each sampling point, soil respiration was measured using a LiCor 6400 (LiCor, Lincoln, NE, USA), air and soil temperature was measured concurrently using a digital thermometer. Moss layers were removed from the PVC rings at the start of the sampling day to allow any build-up of $CO_2$ to escape and avoid inhibition of $CO_2$ efflux from soil during measurements, but reapplied after measurements to minimise soil surface heating and drying from exposure. Soil respiration was

measured using a 781 $cm^3$ dark chamber and was placed on PVC rings with a foam gasket to provide a good seal. Each plot measurement consisted of three cycles of flux measurements, where $CO_2$ was scrubbed to 10 ppm bellow ambient (411 ppm) and then allowed to rise to 10 ppm above ambient. Soil respiration (the $CO_2$ rate of increase of per unit of time) was calculated from the average increase in $CO_2$, from 10 ppm below to 10 ppm above ambient, for the three cycles. $CO_2$ efflux was corrected for air moisture and temperature and calculated as mg $CO_2$ $m^{-2}$ $hr^{-1}$.

Four sub-sample soil cores (2.5 cm diameter) were collected to a depth of 15 cm from each plot, to cover horizontal variation across the plots. The mineral layer was discarded, leaving the organic layer for subsequent analysis (typically ~10cm). This was root picked and stones were removed. The four cores taken from each plot were combined and well mixed by hand until an even colour and consistency was achieved. Soil analysis was conducted on the well-mixed combined soil sample from each plot.

### *4.3.4. Soil chemical analysis*

Dissolved organic carbon was extracted with 35 ml MiliQ water and 5 g fresh soil, shaken for 10 minutes and filtered through a Whatman no. 1 filter paper gravimetrically (Harrison and Bardgett, 2004). Extracts were frozen at -18°C before analysis. Total C and organic C was determined using a Total Organic Carbon analyser (Sievers Portable TOC 5310C, University of Sheffield Geography Department) with a dilution factor of 1:5. Plant available ammonium, nitrate and total nitrogen (N) was extracted using 25 ml 1M potassium chloride and 5 g fresh soil, shaken for 60 minutes and filtered through a Whatman no.1 filter paper gravimetrically (Allen, 1989; Li *et al.*, 2012b). Extractable inorganic nitrogen (nitrate and ammonium) was measured on an auto-analyser (Skalar SAN++ System Continuous Flow Analyser, University of Sheffield, Geography Department). Nitrate was reduced to $NO_2^-$ by a copper-cadmium redactor column, reacted with sulphanilamide to form a diazo compound, and then coupled with N-1-naphthylethylene diamine dihydrochloride to form a purple azo dye. For total nitrogen, 15ml aliquots of potassium chloride extracts, were digested with

potassium persulfate and sodium hydroxide, in an autoclave at 121°C at 20 p.s.i. for 30 minutes (Langner, C.L., 1982; Kalff, J., 1984). The persulfate under alkali conditions oxidises the organic nitrogen and ammonium to nitrate. The digested samples were run on the autoanalyser and measured for nitrate following the method outlined above.

Plant available soil phosphorus (P) was determined using 0.435M acetic acid extraction and analysed using the ascorbic acid microplate method adapted from (Kuo, 1996). Absorbance was measured photometrically at 880nm after 10 minutes incubation time. P and N were corrected for soil moisture content and expressed as mg $kg^{-1}$ dry soil. DOC was calculated as mg of C $Kg^{-1}$ dry soil. Soil moisture content was determined by drying a subsample (48 hrs at 105°C) and weighing.

### *4.3.5. Soil microbial analysis*

Microbial biomass C and N was determined following the chloroform fumigation method (Vance, Brookes and Jenkinson, 1987). Briefly, soil samples were divided into two 5g samples; half underwent chloroform fumigation for 48 hrs to ensure a good fumigation of the peat soil. 5g of the fumigated and non-fumigated soil were extracted with 25 ml of 0.5M $K_2SO_4$, by shaking for 30 minutes and filtering gravimetrically through Whatman no.1 filter paper (Vance, Brookes and Jenkinson, 1987). Extracts were frozen at -18°C before analysis. Extracts were measured for total C by a Total Organic Carbon analyser (Sievers Portable TOC 5310C) with a 1:5 dilution factor. Extracts were measured for total N by cadmium-reduction colourimetry using a Skalar autoanalyser. Sample measurements were corrected for soil moisture content; non-fumigated results were subtracted from fumigated results to obtain microbial measurements (Vance, Brookes and Jenkinson, 1987). Fumigated microbial carbon measurements were corrected with the standard kEC value of 0.35 (Joergensen, 1996). Fumigated microbial nitrogen measurements were corrected with the standard kEN value of 0.45 (Wu et al., 1990). Data is presented as mg microbial N $g^{-1}$ dry soil for microbial nitrogen and mg C $L^{-1}$ dry soil for microbial biomass.

*4.3.6. Enzyme assays*

For five extracellular enzymes involved in the decomposition of SOM (Table 4.1), activity was assessed using the high throughput microplate assay technique following the protocol of Jackson, Tyler and Millar (2013). PNP linked substrates were used to determine the concentration of b-glucosidase, b-N acetylglucosaminidase and acid phosphatase (absorbance measured spectrophotometrically 405nm) using the colourmentric approach. pNP enzymes activity is expressed as nmol pNP $h^{-1}$ mg MCB. DOPA substrate (L-3, 4-dihydroxyphenylalanin) was used to spectrophotometrically measure the concentration of lignin peroxidase and lignin phenol oxidase (absorbance measured spectrophotometrically 405nm) after 0mins, 1.5h, and 18.5hr (total incubation time of 20hrs). Leucine aminopeptidase activity was measured using the leucine p-nitroanilide substrate, incubated for 6 hrs and measured spectrophotometrically at 405nm. Non-pNP enzyme activity is expressed μmol $h^{-1}$ mg MCB. Enzyme activity is expressed per unit of microbial biomass to illustrate the activity of the community relative to the size of the community. The ratio of C-cycling: N-cycling enzyme concentration reflects the investment of microbial resources, suggesting more N-limited to more C-limited with larger ratios.

Table 4. 1. Soil enzyme name, reaction involved with, and significance measured in this experiment (Matsui, Fowler and Walling, 2006; Ch *et al.*, 2017; Luo, Meng and Gu, 2017; Wang *et al.*, 2020)

| **Enzyme name** | **Reaction** | **Source** | **End product** | **Soil function indicated** | **Factors influencing enzyme activity** |
|---|---|---|---|---|---|
| **B-1,4-glucosidase (BG)** | Cellobiose hydrolysis | Bacteria, archaea, fungi, plants, animals | Glucose | C-cycling | Temperature, pH, water, $O_2$, location of organic matter, fungicides, mineral elements |
| **Acid phosphatase (PHO)** | Hydrolysis of esters and anhydrides of phosphoric acid | Plants, fungi, bacteria | Phosphate ($PO_4$) | P-cycling | Organic matter content, pH, management practices, pollution, plant species |
| **B-N-acetylglucosaminidase (NAG)** | Hydrolysis of chitin oligomers | Bacteria, archaea, fungi, plants, animals | N-acetly-B-D-glucosaminide | N- and C-cycling | Availability of N, soil depth, pH, ect. |
| **Phenol oxidase (POX)** | Lignin hydrolysis | Plants, fungi, bacteria | C compounds (humic substances) | C-cycling | Low substrate specificity, Oxygen as final electron acceptor, pH, precipitation, temperature, SOM content, N enrichment, management practices |
| **Leucine amino peptidase (LAP)** | cleave N-terminal residues from proteins and peptides | Plants, fungi, bacteria, animals | Leucine (amino acid) | N- and C-cycling | Temperature, pH, heavy metal contamination and type ect |

### *4.3.7. Statistical analysis*

Statistical analysis was conducted using R with the Lme4 package (Bates, Maechler, B. M. Bolker, *et al.*, 2015). The effects of extreme event proxy damage (clipping treatment) and time were analysed using linear mixed effects models for soil respiration, DOC content, total soil organic carbon, soil temperature, soil moisture, microbial biomass carbon. The effects of extreme event proxy damage (clipping treatment) was analysed on, extractable nitrate, extractable ammonium, extractable total

nitrogen, extractable phosphorus, extracellular enzymes b-glucosidase, b-N acetylglucosaminidase, acid phosphatase, lignin peroxidase and lignin phenol oxidase, and Leucine aminopeptidase. Day 1 – 238 were analysed together and day 379 (following the drought damage) was analysed in isolation with the drought controls.

In the linear mixed effects models, 'plot' was assigned as a random variable to account for the matched-pairs design and repeated measures, and 'treatment' was assigned as a fixed factor. When additional drought controls were added into the study on day 379, they were assigned a unique treatment category and unique plot categories. In all cases, standardised residuals were visually inspected for homogeneity of variance. In cases where model assumptions were not met, data were log-transformed prior to analysis. *Post-hoc* Tukey tests ($\alpha \leq 0.05$) were performed when a main term test was significant.

## 4.4. Results

### *4.4.1. Time series following clipping*

#### 4.4.1.1. Soil respiration

Overall, the EE proxy (clipping) significantly reduced soil respiration between one and 238 days following clipping (LME, df = 1, 77, F = 6.76, p = 0.009). EE proxy mean soil respiration was 46.4 % lower compared to controls 173 days after clipping (t(77) = 4.60, p < 0.001) (Fig. 4.3.a) .

Soil respiration was found to change depending on the time following clipping (LME, df = 5, 77, F = 18.70, p < 0.001). Overall, soil respiration reduced between day one and day 13 (t(77) = 4.40, p < 0.001), and increased between day 98 and 173 (t(77) = 3.01, p = 0.0402) and reduced between day 173 and 238 (t(77) = 8.01, p < 0.001).

#### 4.4.1.2. Soil organic carbon

Soil organic carbon between one and 238 days was not affected by the EE proxy (LME, df = 1, 77, F = 0.80, p = 0.8482). Overall, soil organic carbon did significantly change depending on the day (LME, df = 5, 77, F = 13.77, p < 0.001). In all plots, soil organic carbon reduced between day one and 13 (t(77) = 2.88, p = 0.055) and all days following clipping had reduced organic carbon compared to day one (supplementary material for full contrast significance table S3.2). (Fig. 4.3.b).

#### 4.4.1.3. Microbial biomass carbon

Overall, the microbial biomass carbon was unaffected by the EE proxy (LME, df = 1, 63, F = 1.55, p = 0.213) but on day 238 EE proxy mean microbial biomass carbon was 72.1 % higher compared to controls (t(63) = 2.78, p = 0.0071). In all plots, the microbial biomass carbon did not change depending on the day after clipping (LME, df = 4, 63, F = 2.06, p = 0.21335). (Fig. 4.3.d).

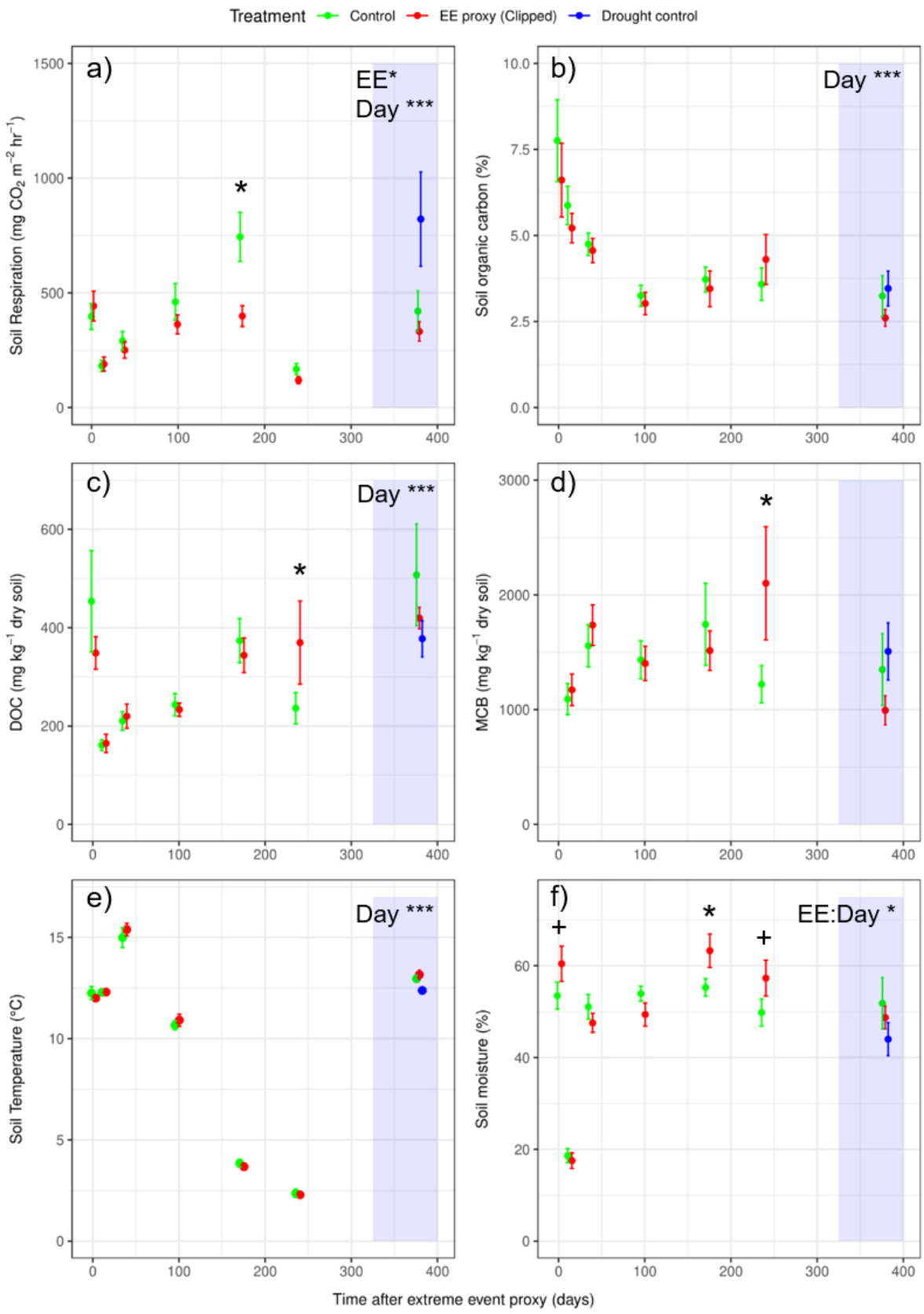


Figure 4. 3. Time series matched pair results following EE proxy: (Clipping) red points are means and standard error are bars (n = 8); Controls: green points are means and standard error are bars (n = 8), a) Soil respiration (mg $CO_2$ $m^{-2}$ $hr^{-1}$) b) Soil organic carbon (%) c) Dissolved organic carbon (mg $kg^{-1}$

dry soil) d) microbial biomass carbon (mg $kg^{-1}$ dry soil) e) soil temperature (°C) f) soil moisture (%). Top right hand corner: EE (overall effect of EE proxy), Day (overall effect of change depending on day), EE:Day (overall effect of EE proxy and Day interaction), * ($p < 0.05$), ** ($p < 0.01$), ***($p < 0.001$) + ($p < 0.08$) above error bars indicates p value of pairwise comparison on day. Blue shaded area is not included in overall statistics and shows results 379 days after EE proxy at the intensive sampling.

#### 4.4.1.4. Dissolved organic carbon

Overall, the dissolved organic carbon was unaffected by the EE proxy (LME, df = 5, 75.74, F = 0.001, p = 0.9790) but on day 238 the mean dissolved organic carbon content was 56.4 % greater in EE proxy plots compared to control plots ($t(76) = 2.08$, $p = 0.04$). In all plots, the dissolved organic carbon content changed depending on the day (LME, df = 5, 75.74, F = 7.99, $p < 0.001$). The dissolved organic carbon content of all plots reduced between day one and 13 ($t(76) = 5.26$, $p < 0.001$). (Fig. 4.3.c).

#### 4.4.1.5. Soil moisture content

Soil moisture was unaffected by the EE proxy (LME, df = 1, 77, F = 1.99, p = 0.15875) but was affected by the EE proxy and days after clipping interaction (LME, df = 5, 77, F = 2.34, p = 0.039). One day after clipping, mean soil moisture was 13.0 % higher in EE proxy plots compared to controls ($t(77) = 1.81$, $p = 0.07$). Mean soil moisture in EE proxy plots was 14.5% higher on day 173 ($t(77) = 2.08$, $p = 0.0412$) and 15.1 % higher on day 238 ($t(77) = 1.95$, $p = 0.055$) compared to control plots. (Fig. 4.3.f).

#### 4.4.1.6. Soil temperature

Soil temperature was unaffected by the EE proxy (LME, df = 1, 77, F = 0.04, p = 0.848) but overall did change depending on the day after clipping (LME, df = 5, 77, F = 886.01, $p < 0.001$). In all plots,

there was no change in soil temperature between day 1 and 13 ($t(77) = 0.68$, $p = 0.9827$), but soil temperature increased from day 13 to 37 ($t(77) = 11.81$, $p < 0.001$) and decreased incrementally until day 238 (supplementary material for full significant contrasts S3.3). (Fig. 4.3.e) .

### *4.4.2. Intensive sampling the following summer (day 379)*

#### 4.4.2.1. Soil respiration

In the following July, the EE proxy reduced soil respiration by 147.4 % compared to the drought controls ($t(8.06) = 2.30$, $p = 0.04$) (Fig. 4.4)

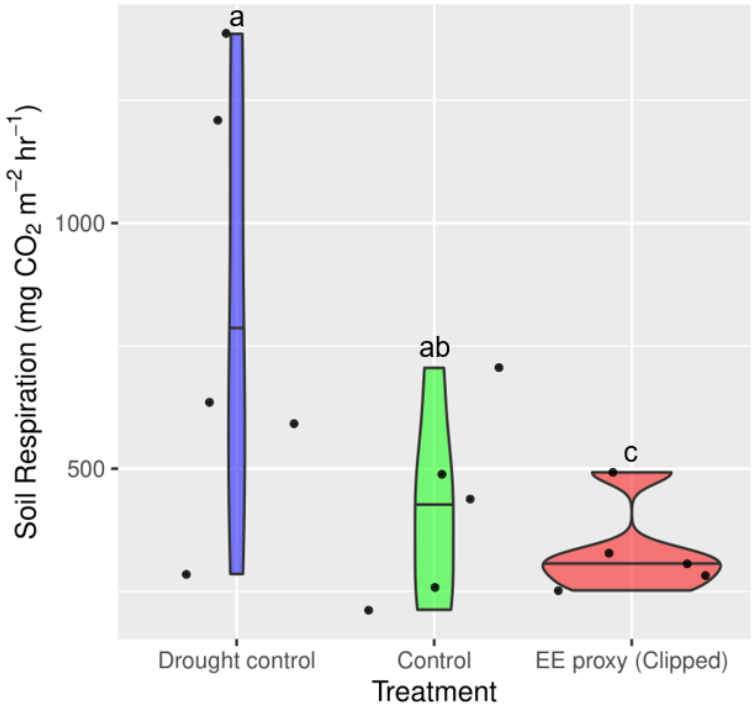


Figure 4. 4. Violin plots of soil respiration (mg $CO_2$ $m^{-2}$ $hr^{-1}$); shape of coloured area describes the distribution of replicate results within the groups, horizontal bar indicates the mean on the replicates (n = 5), black dots jittered show individual replicate results within the group. Blue violin plot are the drought controls (individual additional control plots added into the experiment due to site level drought and control plot drought damage, plots were selected from healthy vegetation. Green violin plots are the original matched pair controls. Red violin plots are the EE proxy (clipped) vegetation matched pair plots. Lettering above violin plots indicate significant differences between groups, shared letters indicate non-significant difference, different letter indicate a significant difference.

4.4.2.2. Soil extractable nutrients

The EE proxy increased extractable ammonium by 64.3 % (t(12) = 2.08, p = 0.059; Fig. 4.5.b) but had no effect on extractable, nitrate (t(12) = 0.65, p = 0.109; Fig 4.5.c), total nitrogen (t(12) = 0.51, p = 0.567; Fig 4.5.d), or phosphorus (t(11.6) = 0.23, p = 0.5199, Fig 4.5.a) compared to the drought controls.

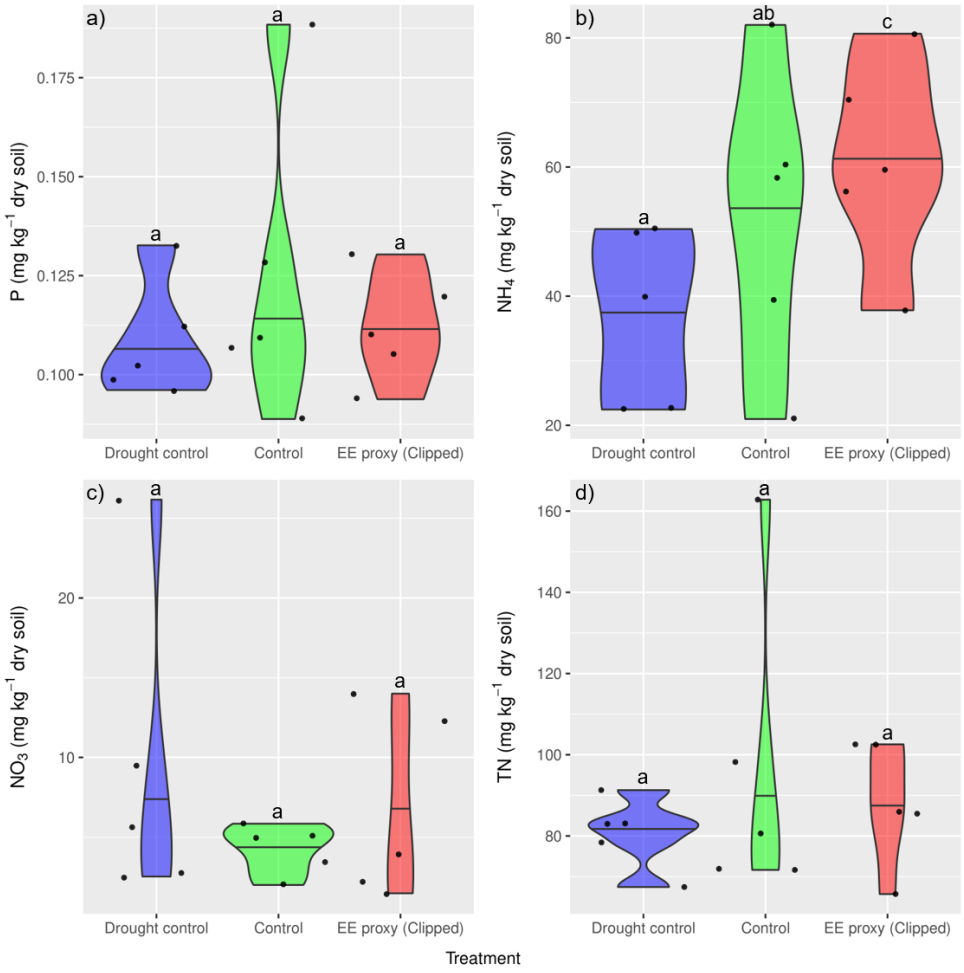


Figure 4. 5. Violin plots of EE proxy effects on extractable nutrients: a) soil extractable phosphorus b) soil extractable ammonium c) soil extractable nitrate d) soil extractable total nitrogen (mg kg$^{-1}$ dry soil). Shape of coloured area describes the distribution of replicate results within the groups, horizontal bar indicates the mean on the replicates (n = 5), black dots jittered show individual replicate results within the group. Blue violin plot are the drought controls (individual additional control plots added into the experiment due to site level drought and control plot drought damage,

plots were selected from healthy vegetation. Green violin plots are the original matched pair controls. Red violin plots are the EE proxy (clipped) vegetation matched pair plots. Lettering above violin plots indicate significant differences between groups, shared letters indicate non-significant difference, different letter indicate a significant difference.

4.4.2.3. Soil temperature and moisture

The EE proxy significantly increased soil temperature by 6.3 % compared to the drought controls ($t(10.93) = 3.21$, $p < 0.01$). The control plots temperature was 4.7 % higher than the drought control plots temperature ($t(10.93) = 2.45$, $p = 0.044$) (Fig. 4.6; left graph). There was no EE proxy effect on soil moisture ($t(11.06) = 0.819$, $p = 0.389$) (Fig. 4.6; right graph).

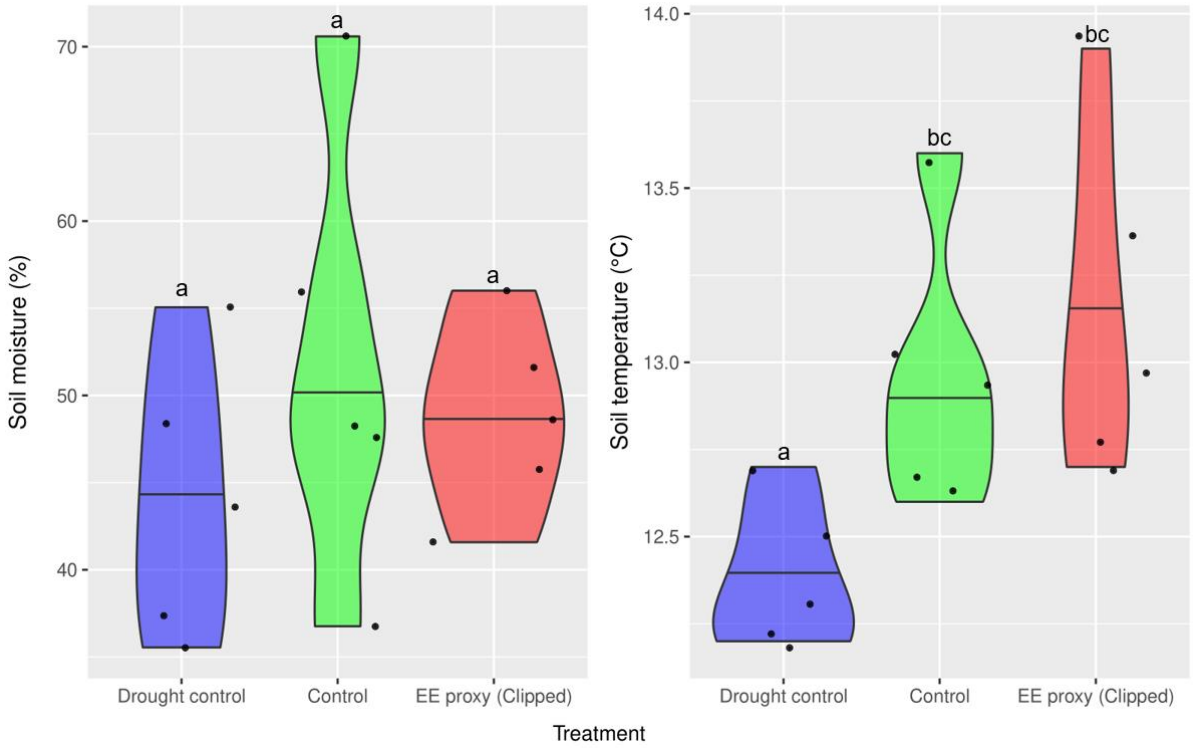


Figure 4. 6. Violin plots of EE proxy effects on soil moisture (%) (On the left) and soil temperature (°C) (on the right). Shape of coloured area describes the distribution of replicate results within the groups, horizontal bar indicates the mean on the replicates (n = 5), black dots jittered show individual replicate results within the group. Blue violin plot are the drought controls (individual additional control plots added into the experiment due to site level drought and control plot drought damage,

plots were selected from healthy vegetation. Green violin plots are the original matched pair controls. Red violin plots are the EE proxy (clipped) vegetation matched pair plots. Lettering above violin plots indicate significant differences between groups, shared letters indicate non-significant difference, different letter indicate a significant difference.

#### 4.4.2.4. Enzymes

The EE proxy had no effect on soil enzyme activity expressed per unit of microbial biomass (supplementary material for full statistics S3.4; Fig 4.8).

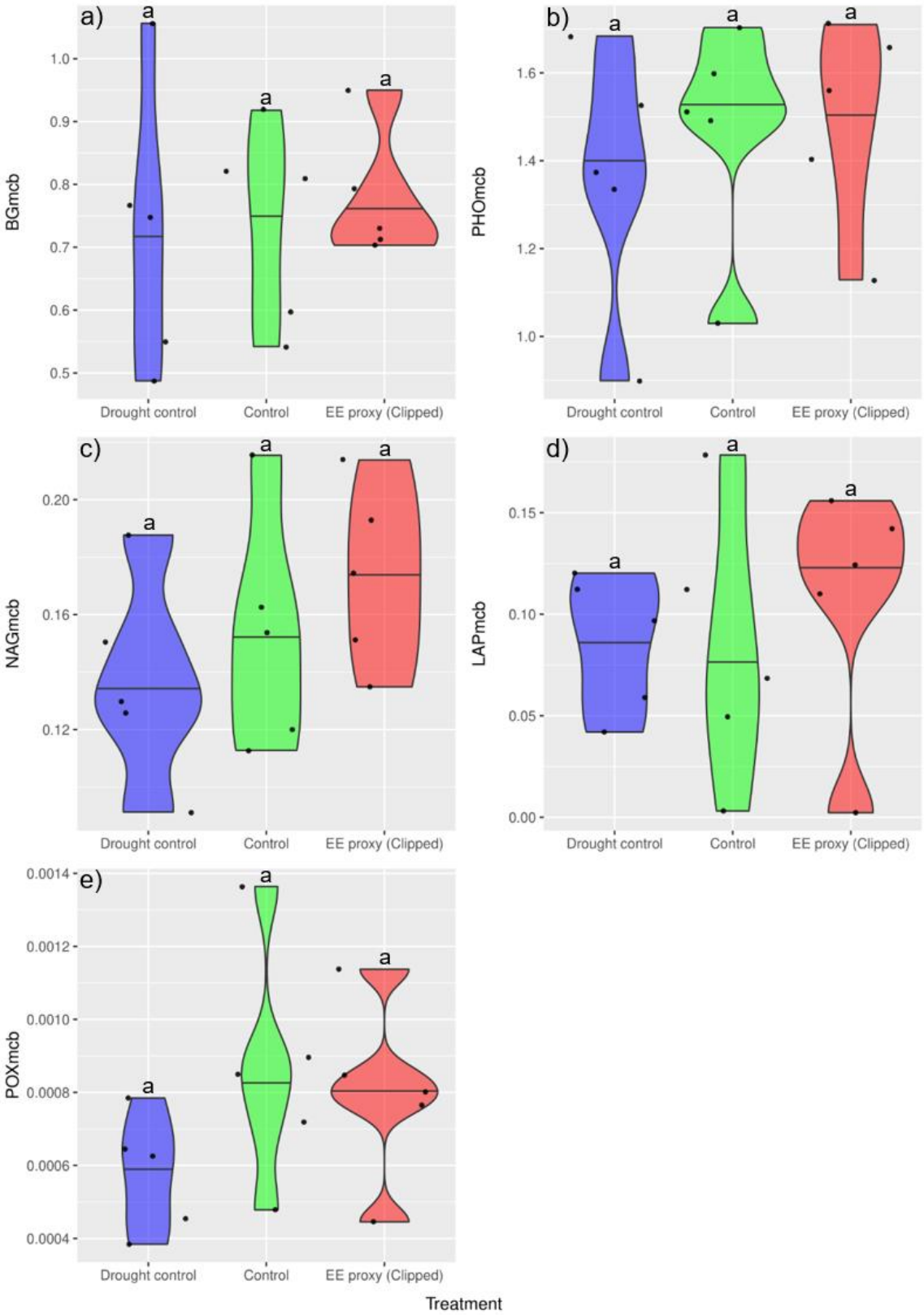


Figure 4. 7. Violin plots of EE proxy effects on soil extracellular enzyme activities expressed per unit of microbial biomass carbon, a) B-1, 4-glucosidase b) Acid phosphatase c) B-N-acetyleglucosaminidase d) Leucine aminopeptidase e) Phenol oxidase. Shape of coloured area describes the distribution of replicate results within the groups, horizontal bar indicates the mean on

the replicates (n = 5), black dots jittered show individual replicate results within the group. Blue violin plot are the drought controls (individual additional control plots added into the experiment due to site level drought and control plot drought damage, plots were selected from healthy vegetation. Green violin plots are the original matched pair controls. Red violin plots are the EE proxy (clipped) vegetation matched pair plots. Lettering above violin plots indicate significant differences between groups, shared letters indicate non-significant difference, different letter indicate a significant difference.

4.4.2.5. Soil organic carbon, dissolved organic carbon and microbial biomass carbon

The EE proxy had no effect on soil organic carbon ($t(9.86) = 0.32$, $p = 0.244$; Fig 4.9.b), dissolved organic carbon ($t(11.53) = 0.46$, $p = 0.291$; Fig 4.9.a), or the microbial biomass carbon ($t(10.01) = 0.46$, $p = 0.17$; Fig 4.9.c)

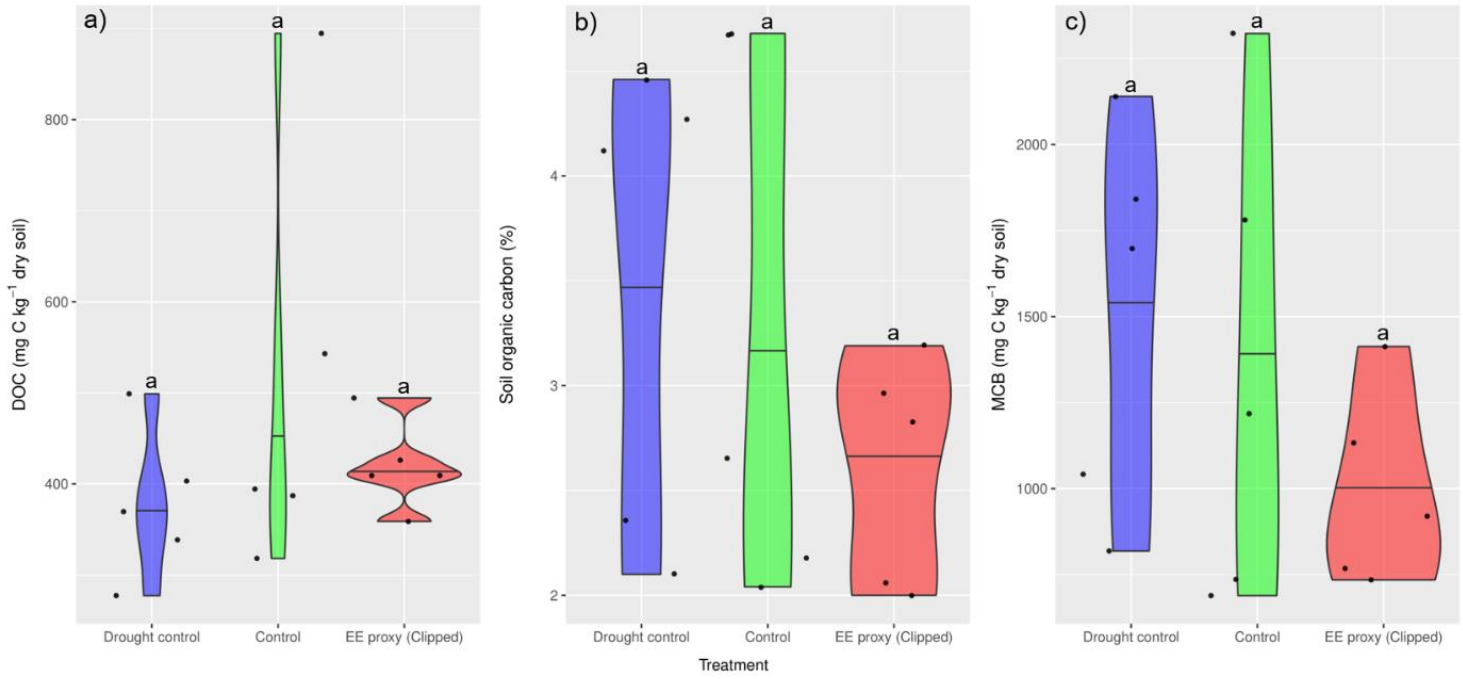

Figure 4. 8. Violin plots of EE proxy effects on a) soil dissolved organic carbon content (mg $kg^{-1}$ dry soil) b) soil organic carbon content (%) c) microbial biomass carbon (mg $kg^{-1}$ dry soil). Shape of coloured area describes the distribution of replicate results within the groups, horizontal bar indicates the mean on the replicates (n = 5), black dots jittered show individual replicate results within the group. Blue violin plot are the drought controls (individual additional control plots added into the experiment due to site level drought and control plot drought damage, plots were selected from healthy vegetation. Green violin plots are the original matched pair controls. Red violin plots are the EE proxy (clipped) vegetation matched pair plots. Lettering above violin plots indicate significant differences between groups, shared letters indicate non-significant difference, different letter indicate a significant difference.

## 4.5. Discussion

### *4.5.1. Time-series following clipping*

#### 4.5.1.1. Summary of main results

Overall, results from this experiment indicate that the soil microbial processes were relatively resistant to the effects of the EE proxy and that site-level/seasonal effects were more substantial in effecting the soil microbial processes rather than the removal of the canopy. Nevertheless, some differences between the EE proxy damaged plots and control plots were found. The EE proxy reduced soil respiration over the time series compares to controls. After 238 days following clipping, although overall effects of EE proxy were not found (i.e. all time points combined), the microbial biomass carbon and the dissolved organic carbon was greater in EE proxy plots compared to controls. In addition, on day 238, 173 and one, the EE proxy increased soil moisture compared to controls. For EE and control plots combined, the transition from the summer season, when clipping occurred, into winter and spring, produced changes in soil respiration, organic carbon, dissolved organic carbon and soil temperature.

4.5.1.2. Interpreting the results in light of trenching impacts

It was hypothesized that the EE proxy would lead to an initial surge in labile carbon availability, subsequently enhancing carbon cycling in comparison to control plots. Following the EE proxy, day one organic carbon measurements were the highest of the experiment and the dissolved organic carbon measurements were among the largest. Between day one and 13 there was a sharp drop in soil organic carbon and dissolved organic carbon that occurred in all plots. The relatively high carbon availability on day one, likely resulted from trenching rather than clipping as all plots were affected. It is probable that trenching was not performed with a sufficient time for severed roots' decomposition not to cause an increase in carbon availability in the soil, or the control plots might not have been large enough to eliminate the boundary effect on carbon availability within the plots. Trenching was likely not performed well in advance of this study because the Covid pandemic caused the study to be designed and started very quickly. Previous research suggests that trenching should be done at least nine months prior (Bowden *et al.*, 1993). However, the comparison between plots that were just trenched (controls) and those that were clipped (EE proxy) should reveal the magnitude of the carbon pulse induced by clipping alone. None-the-less in future experiments, it is recommended that trenching occurs earlier before clipping and/or larger plots are used allowing sampling away from edge effects.

4.5.1.3. The time series effects of the EE proxy

Over the time-series part of the experiment, soil respiration for EE proxy plots and control plots followed a similar pattern, increasing during the autumn – winter transition, indicating that site level/ seasonal effects influence soil respiration strongly rather than canopy cover (assumed root rhizosphere processes). However, the EE proxy reduced soil respiration over the time series compared to control plots; where the largest difference was found 173 days after, when mean soil respiration was 46.38% lower ($p < 0.001$) compared to controls. This suggests that rhizosphere processes although not driving substantial changes in soil respiration do have an effect and that removing the canopy does reduce soil

respiration to some degree. Soil respiration rates depend on the availability of soluble and labile carbon sources in the soil, root respiration rates, and microbial respiration depends on the amount of organic matter entering the soil (Schlesinger and Andrews, 2000; Wardle *et al.*, 2004). The reduction in soil respiration in EE proxy damaged plots compared to controls is likely due to the removal of above ground photosynthetic plant material, and the halting of plant photosynthesis following the damage. This would reduce plant root respiration and labile carbon availability for soil microorganisms. These results are consistent with a grazing experiment, where grassland above ground plant material was reduced to simulate grazing and a reduction in microbial respiration was found (Stark and Kytöviita, 2006). The relative contribution of a lack of root respiration and microbial respiration to the reduction found in EE proxy plots is unknown, but likely varied over the time-series. Root respiration is found to contribute to between 10-90% of soil respiration depending on the ecosystem and season (Hanson *et al.*, 2000). Although, the relative contribution of reduced root respiration and reduced microbial respiration is not known, it is likely that both contributed to the reduced soil respiration under EE proxy damaged plots.

It was hypothesised that over the time series, the EE proxy soil microbial community would reduce in biomass and reduce soil respiration compared to controls, as a response to reduced labile carbon availability and switching to less energetically favourable SOM for their carbon substrate needs (Cheng, 1999; Kramer and Gleixner, 2008; De Graaff *et al.*, 2010). Although, the EE proxy reduced soil respiration, overall it did not affect soil dissolved organic carbon, soil organic carbon or the microbial biomass. This indicates that in this ecosystem rhizosphere processes are not substantial drivers of soil processes. However, on some sampling day's differences in soil processes between EE proxy damaged plots and control plots were found. After, 238 days the EE proxy plots had greater dissolved organic carbon and microbial biomass compared to controls. The upward trend in dissolved organic carbon between day 13 to 238 could have resulted from the gradual decomposition of roots in the EE damaged plots. Fine root decomposition has been cited to stabilise after four months (Bowden *et al.*, 1993) but other studies using coarse roots found decomposition on the yearly scale (Olajuyigbe *et al.*, 2012). Decomposition of labile carbon occurs at shorter time-scales compared to recalcitrant

carbon (Duddigan *et al.*, 2020), so the labile fraction of root decomposition could have contributed to the observed increase in dissolved organic carbon over time. This might help to explain why after 238 days EE proxy plots had greater dissolved organic carbon compared to controls, as controls would have reduced root death compared to EE proxy plots (just from trenching). Root death under EE proxy plots was likely higher (from clipping and trenching), as results from an EE damaged site suggest roots struggle to recover for up to three years following an extreme winter warming event (Bokhorst *et al.*, 2011).

The greater availability of dissolved organic carbon in EE proxy plots compared to controls on day 238 and the general trend for an increase from day 13 onwards was surprising. As plant carbon flow had been cut-off, even with root death decomposition, it was expected that dissolved organic carbon in the EE proxy plots would be lower than in the controls and disappear over time not increase. A large proportion of dissolved organic carbon in soil is considered relatively labile (Neff and Asner, 2001). A greater amount of dissolved organic carbon, in EE proxy plots, on day 238 did not occur with greater soil respiration (lowest respiration rates of the experiment). The microorganism carbon use efficiency (the relative investment in microbial growth compared to the uptake of carbon) of a community shows the relative investment in microbial growth compared to microbial respiration and substrate acquisition (Manzoni *et al.*, 2012; Sinsabaugh *et al.*, 2013). Although the microbial carbon use efficiency was not measured in this experiment other experiments have shown that one of the factors that shift investment to resource acquisition over growth is low resource availability (Ekschmitt *et al.*, 2005; Don, Rödenbeck and Gleixner, 2013; Kaiser *et al.*, 2014). Therefore, this greater availability of dissolved organic carbon, which coincided with high microbial biomass in EE proxy plots on day 238, could represent greater resource availability for microorganisms and the shift to investment in microbial growth over resource acquisition. In fact, a recent microbial trait based strategy has been developed where microorganisms can be classified into three main life history strategies high yield (Y), resource acquisition (A) and stress tolerance (S) depending on resource availability and abiotic stress (Malik *et al.*, 2020). This illustrates how, a greater availability of resources will not be simultaneously invested in microbial biomass growth and soil decomposition

(degradative enzyme production). Contrary to what was hypothesised, the EE proxy soil microbial community, at least on day 238, appeared to have greater resource availability compared to control plots.

The EE proxy did not affect soil organic carbon but overall it did vary considerably over the time-series. This suggests that in this ecosystem soil organic carbon, on the time-scale investigated, is relatively resistant to canopy removal (assumed cessation of rhizosphere processes) and influenced largely by site-level/seasonal effects. Total soil organic carbon is commonly regarded as a stable slow turnover carbon pool, with most of the carbon in a protected recalcitrant form, with several smaller pools of more rapid turnover carbon (Kiem and Kögel-Knabner, 2003; Fierer *et al.*, 2009). However, in this study soil organic carbon after 98 days in all plots had measurements around half of which they started with. It was surprising that either environmental factors, or potentially an initial-trenching-induced carbon pulse, could produce a difference in soil organic carbon so large in a relatively short amount of time. None-the-less, substantial changes in organic carbon have been recorded in other environments, when assessing for seasonal patterns. For example, three different mixed oak forests were individually sampled two months apart in Ohio and time differences in organic carbon for each forest were 28, 40, and 52% (Boerner, Brinkman and Smith, 2005). In addition, when Canadian prairie was sampled for organic carbon in spring, summer, autumn and winter, one grassland varied over time by 26% and another by 53% (Dormaar, Johnston and Smoliak, 1977). Therefore, whether the drop was seasonally driven or/and a drop associated with the initial-trenching-induced carbon pulse, soil organic carbon in this environment is found to be much more dynamic than expected and has the capacity to gain and lose substantial amounts of organic carbon in relatively short time scales. None-the-less, the EE proxy did not seem to induce a large change in organic carbon.

In addition, the soil organic carbon measurements for the site overall appeared lower than expected for heathland soil organic carbon. Soil organic carbon can vary from 1% in desert soils to over 50% in peatland soils (BSSS, 2021). Soil organic carbon was between ~8% to ~2.5% over this experiment (discussion on the variability found in 4.5.2.2. below). A limitation of this experiment is that bulk

density was not measured which would allow greater comparison with the literature as studies tend to report SOC in the units tonnes per hectare. For example, the countryside survey reports average SOC of 205 t ha-1 for dry heaths (Baggaley *et al.*, 2021). However, heathland soils are considered relatively organic and soil organic carbon (%) would be expected to be in the upper range reported for soils, even with the acid stripping of inorganic carbon from soil samples. One explanation for the low soil organic carbon measurements recorded is that the site is unmanaged heathland, where no grazing or burning of Calluna takes place. The grazing and burning of Calluna is a common management practice of UK heathlands and is used to keep the vegetation in a young and productive state (Hobbs and Gimingham, 1987). The Calluna at Bradfield is in the later growth stage where shoots lay outwards and flat along the ground. Older Calluna vegetation may be not be as effective at carbon sequestration and may be why soil organic carbon appeared low at the site level.

Soil moisture increased by the EE proxy and this depended on day of sampling. This is likely due to a balance of loss of canopy transpiration and reduced canopy inception of precipitation depending on the environmental conditions of the day, producing increased soil moisture on day 1, 173 and 238.

### *4.5.2. Intensive sampling one year later (day 379)*

#### 4.5.2.1. Site-level drought disruption

Between The last two sampling points, a drought effected the site. The drought caused visible plant damage (dry brittle stems and a lack of spring re-greening) over the site but particularly for experimental plots. However, by the time the final sampling took place the *Calluna vulgaris* over the site appeared to recover from the drought and the drought appeared to only delay spring re-greening indicating usually *Calluna vulgaris* is relatively resistant to drought. The damage which remained for the experimental plots, was likely due to trenching severing lateral roots of *Calluna vulgaris* reducing ability to access water resources as lateral roots are an important mechanisms for disturbance recovery (Schellenberg and Bergmeier, 2022). The decision was made to discard the most visibly damaged pots and introduce new undisturbed control plots, which appeared less effected by the site drought. The

drought coincided with spring and by the final time point the site appeared in recovery and viably healthy (spring re-greening and new growth).

Overall, the site a year (and possibly before due to the low OC% results for the entire experiment) into the study was relatively carbon limited, as indicated by the low organic carbon measurements found in all plots. A recent global review of the effects of drought indicated that drought reduces soil organic carbon content mainly because of plant litter input but also because of reduced litter decomposition (Deng *et al.*, 2021). This would explain why all final-day plots had very low organic carbon content, compared to the seasonal time-series of organic carbon measurements previously (assumed baseline). By the time the final sampling took place, over a year after the EE proxy, the visual effects of drought on the site appeared to be in recovery. Soil moisture had returned to near the levels of the year before. So, it is likely that in the control and drought control plots, *Calluna vulgaris* cover had resumed some rhizosphere processes to the soil alleviating the low microbial biomass and organic carbon.

An accumulation of dissolved organic carbon in drought affected environments has been reported for a number of environments, suspected to arise from a reduction in decomposition and higher stability of dissolved organic carbon (Deng *et al.*, 2021). At the site level dissolved organic carbon concentrations were among the highest found for the year. Therefore, drought effects could still be influencing overall decomposition. It is difficult to disentangle the effects of the site level drought from the effects of clipping alone. In addition, as the most visibly damaged plots were discarded from the drought statistical power for the final sampling was reduced.

4.5.2.2. EE proxy effects one year later (day 379)

Overall, results from the intensive sampling conducted one year after clipping also suggest that soil processes were relatively resistant to the EE proxy. Similar to the time-series perspective soil respiration appeared to be strongly reduced highlighting the strong link between rhizosphere carbon and soil respiration rates. The EE proxy reduced soil respiration by 147.42 % ($p = 0.04$) compared to

drought-controls. This indicates a substantial drop in carbon cycling and the decomposition of soil organic matter. For the same reasons as stated above this is likely due to reduction in plant root respirations contribution to soil respiration and rhizodeosition reductions reducing microbial respiration due to carbon limitation. Furthermore, root death and the lack of root respiration probably contributed to the substantial reductions in soil respiration. Interestingly, relatively high dissolved organic carbon (compared to the previous time-series and non-significantly higher than drought-controls) was found in EE plots. This greater availability of labile carbon under clipped plots was not invested by the microbial community into relatively greater soil respiration (as expected) or microbial biomass (observed in the earlier part of the experiment). It is likely due to the site level drought that the microbial life history strategies switched to stress tolerance (S) over high yield (Y), resource acquisition (A).

The EE proxy produced a 60% ($p = 0.059$) increase in available ammonium concentration in the soil compared to drought-controls. This is likely due to the cessation of uptake by the dominant *Calluna vulgaris* as it preferentially utilises ammonium as a nitrogen source (De Graaf *et al.*, 1998). The availability of other forms of nitrogen and phosphorus was not increased by the EE proxy, indicating similar nutrient availability for the other nutrients tested between the EE proxy and drought control plots. Alternatively, if the cessation of plant uptake did increase the availability of other nutrients, it is possible that a greater uptake by the microbial community took place, in the absence of plant-microbe competition for resources. The microbial N and P content was not measured so this cannot be determined.

The availability of phosphorus in particular, for all plots, was very low and almost at undetectable levels. The low phosphorus availability at the site level along with acid-phosphatase enzyme activity overall recorded as the enzyme with the greatest activity indicate strong site phosphorus limitation. Nitrogen was historically considered the main limiting nutrient in most northern hemisphere terrestrial ecosystems. However, over the last century considerable nitrogen deposition has taken place within The Peak District Nation Park, where this experiment was located, leading l to experience some of the

highest cumulative N loads recorded for the country and helping drive P-limitation (Fowler *et al.*, 2004; Horswill *et al.*, 2008). It is therefore possible that the site is either P limited or co-N-P limited.

## 4.6. Conclusions

Overall, the soil processes in this ecosystem appeared relatively resistant to the disturbance of the extreme event proxy with the exception of soil respiration. The removal of vegetation in EE proxy plots resulted in a notable reduction in soil respiration, indicating decreased carbon cycling function in the absence of plant activity. Concurrently, we observed an increase in soil extractable ammonium content under clipped vegetation, likely attributed to reduced plant uptake. It is worth noting that the trenching process obscured the true carbon cycling dynamics within the soil. To enhance the accuracy of future studies on heathland ecosystems, we recommend conducting trenching a year in advance and utilizing plastic sheets to inhibit root growth, thereby avoiding the need for re-trenching. In addition, the site was exposed to strong drought conditions that affected all plots carbon dynamics, complicating comparisons with baseline control plots. The drought may have effected the soil organic carbon content of the soil and overall soil organic carbon content was highly seasonally dynamic and appeared low for an ecosystem of this type. Future studies assessing climate driven EE and carbon cycling should consider the co-occurrence of drought conditions as they may reflect future conditions more accurately. Furthermore, it is also recommended that carbon cycling studies do not just sample in the growing season but sample throughout the year as soil carbon cycling appears strongly influenced by seasonality.

# Chapter 5. General discussion

Unless dramatic action is taken, by the end of the century $CO_2$ in the atmosphere could double (IPPC, 2018). Past greenhouse gas increases are already impacting today's climate, including extreme events. Attribution studies have shown that the devastating floods that took place in Pakistan in 2022, which affected 33 million people and killed 1739 (CDP, 2023), were made significantly worse by climate change (Otto *et al.*, 2023). Also, the Australian 2019/2020 bushfires, which burned over 46 million acres (72,000 square miles) and destroyed around 3500 homes (Webster, 2019), were made worse by climate change drivers (Jan Van Oldenborgh *et al.*, 2021).

Over this century, depending on the action we take now, global temperatures could rise between 1.5 - 5.8 °C. Global air currents that drive regional weather could alter, upsetting precipitation patterns, and the frequency and intensity of extreme events will increase to an uncertain degree (Easterling *et al.*, 2000; IPPC, 2018). Nitrogen deposition, caused by the burning of fossil fuels (NO, $HNO_3$ and $NO_2$ – referred to as $NO_y$) and agricultural emissions (reduced N – referred to as $NH_x$), have already caused widespread reductions in plant biodiversity (Payne *et al.*, 2017). Global climate and regional environmental changes are likely to strongly affect terrestrial ecosystems and their organisms, creating complex response patterns, causing unpredictable effects on ecosystem processes and functionality, which will be exceeding difficult to predict.

In each experimental chapter of this thesis, a disturbance was applied and the resulting effects can be viewed through a resilience and resistance approach. Resistance refers to the ability of an ecosystem to remain unchanged when subjected to a disturbance. An ecosystem with high resistance can withstand environmental pressures and maintain its structure and functions despite external shocks. Pimm (1984) introduced how resistance is an essential component of ecological stability, highlighting that ecosystems with high species diversity tend to exhibit greater resistance to disturbances. Resistance in soil science can refer to the ability of soil to withstand disturbances without undergoing significant changes in structure, composition, or function. For example, Soils with high organic matter

content and aggregate stability are more resistant to erosion and compaction (Lal, 1993). Resilience, on the other hand, refers to the ability of an ecosystem to recover after a disturbance (Tilman and Downing, 1994). It focuses on the speed and extent of recovery, allowing the ecosystem to return to its original state or adapt to a new stable state. Holling (1973) introduced the concept of ecological resilience, emphasizing that resilient ecosystems can absorb shocks and reorganize while undergoing change, thus maintaining their core functions and structures. While resistance and resilience are distinct concepts, they are interconnected. An ecosystem with high resistance may not necessarily be resilient. For example, a forest that initially resists drought (high resistance) might suffer long-term damage if the drought persists and it cannot recover quickly (low resilience). Conversely, an ecosystem with low resistance might still be highly resilient if it can recover quickly from disturbances.

Press and pulse disturbances are two distinct types of ecological perturbations that impact ecosystems in different ways (Bender, Case and Gilpin, 1984). Press disturbances are continuous, long-term changes that exert a persistent influence on an ecosystem. These disturbances are characterized by their sustained nature, which can lead to gradual but significant alterations in the structure and function of ecosystems over time (Bender, Case and Gilpin, 1984). For example, climate change (Long-term shifts in temperature, precipitation patterns, atmospheric $CO_2$ levels), pollution (continuous exposure to pollutants such as heavy metals, pesticides, or industrial emissions) and nutrient enrichment (ongoing addition of nutrients (e.g., nitrogen and phosphorus) from agricultural runoff, or nitrogen deposition). Pulse disturbances are short-term, discrete events that cause immediate and often dramatic impacts on ecosystems. These disturbances are typically episodic and can result in rapid changes, but their effects may not be long-lasting if the ecosystem can recover quickly (Bender, Case and Gilpin, 1984). For example, droughts, floods, heatwaves, wildfires, and other extreme events.

In the second chapter of this thesis, the experiment investigated how the press disturbances of increased $CO_2$ and nitrogen deposition applied to phosphorous limited grasslands altered microbial

community biodiversity and community structure through time. In this experiment, resistance is specifically measured by how well the grassland microbial communities maintained their biodiversity, species richness and community structure, compared to the control treatments (ambient $CO_2$ and no nutrient addition per each grassland) over time. Overall, fungi were more resistant in comparison to bacteria to the effects of increased $CO_2$. Elevated $CO_2$ reduced bacterial Shannon diversity over the year, specifically spring bacterial species richness (acid Grassland), and fungal and bacterial species richness (limestone grassland). This was attributed to a potential relative increase in dominance of certain species driven by the alleviation of carbon limitation via greater plant carbon allocation belowground. In addition, $eCO_2$ produced bacterial communities that significantly differed to those in the ambient $CO_2$ in both grasslands (over full the year), whilst fungal communities were resistant to community change effects. In this experiment, fungi were resistant to the effects of $eCO_2$, in both grasslands, and bacteria were not resistant, particularly in the acidic grassland.

The acidic grassland, had a bacterial community that seasonally changed (control treatment), lower bacterial species richness, and $eCO_2$ significantly increased soil organic carbon. Furthermore, previous research on the same long-term experiment found that $eCO_2$ reduced aboveground plant biomass and that the plant biodiversity was overall lower compared to the limestone grassland (Taylor *et al.*, in press). In comparison, the limestone grassland had greater bacterial species richness, did not change community structure seasonally and had a trend of reduced organic carbon with $eCO_2$. Previous research found that $eCO_2$ increased the limestone grassland above ground plant biomass, plant biodiversity was overall greater compared the acidic grassland and $eCO_2$ did not change but restructured plant biodiversity (Taylor *et al.*, in press). In terms of resilience, the limestone grassland was more resilient to the disturbance of $eCO_2$ as biodiversity (above and belowground) was maintained, soil organic carbon was reduced, and plant biomass increased. This supports the diversity-stability hypothesis where more biodiverse communities are more resilient to disturbance as they are more likely to contain species that can compensate for the loss or decline of others during environmental perturbations, thereby maintaining overall ecosystem stability (Tilman and Downing, 1994). However, as $eCO_2$ represents a ‘press’ disturbance (Bender, Case and Gilpin, 1984), which are

sustained long-term changes in environmental conditions, such as climate change, pollution, or habitat alteration. Resilience in this context refers to the ecosystem's ability to absorb these disturbances while maintaining its essential structure, function, and processes; rather than a return to a pre-disturbed condition (Bardgett and Caruso, 2020). However, the non-significant reduction in soil organic carbon may be cause for concern if this trend continues and worsens in the future (with additional $eCO_2$ fumigation), which would indicate a lack of ecosystem resilience in the limestone grassland.

In the third chapter, a soil storage pulse disturbance was applied to soil samples to assess their resistance to change under storage. In this chapter, commonly used soil sample storage methods were overall found to not maintain soil properties similar to those of fresh un-stored samples. With the exception of the $^{13}C$ signature of OC. Although, overall soil sample storage was found to not be appropriate (maintain significant similarity), the drying storage methods (freeze-drying and air-drying) were found to produce a much greater disturbance effect. The soil analysed in the experiment, was less resistant to drying methods compared to the cooling methods (freezing and refrigeration). In this chapter, the soil was not resistant to storage and it was concluded that storage should be avoided when possible, with the exception of $^{13}C$ signature of OC.

In the fourth chapter, a climatic extreme event proxy (the clipping of above ground vegetation) pulse disturbance was applied to *Calluna vulgaris* heathland. This experiment aimed to investigate how plant activity affects soil microbial carbon cycling and to understand the changes in microbial carbon cycling when plant activity ceases, which is common in extensively damaged ecosystems following extreme events (Treharne *et al.*, 2019). Most soil processes in this ecosystem were resistant to the disturbance effect of the extreme event proxy. For example, soil processes such as enzyme activities, extractable phosphorus content and microbial biomass carbon were unchanged one year after the extreme event proxy took place. This indicates strong soil ecosystem resistance to possible extreme event damage. However, soil respiration was reduced and soil extractable ammonium concentration was increased one year later, indicating that plant carbon transfer to the soil and plant uptake of

ammonium had reduced in the absence of plant cover. These results are consistent with girdling experiments where reduced soil respiration and increased nitrogen availability in the soil have been found in other ecosystems (Parker *et al.*, 2017; Hogberg *et al.*, 2010; Brözsterg *et al.*, 2015) and the understanding that soil respiration is dominated by recently fixed plant carbon.

In the fourth chapter, the experiment was not continued past one year. During the course of the experiment if new plant growth emerged it was clipped, to mimic the duration of recovery usually found in extreme event damaged ecosystems (2-4 years) (Bokhorst *et al.*, 2011; Bret-Harte *et al.*, 2013). Therefore, resilience, in terms of the recovery of the ecosystem to the pre-disturbance condition, cannot be fully assessed. In addition, the occurrence of the site-level drought, which interacted with the trenching to damage control plots, made disentangling the response of the extreme event proxy more difficult and additionally assessing the resilience of the ecosystem to the extreme event proxy.

The interaction between pulse perturbations (e.g., droughts, heat waves) and press perturbations (e.g., nutrient enrichment, elevated $CO_2$) (Bender, Case and Gilpin, 1984) is critical. Sustained press perturbations can progressively alter microbial communities, making them more vulnerable to abrupt changes when combined with climate extremes (Bardgett and Caruso, 2020). For example, nutrient enrichment reduces fungal abundance relative to bacteria (de Vries *et al.*, 2018), which decreases community resistance to drought and increases vulnerability to abrupt transitions (Bardgett and Caruso, 2020).

In this thesis, a site level drought affected the extreme event proxy experiment, and after the site was expected to be in recovery, soil organic carbon measurements were the lowest of the experiment, indicating that soil moisture is a strong controller of soil carbon sequestration. It seems ironic that an experiment set up to simulate the effects of an extreme event (plant carbon cut-off), would have its control treatment damaged by a site level drought. However, this highlights how the investigation of single experimental manipulation, to help predict future ecosystem response, can be less valuable than

experiments investigating multiple interacting factors. The vast majority of studies that have aimed to increase our understanding of climate change have, understandably, focussed on the effect of a single predictor, such as elevated $CO_2$ (Hungate *et al.*, 1997; Niklaus *et al.*, 2001; Neher *et al.*, 2004; Pendall, Mosier and Morgan, 2004; Reich and Hobbie, 2013; Lipson *et al.*, 2014; Qiao *et al.*, 2014; Feng *et al.*, 2015), warming (Pelini *et al.*, 2011; Crowther *et al.*, 2016; Chen, Elsgaard, *et al.*, 2020), or drought (Toberman *et al.*, 2008; Deng *et al.*, 2021). Although, more recently there is a trend to include a combination of effects, like the combined effect of plant diversity and $eCO_2$ (Grüter, Schmid and Brandl, 2006); warming and soil moisture (Dan *et al.*, 2016); $eCO_2$ warming, and drought (Arndal *et al.*, 2018); and $eCO_2$, phosphorus and nitrogen addition (Keane *et al.*, 2020). However, the combination of multiple trend induced climate change effects; such as warming and $eCO_2$, with multiple extreme event driven effects; such as drought, fire, and flooding is rare.

In science, the simplest experiments often yield the most insightful response. However, here I argue that the more complicated experiment will be more insightful of future change experiments will be. It is a hard ask for researchers, who have set up long-term trend experiments, to expose their experiments to extreme event simulations. However, when long-term experiments have been exposed to extreme events naturally, the discoveries have been rather remarkable. For instance, the long-term grassland plant biodiversity experiment in Germany, The Jena Experiment (Roscher *et al.*, 2004), was hit by a natural flooding event in 2013. From conversations with some of the researchers that worked on this experiment, they were concerned that the experiment would be destroyed when the flooding hit. However, the natural occurrence of flooding demonstrated that the plots with the greatest species richness had plants that were less negatively affected by the flooding (plant height, specific leaf area (SLA), root aerenchyma, starch content) (Wright *et al.*, 2017). However, the microbial biomass was more severely reduced in high, but not low, plant functional group richness; indicating, that in the short-term high plant diversity plots had reduced stability of soil processes (Macé *et al.*, 2016). These interesting discoveries would not have been uncovered if it was not for the flooding. Furthermore, the findings from the flooding event provide valuable insight into how biodiversity drivers (such as

nitrogen deposition reducing plant diversity) may interact with climate change (increased frequency and intensity of flooding) in the future.

In the FACE chapter in this thesis, an unusual response of $eCO_2$ reducing microbial alpha diversity was observed. This contradicted reports in the literature of increased microbial alpha diversity under $eCO_2$ (Lipson *et al.*, 2014) or no change (Lipson *et al.*, 2006; Austin *et al.*, 2009; De Graaff *et al.*, 2010; Hayden *et al.*, 2012; Naylor *et al.*, 2020). However, the inclusion of two phosphorus limited grasslands with contrasting soil pH in this thesis's work, and the inclusion of a phosphorus treatment, eluded to the fact that soil pH and phosphorus limitation may have been controlling this response. There was evidence that, in the limestone grassland, which had a more neutral pH, the alleviation of phosphorus limitation increased in alpha diversity. Therefore, the complicated nature of the experiment (two grasslands, three nutrient treatments, two $CO_2$ treatments and four time points) allowed a greater discernment of the drivers of contrasting results found in the different treatment combinations. Complicated experiments, with factorial designs, may offer greater insight into ecosystem response to future conditions and help to disentangle the drivers of their response.

## 5.1. Seasonal studies are important

Time series generated through manipulative experiments are essential to quantify the resistance and resilience of soil microbial communities and their functions. In this thesis, I took a time-series approach spanning seasons to investigating the effects of trend climate change and extreme events. This approach provided insight into the FACE chapter, where there was evidence that $eCO_2$ extended the summer microbial communities into autumn (acidic grassland) when it would usually undergo change at this time. In addition, the approached showed that more seasonally dynamic communities may be more sensitive to $eCO_2$, as they are shaped to a greater extend by rhizosphere carbon. In the extreme event (EE) proxy chapter, a time-series approach uncovered that, contrary to what was hypothesised, soil dissolved organic carbon and the microbial biomass carbon increased under damaged plots compared to controls at 238 days after clipping. Whereas, a reduction compared to

controls was expected due to hypothesised reduced carbon availability, it was suggested that this indicated a possible shift to investment into microbial growth, over resource acquisition, potentially driven by greater microbial resource availability. If in these chapters, sampling was only conducted in the growing season, these microbial processes and responses to treatments would have been missed.

The emphasis of environmental sciences on the growing season may be rooted in the historical connection between plant research and agriculture, where the primary focus was on optimizing crop growth. Traditional forms of plant research, including botany, likely concentrated on periods when plants were visibly active, such as during the summer. A recent review on the future of soil microbiome research, indicated that although great advancements in geographical sampling have provided valuable insights into the spatial variability of soil organisms, a major limitation is that all major large-scale biodiversity investigations have been assessed at one point in time (Geisen, 2021). Whereas, local studies with multiple time points have provided valuable insights into how seasonal factors shape the soil microbiome (Vervoort *et al.*, 2012). The authors of the review suggested that just as environmental conditions (moisture, temperature, the availability of different carbon and nutrients) shape the soil microbiome across geographical regions, they also shape the soil microbiome at temporal scales (Geisen, 2021). It is well established, that soil microbial communities are highly dynamic and respond to changes in resource availability (Fontaine *et al.*, 2007). So, if the future aim is to asses soil microbial functioning from microbial communities, it is important to recognise that they are under constant temporal change and secondly, to incorporate a temporal strategy in microbial surveys (Geisen, 2021).

This is especially important considering the context of climate change. Soil microbial communities are able to influence the uptake and storage of greenhouse gasses at the ecosystem level (Bardgett, Freeman and Ostle, 2008) and soil microbial activity does not cease to exist outside of the growing season. In a grassland $eCO_2$ experiment, the authors took a seasonal time-series approach and discovered that $eCO_2$ induced a priming effect that increased the decomposition of SOM increasing the activity of bacterial nitrite reductase (Keidel, 2019). This was discovered by monitoring $N_2O$ and

$CO_2$ soil emissions in autumn and winter where an increase in both was found under $eCO_2$. The authors suggested that this showed a positive feedback loop, where $eCO_2$ stimulated greater loss of $CO_2$ and $N_2O$ from the grassland, which could further accelerate global warming (Keidel, 2019). Without the inclusion of autumn and winter in this study, this priming effect would not have been discovered, and the important consequence for a potential climate change inducing feedback would have been missed.

Important soil microbial processes occur outside of the growing season in other ecosystems too. In the Arctic, the belowground growing season is twice as long as the above ground season (Blume-Werry *et al.*, 2016) and microbial processes which occur in winter feedback to affect plant growth in summer. A shift from immobilisation in summer to nitrogen mineralisation occurs in winter (Giblin *et al.*, 1991; Schimel, Bilbrough and Welker, 2004). Soil enzyme concentrations are highest over winter, particularly before spring thaw (Wallenstein, Mcmahon and Schimel, 2009), as a larger pool of nitrogen is available (Schimel, Bilbrough and Welker, 2004) for microorganisms to allocate to enzyme production. In the Arctic, soil respiration continues over winter, despite cold weather constraints (Zimov *et al.*, 1996; Oechel, Vourlitis and Hastings, 1997; Fahnestock, Jones and Welker, 1999) and winter activity accounts for ~20% of annual $CO_2$ emissions (Hobbie, Schimel, Trumbore and Randerson, 2000), representing a substantial proportion of emitted carbon. Often extreme climatic events are in winter, and the greatest climate change is occurring over winter in the Arctic (AMAP, 2017). Therefore, to focus research solely on the summer season in the context of climate change makes no sense. Even in ecosystems where winter temperatures reach – 20 °C and there is no sunlight, important ecosystem processes, driven by microbial activity take place and feedback into the ability of ecosystems in summer to sequester carbon.

Here I argue that a time-series approach, spanning seasons, should be the dominant approach taken in non-seasonal ecosystems when investigating ecosystem response to climate change. There is evidence that climate change is altering the timing of seasons, with a the onset of spring becoming earlier and a delay in the onset of autumn, shrinking winter in the middle (Sparks and Menzel, 2002), as found in

this thesis with $eCO_2$ changing the seasonal pattern in microbial communities. A time-series approach that spans seasons also helps to identify how plants damage from extreme events can be variable, as favourable summer conditions have been found to buffer the worst effects of extreme event damage that occurred in winter (Treharne *et al.*, 2019). Moreover, ecosystems exhibit seasonal feedback loops that contribute to overall ecosystem productivity. For example, plants can alter the seasonal variations in soil nutrient availability (Kaiser *et al.*, 2011). To comprehensively grasp the impacts of climate change on ecosystem processes, it is crucial to understand how these feedback mechanisms operate both in the absence and presence of climate change.

Therefore, it is recommended that global change research, where feasible, adopts a time-series sampling approach to investigate ecosystem functioning throughout all seasons, not solely the growing season. Recognizing the financial and logistical challenges, particularly for international fieldwork, two solutions are proposed. Firstly, foster meaningful collaboration with local scientists, students, or technicians who can contribute to sample collection, preparation, and shipping, and receive fair credit for their contributions. Local knowledge, often undervalued, can offer valuable insights into ecosystems. This recommendation additionally aims to steer away from the practice of 'helicopter science,' (Adame, 2021) offering a comprehensive solution for how well-funded Western universities conduct fieldwork abroad. Secondly, instead of a single sampling (on the same day) during a short fieldwork trip, consider a time series with samples taken, for example, every other day over a week, striking a balance between geographical and temporal coverage. This approach can provide a more comprehensive understanding of dynamic communities in the face of ongoing changes.

## 5.3. Concluding statement

In chapter 2 (FACE chapter), this thesis revealed that $eCO_2$ tended to decrease the alpha diversity of microbial communities compared to ambient $CO_2$, with a similar trend observed for reduced alpha diversity in spring and summer across all treatment compared to autumn and winter. The suggestion

was that greater rhizodepostion, in the $eCO_2$ treatment and during spring and summer more generally, was increasing soil carbon availability and promoting the dominance of certain species. This chapter found evidence that soil biodiversity can be reduced by $eCO_2$, which could have unknown consequences for soil ecosystem functionality in a future climate. Future work in this area, specifically on this long-term experiment, could benefit from linking aboveground and belowground biodiversity and species presence/absence in each treatment to asses whether changes in plant biodiversity or other response variables (e.g plant-carbon quantity and quality, nitrogen deposition or soil chemistry) are driving the changes in soil microbial community structure. Furthermore, an analysis into the specific microbial taxa driving changes in microbial biodiversity would indicate if the suggestion of increased microbial dominance of certain taxa was the correct explanation suggested for reduced biodiversity.

In chapter 3 (soil storage chapter), this thesis demonstrated that that routine soil sample storage methods, lasting one and three months, did not preserve soil characteristics to similar levels as found in fresh soil, except for $\delta^{13}$ of organic carbon, which was preserved under storage. To further the work in this chapter, a repeated experiment with different types of soil would be beneficial to assess if trends in soil storage property change were consistent or not across soil types. This work would help researchers choose soil storage methods and durations appropriate for their work when storage is unavoidable.

In chapter 4 (EE proxy chapter), this thesis found that an extreme event simulation (the clipping aboveground plant material) reduced soil respiration over the following year, including a year later when soil ammonium availability increased. Indicating that extreme event damage that results in high plant mortality can reduce soil respiration and alter nutrient dynamics a year later. Although, changes to soil respiration and soil ammonium concentration were found, generally the ecosystem appeared resistant to dramatic changes in soil microbial processes. This indicates that extreme events, with high plant damage as the predominant impact, may not be a major concern in this environment. Moreover, a natural drought at the site level likely reduced soil organic carbon content to the lowest

measurements of the experiment, indicating that site level water availability may more strongly effect soil carbon sequestration rather than plant driven processes. Future work in this area could benefit from including soil bulk density measurements to allow the scale-up and comparison of the effects on soil organic carbon to other heathlands, as this was a major limitation of this work. In addition, repeating this experiment in different ecosystems would help in our understanding of how species specific plant processes drive soil microbial carbon cycling across environments, and aid in our understanding of the resilience of different ecosystems to extreme events to better inform land management.

Despite the diverse topics covered in these chapters, several key points emerged across multiple chapters, underscoring the importance of time-series sampling, the significant short-term variability in organic carbon from upper soil horizons, and the necessity for complex global change experiments to enhance predictions of future conditions. The primary aim of this thesis was to investigate soil microbial carbon cycling and diversity under climate change. The findings reveal the complex and unpredictable responses of soil microbial cycling to climate change. Elevated $CO_2$ levels applied to two phosphorus-limited grasslands produced contrasting results, suggesting a potential trade-off between increased plant growth and enhanced soil carbon sequestration under future climate conditions. Additionally, there was evidence that $eCO_2$ can reduce microbial biodiversity and alter the timing of belowground seasonal community changes. Furthermore, an extreme event proxy mimicking high plant mortality appeared to have no short-term effect on soil organic carbon. However, soil organic carbon in the upper horizon showed high variability over the course of a year, likely due to seasonal changes. In conclusion, this thesis highlights the critical need for further research to understand the changes in microbial carbon cycling under the influence of climate change.

# 6. References


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# S. Supplementary material

S1. Soil microbial communities biodiversity responses to elevated $CO_2$, nitrogen deposition and the alleviation of phosphorus limitation, in two phosphorus limited grasslands

*S1.1. Acidic grassland bacterial Shannon diversity time full contrasts*

$contrasts
 contrast estimate SE df t.ratio p.value
 T1 - T2 -0.0436 0.0473 90.7 -0.921 0.7939
 T1 - T3 -0.6839 0.0469 93.3 -14.579 <.0001
 T1 - T4 -0.7539 0.0464 91.7 -16.251 <.0001
 T2 - T3 -0.6404 0.0498 95.4 -12.855 <.0001
 T2 - T4 -0.7103 0.0489 93.1 -14.536 <.0001
 T3 - T4 -0.0699 0.0480 92.6 -1.458 0.4671

*S1.2. Acidic grassland bacterial Chao species richness time full contrasts*

$contrasts
 contrast estimate SE df t.ratio p.value
 T1 - T2 -64.5 85.9 91.1 -0.752 0.8758
 T1 - T3 -380.3 84.7 94.4 -4.492 0.0001
 T1 - T4 -410.8 84.0 91.5 -4.889 <.0001
 T2 - T3 -315.8 89.5 98.8 -3.530 0.0035
 T2 - T4 -346.2 88.2 94.0 -3.924 0.0009
 T3 - T4 -30.5 86.7 94.2 -0.351 0.9850

*S1.3. Limestone grassland bacterial Shannon diversity time full contrasts*

$contrasts
 contrast estimate SE df t.ratio p.value
 T1 - T2 -0.0355 0.0693 86.3 -0.512 0.9560
 T1 - T3 -0.2979 0.0706 86.0 -4.217 0.0004
 T1 - T4 -0.4411 0.0698 85.2 -6.321 <.0001
 T2 - T3 -0.2623 0.0707 85.6 -3.711 0.0020
 T2 - T4 -0.4056 0.0701 87.1 -5.783 <.0001
 T3 - T4 -0.1433 0.0715 86.0 -2.004 0.1945

*S1.4. Acidic grassland bacterial beta diversity time full contrasts*

| pairs | Df | SumsOfSqs | F.Model | R2 | p.value | p.adjusted | sig |
|---|---|---|---|---|---|---|---|
| 1 T1 vs T2 | 1 | 0.007055976 | 1.030618 | 0.01839384 | 0.341 | 1.000 | |
| 2 T1 vs T3 | 1 | 0.175520296 | 27.908688 | 0.32869061 | 0.001 | 0.006 | * |
| 3 T1 vs T4 | 1 | 0.312844927 | 47.953409 | 0.45258958 | 0.001 | 0.006 | * |
| 4 T2 vs T3 | 1 | 0.150502582 | 21.675412 | 0.29420143 | 0.001 | 0.006 | * |
| 5 T2 vs T4 | 1 | 0.271357824 | 37.750864 | 0.41598352 | 0.001 | 0.006 | * |
| 6 T3 vs T4 | 1 | 0.028401427 | 4.304440 | 0.07258209 | 0.004 | 0.024 | . |

*S1.5. Limestone grassland bacterial beta diversity time full contrasts*

| pairs | Df | SumsOfSqs | F.Model | R2 | p.value | p.adjusted | sig |
|---|---|---|---|---|---|---|---|
| 1 T1 vs T2 | 1 | 0.01151305 | 1.415907 | 0.02650722 | 0.187 | 1.000 | |
| 2 T1 vs T3 | 1 | 0.12193212 | 22.338093 | 0.30880124 | 0.001 | 0.006 | * |
| 3 T1 vs T4 | 1 | 0.19430775 | 30.781621 | 0.37638800 | 0.001 | 0.006 | * |
| 4 T2 vs T3 | 1 | 0.06573577 | 9.553528 | 0.16041918 | 0.001 | 0.006 | * |
| 5 T2 vs T4 | 1 | 0.11551576 | 14.988668 | 0.22714003 | 0.001 | 0.006 | * |
| 6 T3 vs T4 | 1 | 0.01303689 | 2.627199 | 0.05088789 | 0.036 | 0.216 | |

# S2. Quantification of storage effects in analysis of upland soil samples

## *S2.1. Soil sample dry weight (%)*

Table S. 1. Dry soil weights as percentage of total mass for each storage sample. ST = storage experiment, number = replicate number, FD = freeze-dried, F = frozen, R = refrigerated, AD = air-dried.

| Sample code | Rounded to two decimal place – soil dry weight content |
|---|---|
| ST2 FD1 | 100 |
| ST2 FD2 | 98.99 |
| ST2 FD3 | 100.33 |
| ST2 FD4 | 100 |
| ST2 FD5 | 99.33 |
| ST2 FD6 | 99 |
| ST2 AD1 | 96.37 |
| ST2 AD2 | 95.65 |
| ST2 AD3 | 95.35 |
| ST2 AD4 | 95.59 |
| ST2 AD5 | 95.64 |
| ST2 AD6 | 95.72 |
| ST2 R1 | 52.24 |
| ST2 R2 | 52.85 |
| ST2 R3 | 52.38 |
| ST2 R4 | 50.24 |
| ST2 R5 | 53.47 |
| ST2 R6 | 52.59 |
| ST2 F1 | 51.64 |
| ST2 F2 | 51.96 |
| ST2 F3 | 52.32 |
| ST2 F4 | 52.36 |
| ST2 F5 | 54.77 |
| ST2 F6 | 53.98 |
| ST3 FD1 | 98.4 |
| ST3 FD2 | 98.27 |
| ST3 FD3 | 98.96 |
| ST3 FD4 | 98.91 |
| ST3 FD5 | 98.93 |

| | |
|---|---|
| ST3 FD6 | 98.9 |
| ST3 ADI | 95.75 |
| ST3 AD2 | 95.68 |
| ST3 AD3 | 95.81 |
| ST3 AD4 | 95.54 |
| ST3 AD5 | 95.76 |
| ST3 AD6 | 95.87 |
| ST3 F1 | 52.47 |
| ST3 F2 | 51.93 |
| ST3 F3 | 53.97 |
| ST3 F4 | 52.74 |
| ST3 F5 | 53.85 |
| ST3 F6 | 53.23 |
| ST3 R1 | 51.99 |
| ST3 R2 | 52.61 |
| ST3 R3 | 51.43 |
| ST3 R4 | 52.2 |
| ST3 R5 | 51.24 |
| ST3 R6 | 52.75 |

S3. Consequences of plant-to-soil carbon cut-off following an extreme climatic event simulation

*S3.1. Photographs of discarded drought effected matched paired experimental plots*

Figure S. 1. Photographs of discarded drought plots

*S3.2. Soil organic carbon significantly lower overall compared to day 1 in seasonal analysis*

Fixed effects:

| | Estimate | Std. Error | df | t value | Pr(>\|t\|) | |
|---|---|---|---|---|---|---|
| (Intercept) | 7.7563 | 0.6213 | 64.8801 | 12.483 | < 2e-16 | *** |
| treatmentB | -1.1463 | 0.8036 | 77.0000 | -1.426 | 0.157779 | |
| time13 | -1.8825 | 0.8036 | 77.0000 | -2.343 | 0.021730 | * |
| time37 | -3.0075 | 0.8036 | 77.0000 | -3.743 | 0.000349 | *** |
| time98 | -4.5046 | 0.8036 | 77.0000 | -5.606 | 3.10e-07 | *** |
| time173 | -4.0325 | 0.8036 | 77.0000 | -5.018 | 3.27e-06 | *** |
| time238 | -4.1688 | 0.8036 | 77.0000 | -5.188 | 1.68e-06 | *** |

*S3.3. Soil temperature significantly decreased incrementally with time tukey*

$`pairwise differences of time`

| 1 | estimate | SE | df | t.ratio | p.value |
|---|---|---|---|---|---|
| time1 - time13 | -0.169 | 0.245 | 77 | -0.689 | 0.9827 |
| time1 - time37 | -3.062 | 0.245 | 77 | -12.495 | <.0001 |
| time1 - time98 | 1.337 | 0.245 | 77 | 5.457 | <.0001 |
| time1 - time173 | 8.363 | 0.245 | 77 | 34.120 | <.0001 |
| time1 - time238 | 9.800 | 0.245 | 77 | 39.985 | <.0001 |
| time13 - time37 | -2.894 | 0.245 | 77 | -11.807 | <.0001 |
| time13 - time98 | 1.506 | 0.245 | 77 | 6.146 | <.0001 |

time13 - time173 8.531 0.245 77 34.808 <.0001

time13 - time238 9.969 0.245 77 40.674 <.0001

time37 - time98 4.400 0.245 77 17.952 <.0001

time37 - time173 11.425 0.245 77 46.615 <.0001

time37 - time238 12.863 0.245 77 52.480 <.0001

time98 - time173 7.025 0.245 77 28.663 <.0001

time98 - time238 8.463 0.245 77 34.528 <.0001

time173 - time238 1.438 0.245 77 5.865 <.0001

*S3.4. Enzymes expressed per unit of microbial biomass non-significant effects of treatment (clipping)*

**BG**

bg.mod = lmer(BGmcb~treatment+ (1|plot), data = intensive)

boundary (singular) fit: see help('isSingular')

> anova(bg.mod)

Type III Analysis of Variance Table with Satterthwaite's method

Sum Sq Mean Sq NumDF DenDF F value Pr(>F)

treatment 0.0082762 0.0041381 2 12 0.144 0.8673

**PHO**

PHO.mod = lmer(PHOmcb~treatment+ (1|plot), data = intensive)

anova(PHO.mod)

Type III Analysis of Variance Table with Satterthwaite's method

Sum Sq  Mean Sq NumDF  DenDF F value Pr(>F)

treatment 0.021545 0.010773  2 4.8398  0.2998 0.7538

**NAG**

NAG.mod = lmer(NAGmcb~treatment+ (1|plot), data = intensive)

> anova(NAG.mod)

Type III Analysis of Variance Table with Satterthwaite's method

Sum Sq Mean Sq NumDF DenDF F value Pr(>F)

treatment 0.00266 0.00133  2 6.322  1.3391 0.3274

**LAP**

LAP.mod = lmer(LAPmcb~treatment+ (1|plot), data = intensive)

> anova(LAP.mod)

Type III Analysis of Variance Table with Satterthwaite's method

Sum Sq  Mean Sq NumDF  DenDF F value Pr(>F)

treatment 0.0015553 0.00077767  2 5.6513  1.2801 0.3479

**POX**

POX.mod = lmer(POXmcb~treatment+ (1|plot), data = intensive)

> anova(POX.mod)

Type III Analysis of Variance Table with Satterthwaite's method

Sum Sq  Mean Sq NumDF  DenDF F value Pr(>F)

treatment 2.972e-08 1.486e-08  2 5.3481  2.1453  0.207

**THE UNIVERSITY OF SHEFFIELD: COVID-19 IMPACT FORM**

This form is not compulsory but is intended to be a helpful note to examiners. You may submit this form alongside (not in) your thesis. The purpose of the form is to detail how your thesis has been impacted by the Covid-19 disruption.

| **STUDENT'S DETAILS** | |
|---|---|
| Name:<br><br>Jasmine Roha Wakefield | Registration Number:<br><br>190182836 |
| Department:<br><br>School of Bioscience | Faculty:<br><br>Faculty of Science |
| **Please provide a brief summary of the work you were planning to complete before covid-19 restrictions were implemented (max 300 words)** | |
| I was planning on a PhD investigating the effects of extreme event damage in arctic ecosystems. This would have included 2/3 arctic field seasons in Svalbard and Norway/Sweden. My research topic would have solely focussed on these geographic areas and the investigation of how microbial carbon cycling and ecosystem function is changed in the damaged landscapes. | |
| **Please provide a summary of plans for specific studies you were intending to conduct in the future (see note 1) (max 300 words)** | |
| | |

Due to multiple lockdowns and covid restrictions, I was unable to attend fieldwork abroad. To start with we thought this may only impact one year of fieldwork so I conducted some UK based research to make use of the extra time (Chapter 3 and 4), as field seasons were conducted in summer. I also conducted a full literature review of the existing arctic research and wrote a 40 page theoretical framework on microbial carbon cycling in arctic extreme event damaged ecosystems during this time (not included in the thesis).

After the second failed arctic fieldwork season, it was decided I should change the focus of my PhD as it was unlikely I would have time to conduct research abroad. I made use of a long-term $CO_2$ FACE experiment my supervisor had set-up locally to the university. I used techniques, such as amplicon sequencing, that would have been used in the arctic research.

My thesis therefore has three varying topics as a result but broadly fits together by assessing how climate change is affecting the microbial cycling of carbon in upland and boreal ecosystems.

**Where relevant, please provide details of changes to your personal circumstances (see note 2) (max 150 words)**

**If your thesis includes Covid-related research, please include a brief statement of how it relates to your overall research aims (see note 3) (max 150 words)**

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| **Please provide details of any previous funded extension, tuition-free extension or non-medical Covid-related leave of absence approved since March 2020** |
| I was extremely lucky to receive a 6 month funding extension as my PhD was strongly impacted by Covid. This gave me extra funding to continue research into the last 6 month of my PhD. |

**Note 1.** This information could form a basis for discussion at the viva examination and give Examiners additional means to assess the volume and standard of the work completed. Detailed information could be included in a future work section in the thesis itself.

**Note 2.** For example, ill health or additional caring responsibilities. additional difficulty related to an underlying disability, returned to clinical service, or has worked in a voluntary capacity for Covid-related research. These data could contextualise the judgement made by Examiners as to the most appropriate outcome.

**Note 3.** If in doubt, consult with your supervisor and Departmental PGR Lead.