Biologically-driven rock weathering: roles of bacterial carbonic anhydrase in arable soils, and mycorrhiza and roots in a floristically diverse grassland.

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i. Acknowledgments

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ii. Specific scientific contributions

Dimitar Epihov – provided the *Burkholderia thailandensis* knockout library (for Chapter 2) and collected and provided raw metagenomic data from the Energy Farm Field site for the years 2019 and 2020 (for Chapter 3). Along with contributing to the conceptualisation and editing of all chapters.

Jonathan Leake – provided the used mycorrhizal exclusion columns for modification and preparation (Chapter 4). Along with contributing to the conceptualisation and editing of all chapters.

David Beerling – Aided in the conceptualisation of Chapters 2 and 5. Along with the editing all chapters.

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Sarah Thorne – was paid to perform pXRF on basalt rock grains (Chapter 4) and powdered hay samples (Chapter 5).

David Martin – Provided laboratory technical support from 2022-2024.

Xavier Dupla – Collaborated on some of the soil trace metal analysis in Chapter 5 and aided in formulating the statistical analysis.

iii. Abbreviations

Terms

CDR – Carbon dioxide removal.

ERW – Enhanced rock weathering.

LC3M – Leverhulme Centre for Climate Change Mitigation

CO_2 – Carbon dioxide

HCO_3^- – Bicarbonate

$CaCO_3$ – Calcium Carbonate

$GtCO_2$ – Gigatonnes carbon dioxide

$MtCO_2$ – Megatonnes carbon dioxide

DEC – Dissolved elemental concentration

Ma – Million years

ha – Hectare

Mha – Million hectare

UK – United Kingdom

US – United States of America

N/A – Not Available

K-EDDHA – Potassium-ethylenediamine-*N*,*N*′-bis-2-hydroxyphenylacetic acid

Techniques

pXRF – portable X-ray fluorescence.

ICP-MS - Inductively coupled plasma mass spectrometry.

iv. Declaration

I, Derek Stuart Bell, confirm that the contents of this thesis are my own work. I acknowledge the universities rules on the Use of Unfair Means (Unfair means, cheating and plagiarism | Support for new students | The University of Sheffield). None of the presented works have been used for any other academic accreditation or industrial purposes, other than the presentation of some of the data at annual LC3M conferences, Canadian Society for Soil Science 2024 conference and the European Geosciences Union 2024 conference.

The contents of Chapter 5 have been submitted for publication at the journal Agriculture Ecosystems and Environment and is currently in pre-print.

Plans to submit the results of Chapters 2, 3, and 4 are in progress.

Biologically-driven rock weathering: roles of bacterial carbonic anhydrase in arable soils, and mycorrhiza and roots in a floristically diverse grassland.

v. Summary

Atmospheric carbon dioxide (CO_2) removal strategies are needed to limit global temperature rise to 1.5 °C above pre-industrial levels. Enhanced rock weathering (ERW), which uses silicate rock dust spread onto agricultural soils to accelerate the inorganic carbon cycle, can theoretically absorb gigatonnes of CO_2 globally. Rates of weathering are affected by soil biology, but the importance of the enzyme carbonic anhydrase (CA), bacteria, fungi, and plants, remain unresolved, and ERW potential in grasslands needs to be investigated.

To address these issues, this thesis investigated the potential to accelerate ERW rates by CA and soil organisms. CA's role in bacterial weathering was studied by growing *Burkholderia thailandensis* strains with different CA genes inactivated, in a basalt supplemented liquid medium. One of these *B. thailandensis* mutants had reduced weathering under certain conditions. However, metagenomic analysis of CA abundance in a long-term ERW trial, on North American maize-soybean cropland, found no evidence of selective pressure for CA genes in basalt-treated soils in which ERW was occurring, suggesting it does not play a major role in mineral dissolution *in situ*.

The first grassland field trial of ERW was conducted on a floristically diverse mildly acidic upland UK site. Basalt amendment increased soil pH and exchangeable cations, indicative of CO_2 removal, and raised phosphorus, magnesium, and sodium concentrations in hay, without adversely impacting hay yields or floristic diversity. By combining a novel magnetic rock extraction method with mesh-walled biological simplification columns incubated over the growing season in this grassland, mesh permitting non-saprotrophic filamentous fungal colonisation such as by mycorrhizas appeared to enhance mineral weathering rates *in situ,* increasing soil concentrations of calcium, strontium, titanium, and iron, whereas plant roots appeared to reduce weathering.

The thesis highlights the complexity of biota in weathering in soils, demonstrating how their contributions can be identified at various scales, and explores the potential of floristically diverse hay meadows for ERW using basalt additions.

Chapter 1: Introduction

1.1 Climate change and the need for carbon dioxide removal

Environmental damage due to anthropogenic climate change is viewed as one of the greatest threats to humanity. As part of the fight to combat this change and stabilize the climate, internationally agreed targets of limiting temperature increase to 1.5 °C above pre-industrial levels have been established (UNEP, 2018). Atmospheric carbon dioxide (CO_2) is considered one of the most important greenhouse gases as it is one of the primary gasses emitted during fossil fuel burning and land use change (IPCC 2014). Alone it contributed to approximately 64% (38 ± 3.0 gigatonnes (Gt) CO_2) of the total 59 ± 6.6 Gt CO_2e of greenhouse gas emissions in 2019 alone (IPCC, 2023).

With the rise in global atmospheric CO_2 concentrations, an increase of 1.1 °C, averaged from 2011-2020, was seen in global surface temperatures relative to the pre-industrial averages (IPCC, 2023). Increases in global population, demand for energy, and the limits of clean fuel technologies necessitate the need for carbon dioxide removal (CDR) strategies to offset continued emissions and prevent further warming (The Royal Society, 2018; UNEP, 2018; Smith et al., 2024). Continued global warming, even to IPCC's more ambitious goal of limiting this to 1.5 °C, will still likely result in changes in global precipitation, rising sea levels and ocean acidification, more frequent extreme weather events, and large scale ecosystem loss or damage among many other estimated detrimental effects (Hoegh-Guldberg *et al.*, 2018).

Estimates of the cumulative CDR needed by the end of the century to meet temperature limitation targets range greatly from 260 Gt of CO_2 (Smith et al., 2024) to 1000 Gt of CO_2 (Pagano et al., 2020). Currently under most climate mitigation scenarios, produced by the IPCC, all countries have a "CDR gap", though importantly this shortfall in CDR could be addressed

through greater emission reductions, along with the scaling up of novel and conventional CDR techniques (Smith et al., 2024). The conventional CDR techniques, which received almost all the national funds for deployment of CDR globally, include natural and managed afforestation and reforestation (Smith et al., 2024). In 2023 natural and managed afforestation/reforestation was expected to account for CDRs of 1.86 and 2.01 Gt CO_2, respectively; novel methods such as biochar, bioenergy carbon capture and storage (BECCS), direct air carbon capture and storage (DACCS), and enhanced rock weathering (ERW) were estimated as delivering 0.790, 0.510, 0.004, and 0.030 Mt CO_2 removals respectively, in 2023 (Pongratz et al., 2024). This highlights the differences in potential effectiveness of deployment of these technologies globally. However, progress in deployment of CDR is lagging, even for the most basic of approaches such as tree planting, for example the UK governments stated goal in 2019 of creating 30,000 hectares of new woodland per year by 2025 has not been met, with an area the size of Birmingham not yet being planted (Gabbatiss and Viisainen, 2024; UK-GOV, 2023). By 2050, those unplanted trees would have removed some 8.5 Mt CO_2 from the atmosphere, which is ~2% of the UK's annual emissions in 2023, and the resulting shortfall will need to be met by additional CDR, if we are to achieve the net zero target by 2050.

1.2 Enhanced rock weathering as a CDR strategy

Enhanced rock weathering (ERW), also known as enhanced terrestrial weathering or enhanced weathering (EW), is an approach in which crushed silicate rocks with a high potential for weathering are applied to typically agricultural soils (Schuiling and Krijgsman, 2006; Moosdorf et al., 2011; Hartmann et al., 2013; Taylor et al., 2016; Beerling et al., 2024). Amongst the most abundant and suitable rock types are those rich in calcium and magnesium-bearing silicate minerals, such as olivine and pyroxenes including basic igneous rocks like basalt, dunite, and pyroxenite that have high rates of weathering (Renforth, 2012; Hartmann et al., 2013; Lewis et al.,

2021). This technique is based on accelerating the natural processes of the inorganic carbon cycle (Figures 1 and 2) which is one of the primary regulators of the global climate (Hartmann et al., 2013).

CO_2 Hydration:	$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$
Wollastonite dissolution:	$CaSiO_3 + 2CO_2 + 3H_2O \rightleftharpoons Ca^{2+} + 2HCO_3^- + H_4SiO_4$
Calcium Carbonate Precipitation:	$Ca^{2+} + 2HCO_3^- \rightleftharpoons CaCO_3 + H_2O + CO_2$

Figure 1. Carbonic acid formation and wollastonite weathering reaction.

The chemical process of CO_2 absorption via weathering, described above in Figure 1, is dependent on the concentration of CO_2 in water. When CO_2 dissolves in water it forms the weak acid carbonic acid. The formation of carbonic acid is favourable in soils (Amundson and Davidson, 1990) as CO_2 concentrations greatly exceed the 380 ppm concentrations found in the low troposphere (Satish et al., 2012). In one instance Yonemura et al. (2009) found CO_2 concentrations of greater than 25,000 ppm in some arable fields after tillage. In soils 2 moles of carbonic acid can then weather 1 mole of calcium or magnesium silicate, where this is present in soil minerals, producing 2 moles of bicarbonate (HCO_3^-) along with releasing the cations from the rock matrix. This initial reaction in soils results in the drawdown of 2 moles of atmospheric CO_2. Once in the HCO_3^- form the carbon is considered unstable as it could degas back to the atmosphere if it enters a region of high acidity (S. Zhang et al., 2022), or it could precipitate as carbonate either in the soils or water resulting in 50% of the absorbed carbon being degassed (Harrington et al., 2023). However, if the HCO_3^- makes it to the ocean and is not converted to carbonate it could effectively sequester the two moles of CO_2 as alkalinity for ~0.1 – 1.0 million years (Renforth and Henderson, 2017).

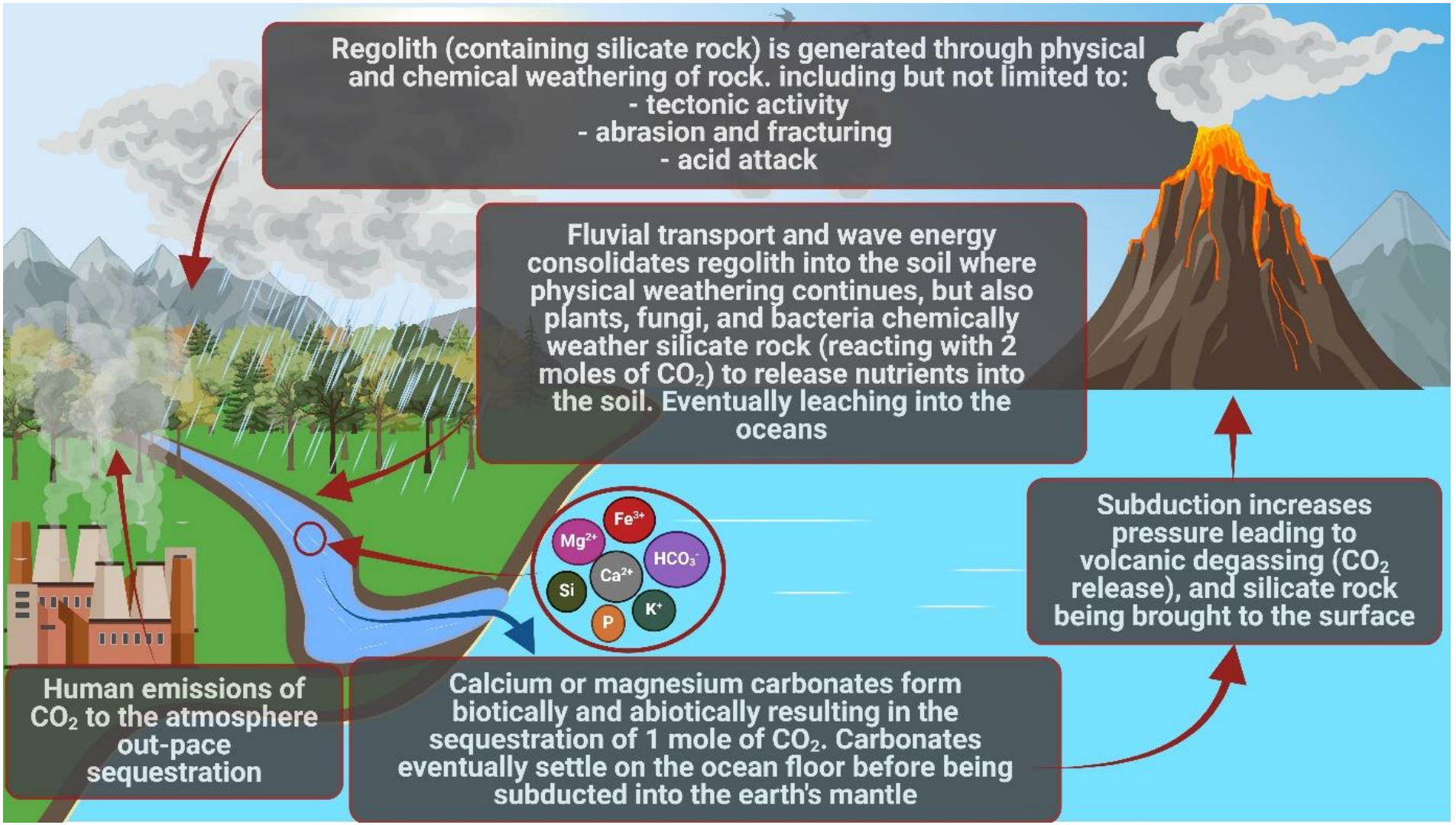

Figure 2. The carbonate-silicate geochemical cycle also known as the inorganic carbon cycle. Based on Berner et al. (1983) and Walker et al. (1981). Created in biorender.com.

Geochemical modelling has highlighted the potential for ERW to contribute to CDR, with estimates assuming rapid deployment of rock dust across global croplands to be able to reach 0.5 – 2.0 Gt CO_2 sequestered annually by the year 2050 (Beerling et al., 2020). United Kingdom (UK) geochemical models predict that if rapidly deployed to UK croplands, rock dust weathering could sequester 6 – 30 Mt CO_2 annually by the year 2050 (Kantzas et al., 2022). Although Harrington et al. (2023) estimated that carbonate precipitation in rivers could reduce this potential CDR by 16 – 27%. ERW could deliver ecosystem benefits as well, since historic acid deposition in UK waters and soils has caused significant damage to both terrestrial and aquatic ecosystems (Monteith and Evans, 2005; Edmunds and Kinniburgh, 1986). Any net alkalinity increases in UK soils or waters resulting from ERW may act to remediate impacts of acid deposition on streams, rivers, and lakes. ERW may also aid in reducing ocean acidification, models have estimated that co-deployment of carbon capture and storage with ERW could reduce ocean acidification by a third and protect existing coral reefs through increasing aragonite concentrations in waters (Vakilifard et al., 2021).

1.3 Basalt for ERW and potential co-benefits and risks

The potential co-benefits of ERW in agriculture was reviewed by Abdalqadir et al. (2024), who found research has covered a wide range of different rock substrates with each carrying different risks, co-benefits, and CDR potentials. Basalt, although less reactive than other mafic rocks such as phonolite or trachy-andesite (Van Der Bauwhede et al., 2024) and ultramafic rocks such as dunite and harzburgite (Taylor et al., 2016), has been recommended as a mineral substrate for ERW due to its low concentrations of the potentially toxic trace metals nickel and chromium and its high abundance globally (Beerling et al., 2018, 2020; Kantola et al., 2017). Globally, naturally occurring basaltic weathering reactions are likely responsible for sequestering approximately 179 Mt of CO_2 annually (Dessert

et al., 2003) as part of the inorganic carbon cycle; however, this only amounts to 0.47% of the ~38 Gt of CO_2 emitted in 2019 alone (IPCC, 2023). By increasing the total surface area and by adding to soils where concentrations of reactive CO_2 are high, ground basalt rock utilized for ERW could substantially augment this natural rate of weathering and increase CO_2 removal from the atmosphere.

Basaltic rock is especially suitable for ERW as it also contains many plant-essential macro- and micro-nutrient elements (P, K, Ca, Mg, Fe, Si, Cu, Ni, and Zn) and its alkalinity can act as a liming agent (Kantola et al., 2017; Beerling et al., 2018; Kelland et al., 2020; Lewis et al., 2021). However, concerns have been expressed regarding possible excessive bioaccumulation of the trace metals nickel and copper in soils with repeated basalt applications (Dupla et al., 2023). Basalts can vary considerably in their mineralogy and trace metal content, so careful selection of sources used for additions to agricultural land is potentially important to minimize risks. To date, short- (1-2 years) and long-term (>3 years) field and lab studies have not substantiated the concerns about toxic elements entering the food chain, but they have demonstrated yield, and sometimes nutritional quality enhancement in major crops. Greater than 10% yield increases in basalt amended fields were seen in trials for maize (*Zea mays)* (Beerling et al., 2024; Guo et al., 2023), soybeans (*Glycine max*) (Beerling et al., 2024; Guo et al., 2023), and wheat (*Triticum aestivum*) (Guo et al., 2023), while increases in cacao tree (*Theobroma cacao)* height and girth were also seen (Shamshuddin et al., 2011). Pot experiments found growth or yield increases in potato (*Solanum tuberosum)* (Vienne et al., 2022), and millet (*Sorghum bicolor)* (Kelland et al., 2020). When the hybrid grass *Lolium perenne x Festuca* 'Festulolium' was grown in mesocosms, using a 30 day wet then dry precipitation regime over 120 days, basalt increased shoot biomass by 14% (Reynaert et al., 2023). In addition, mesocosm experiments with basalt addition to soil all showed significant increases in soil pH (Gillman et

al., 2002; Anda et al., 2015) or leachate alkalinity (Buckingham and Henderson, 2024). Similar trends of increasing soil pH and/or leachate alkalinity were seen in all the aforementioned mesocosm and field trials, with the exception of Reynaert et al. (2023) where it was not reported, highlighting basalts potential as lime replacement (Beerling et al., 2018).

In addition to yield enhancement, some trials have found significant increases in the human nutritional value of grain crops. For instance, Beerling et al. (2023) reported after 4 years growing a *Z. mays*/*G. max* rotation with 50 tonne ha^{-1} y^{-1} basalt amendments, significant increases in the plant grain concentrations of potassium, magnesium, manganese, phosphorus, and zinc. At the same time, there were no significant increases in the concentration of toxic trace metals of concern in both plant species, which is especially relevant considering this is the longest running field trial of basalt ERW with these crops. Similarly, *S. bicolor* grain concentrations of silicon, calcium, strontium, and potassium all significantly increased with basalt amendment (Kelland et al., 2020). A final potential co-benefit to basalt application is the reduction of nitrous oxide (N_2O) emissions (Blanc-Betes et al., 2021). When maize was grown in mesocosms with basalt there were significant cumulative reductions in N_2O of >28% in two runs spanning 24-29 days (Chiaravalloti et al., 2023).

Interestingly, there has been little research attempting to characterise ERW's potential in perennial crops or grasslands. The only reported field trial of ERW in a grassland was with the bioenergy grass *Miscanthus*. This trial, performed in the US corn belt, reported significantly greater carbon removal potentials with basalt application to *Miscanthus* relative to basalt application to a maize and soy rotation (Kantola et al., 2023). Furthermore, in a separate *in silico* assessment they estimated that basalt amendment to *Miscanthus* could reduce N_2O emissions by 9% (Blanc-Betes et al., 2021). This research highlights the potential of perennial vegetation in ERW and is

especially relevant when considering that there is >2 fold more grasslands (9.7 Mha) in the UK relative to croplands (DEFRA, 2023). The perennial nature of grasslands could have a greater ERW derived CDR than croplands through their more substantial root biomass relative to other crops (DuPont et al., 2014) and also, depending on fertiliser usage (Bradley et al., 2006), they can have a highly diverse and abundant mycorrhizal network within the soil (Ma et al., 2023). Grassland soils are also rich in organic matter compared to arable soils (Bradley et al., 2005), giving greater cation-exchange capacity than most mineral soils, with colloidal organic matter having a high pH dependent charge (Illés and Tombácz, 2006). Calcium-rich grassland soils are often associated with high biodiversity relative to acidic grasslands, and increasing grassland soil pH could help to maintain or promote biodiversity (Crawley et al., 2005). Importantly though as no research has been performed on ERW in grasslands there is no understanding of how forage yields and nutrition, along with floristic diversity may be affected. As more UK sustainable farming incentives are made available for managing and creating floristically diverse grasslands (DEFRA, 2024a) future ERW research should be assessed with that in mind.

1.4 Biological weathering mechanisms

Many variables can affect ERW outcomes, including crop type, weather, soil composition, hydrology, seasonality, mineral type, particle size distribution, and soil biology (Vandeginste et al., 2024). These factors collectively contribute to the varied mineral weathering rates and responses to rock additions reported in the literature. As argued by Vicca et al. (2022) the different biological components of soils, including: fauna, plants, fungi, and bacteria, that can directly contribute to rock weathering, could be one of the most important variables to consider for ERW effectiveness. Members of these different biological groups can have multiple distinct mechanisms that can affect rates of rock dissolution.

Research into the potential role of soil fauna in rock weathering has found that earth worm casts increase the availability of some nutrients, including phosphorus, relative to the surrounding bulk soil indicating organic matter mineralization or mineral weathering (Ros et al., 2017). Further research has shown that when a silica solubilising bacteria, isolated from an earthworms gut microbiome, was applied to soils it significantly increased soluble silicon in the soil and resulted in accumulation of silicon in *Z. mays* plants (Hu et al., 2018). In addition to directly weathering the rock, earthworms could indirectly affect weathering through bioturbating rock grains into the soil and affecting water flow through macropore flow and the assembly of soil water-stable aggregates (Vicca et al., 2022; Blouin et al., 2013). Recent research has shown that basalt amendment to three geochemically distinct vineyards significantly affected earthworm populations in soils resulting in an average increase of 71% in earthworm abundance across amended plots (Dupla et al., 2024). Further research has estimated that when olivine and plagioclase are placed in ant colonies and recovered over 25 years later, eight different ant species increased rates of rock dissolution by 50 to 300 fold relative to the controls (Dorn, 2014). This indicates that other invertebrates within the soils may also play an important role in mineral dissolution.

The role of plants in facilitating rock weathering and the formation of soil from rock has been extensively researched and found to arise from multiple processes, as reviewed by Taylor *et al.* (2009), Finlay et al. (2020), and Dontsova et al. (2020), and summarised below in Table 1. Importantly though, plant functional types differ in their intensity of increasing weathering, for instance dinitrogen fixing trees, which generate additional soil acidity have twice the weathering rate relative to non-nitrogen fixing trees in the same nutrient-poor tropical soils (Epihov et al., 2021). Similarly, legume crops with nitrogen-fixing rhizobial root nodule symbionts have much higher rates of weathering than cereals, as demonstrated in a pot

experiment with wollastonite supplied to soil with *G. max* and *Z. mays* where the *G. max* on harvest had nearly 5 fold greater total soil inorganic carbon, indicating higher rates of CDR (Haque et al., 2019).

In addition to directly weathering rock most plants also form partnerships with fungi (Table 1), which in exchange for photosynthates, extend out into the soil and play an active role in the acquisition and transport of nutrients from the soil to the plant, these root-fungal symbionts are called mycorrhizas (Smith & Read, 2008). There are two major types of mycorrhiza, arbuscular mycorrhiza (AM) in which the fungi grow into root cells and form branched structures called arbuscules, and ectomycorrhiza (EM) in which the fungi form a sheath covering the absorptive fine-root surfaces thereby mediating the nutrient and water uptake by their host plants (Smith and Read, 2008). Both types of mycorrhiza have been shown to increase rates of silicate mineral dissolution and for these effects to be amplified by higher atmospheric CO_2 concentrations (Quirk *et al.*, 2014). AM could affect rock weathering in soils directly or indirectly, as reviewed by Verbruggen et al. (2021). Briefly, AM in exchange for photosynthates from the plant form hyphal networks that traverse the soil and colonise rock, effectively increasing the soil volume that plants can gain nutrients from (Leake *et al.*, 2008; Smith & Read, 2008; Quirk *et al.*, 2012). The hyphae then liberates valuable elements from the rock by respiration, which can lead to the formation of carbonic acids, and releasing protons which lower the pH around the root tip as a means of absorbing nutrients (Jennings and Lysek, 1999). When basalt grains were incubated in the presence of liverworts grown with and without AM symbionts, AM partnered plants increased phosphate weathering by 5 to 7 times relative to the non-AM colonised plant, this coincided with visibly more trenching and etching on the surface of the basalt grains (Quirk et al., 2015). EM are often found to accelerate mineral weathering to a greater extent than AM (Quirk *et al.*, 2014), likely due to some of these fungi

secreting copious amounts or low molecular weight organic acids that release phosphorus from calcium bearing minerals like apatite (Smits *et al.*, 2012; Schmalenberger *et al.*, 2015). In addition EM initiates phyllosilicate, i.e. biotite, weathering by biomechanically forcing the rock distorting the lattice and then activating biochemical pathways responsible for chemical weathering, including oxidation of iron and the selective removal of potassium from the silicate (Bonneville et al., 2009). It is also hypothesized that EM's resulting chemical weathering leads to the formation of mineral tunnels that have been seen within rock crystal lattices (Koele *et al.*, 2014).

Table 1. Summary of plant weathering mechanisms from Taylor et al. (2009), Dontsova et al. (2020), and Finlay et al. (2020) with references therein.

Plant weathering mechanism:	Methods of Weathering:
Mechanical forcing	Growing roots force apart rock, which increases the total available surface area for weathering.
Acidic and complexing exudates	Organic exudates from the root can cause weathering through the formation of chelation complexes with metals ions within the rock lattice. In addition, H^+ released from roots to facilitate the uptake of cations from soil solution increase the acidity around the root while simultaneously creating a gradient to absorb alkaline cations from the surrounding soil.
Respiration	Plant root respiration and microbial respiration of plant root exudates and litter increases the concentration of CO_2 within the soil driving the formation of carbonic acid.
Evapotranspiration	The force of evapotranspiration draws water towards the root, the water takes solutes away from weathering sites increasing the total available weathering sites on the rock.
Litter decomposition	A few explanations exist with microbes breaking down the litter and organic compounds within the litter producing acidity. Also, through the leaching of cations from the litter a net acidity would be reached by the plant thereby further driving weathering.
Soil stabilization	Root growth within the soil prevents erosion and binds the rock within the soil so further weathering can take place.
Mycorrhizal symbiosis	The roots of about 80% of plant species, including most crop plants, form symbiotic partnerships with soil fungi that receive organic carbon from plant roots and in return take up and transport water and

	nutrients from the soil into plant roots. These fungi, sometimes in association with phosphate solubilizing bacteria, can dissolve silicate minerals and minerals such as apatite through acid exudates and element depletion.
N-cycle feedback (Epihov et al., 2017, 2021)	By affecting the input of fixed N and nitrate leaching, N_2-fixing plants may generate additional acidity relative to nitrate-fed plants through roots and through their effect on net primary productivity (NPP). Ammonia and nitrite oxidizing lithoautotrophic bacteria that catalyse the conversion of ammonia to nitrate in soil also generate strong acidity that can affect weathering rates.

Bacterial communities are found ubiquitously in both extreme and mild environments with community composition determined by a number of factors. Within soil, microbial community structure and biomass are dependent upon variables including most importantly: pH (Rousk *et al.*, 2009), soil depth (Hao *et al.*, 2021), nutrient (Demoling *et al.*, 2007) and water availability (Williams and Rice, 2007; Hueso et al., 2012), temperature (Pietikäinen *et al.*, 2005), host plant community (Schmid et al., 2021; Schlatter et al., 2015), and heavy metal contamination (Cui *et al.*, 2018). Bacteria can also form symbiotic relationships with plants and fungi, with research finding that when AM and rhizobia bacteria were inoculated together on legumes in a perennial grassland there was higher plant productivity and diversity, nutrient uptake, and seedling establishment (van der Heijden et al., 2016). As part of this symbiosis bacteria can also stimulate other organisms such as fungi to release acid or mineral transporting factors by providing nutrients. For instance, when the diazotrophic nodulating *Bradyrhizobium elkanni* was grown in the presence of *Xanthoparmelia mexicana*, *Botrydiplodia theobromae*, or *Syncephalastrum racemosum* with phosphorite, *B. elkanni* induced increased organic acid production in all of the fungi by making nitrogen available (Seneviratne and

Indrasena, 2006). Further research has shown when AM fungi *Rhizophagus irregularis* was grown with the phosphate solubilising bacteria *Pseudomonas alcaligenes* in soils containing phytate when combined they had a greater weathering potential then when inoculated alone (Zhang et al., 2014). Currently the key genetic drivers of bacterial derived weathering are not well characterized. Some of the potential mechanisms identified, which are also seen in plants and fungi, include: carbonic anhydrases (CA) (Xiao et al., 2015; Sun and Lian, 2019), siderophores (Kraemer, 2004; Winkelmann, 2017; Van Den Berghe et al., 2021; Epihov et al., 2024), organic acid production (Barman *et al.*, 1992), Fe(III)-reductive dissolution (Epihov, 2018; Kawa *et al.*, 2019; Epihov *et al.*, 2021), and lithoautotrophic traits including oxidation of Fe(II), reduced sulfur, and ammonia.

CA stands out as potentially important for rock weathering as it can catalyse the formation of carbonic acid from CO_2 dissolved in water. The un-catalysed formation of carbonic acid is a slow reaction which is determined by pH, temperature, and constituent concentrations (Liu and Dreybrodt, 1997). CA is a metalloenzyme and is currently organized into eight distinct classes: α, β, γ, δ, ζ, η, θ and ι, that are unique in their amino acid sequences and structure (Akocak and Supuran, 2019). Although specific evidence as to the physiological roles of CA in soil organisms are lacking, understanding its impact in chemical weathering is underway. The first evidence that CA could play a role in chemical weathering came from a study that found that the addition of bovine α-CA increased the rate of limestone dissolution in an *in vitro* system (Liu and Dreybrodt, 1997). Li et al. (2007) further demonstrated that when a known CA inhibitor, acetazolamide, was added to a bacterial or a crude enzyme mixture there was a less soluble Ca released from the column over the course of 9 hours. These results do not provide definitive evidence that CA in soils increases the rate of chemical weathering, primarily due to there not being enough literature to support that acetazolamide, a non-competitive inhibitor, only affects CA in

microbes. Xiao et al. (2015) later demonstrated that all 5 CA encoding genes within *Bacillus mucilaginosus* were significantly upregulated following six days of growth in a wollastonite amended medium; they further showed that a purified CA protein (PCA4) when incubated with wollastonite led to increased liberation of Ca. More recent research has shown that when bovine α-CA was incubated in soil mesocosms with soils ranging from acidic to alkaline pH, CA activity in the alkaline pH significantly increased the CO_2 dissolution rate, which should theoretically enhance carbonic acid-driven weathering (Sauze et al., 2018). However, there is no evidence to suggest that any naturally occurring soil CA exists in concentrations high enough to meaningfully contribute to weathering processes.

1.5 Geological precedence for biological rock weathering

The geological record provides proxy indicators of atmospheric CO_2 concentrations and temperatures that suggest biological weathering through plant and mycorrhiza evolution has played an important role in the global climate and has further provided insights into how increases to rock weathering globally could affect surface temperatures (Leake and Read 2017). During the rise of deep rooting vascular plants with mycorrhizal fungal partners in the middle to late Devonian period atmospheric CO_2 concentrations fell rapidly. This corresponded with the sequestration of organic carbon into biomass and soils, and the formation or carbonate minerals such as limestone in the oceans derived from calcium and magnesium weathered from silicate rocks on the continents by increasingly deep-rooted plants. This brought about major changes to the carbon cycle and global cooling (Berner, 1998, 2004, 2006; Beerling and Berner, 2005). The plants themselves which began as bryophyte-like organisms without roots likely found in wetlands (Greb et al., 2006; Qiu et al., 2006) arose in the mid-Ordovician approximately 470 Ma but later diversified to form more complex vascular plants with modern root systems such as *Archaeopteris*, by the mid to late Devonian period (Stein *et al.*, 2020). With

this finding it has supported the hypothesis that the evolution of trees capable of forming fungal associations played a more significant role in global weathering (Pawlik *et al.*, 2020). As the first definitive evidence of mycorrhizal symbiosis with plants appears 411.5 Ma (Remy et al., 1994) it highlights the potential co-evolution of plants with their mycorrhizal partners. Recent research has suggested that land plants, and likely their fungal partners, may have contributed to the late Devonian mass extinction event 372 Ma, as models have suggested that a flush of phosphorus derived from terrestrial mineral weathering could have created a mass eutrophication event causing widespread marine anoxia (Smart et al., 2023).

The expansion of land plants and their fungal partners may not entirely explain the global cooling seen, as there are potentially feedback mechanisms that could have prevented further cooling (Beerling and Berner, 2005). For instance, with cooling temperatures there may have been increased albedo (Le Hir et al., 2011), reduced terrestrial biomass activity, and changes to global precipitation, resulting in a reduction of carbon drawn down by plants and a reduced weathering efficiency (Berner, 2004; Beerling and Berner, 2005). Some of the global cooling will have arisen from the accumulation of recalcitrant organic matter, such as lignin (Boyce and Lee, 2017), but ocean extinction events (Qie *et al.*, 2019), and volcanic activity are also likely to have contributed (Smart et al., 2023). The evolution and expansion of legume-rich tropical forests in tropical regions worldwide in the early Cenozoic has also been hypothesized to have contribute to regimes of enhanced rock weathering by the effects of N_2-fixing trees on N availability, net primary productivity, and ultimately soil acidity (Epihov et al., 2017).

Combined, these geological events and their interactions are a reminder of the long-time scales over which such global changes occurred, raising uncertainty about whether ERW can have a meaningful impact in the short term. However, it also highlights the significant role biology played in

soil formation, mineral weathering, and subsequent global cooling and that thoughtful consideration of biology's role in ERW and its application to agricultural land management could potentially improve CDR outcomes.

1.6 Biological acceleration of ERW

Research into biologically accelerating rates of rock weathering in soils has already begun with mesocosm experiments testing the efficacy of adding weathering organisms or chemical additives to soil and rock mixtures. Generally, research has focused on using either bacteria or metabolic products of these organisms to stimulate weathering. One organism which has been a focus of research recently is the plant growth promoting bacterium *Bacillus subtilis* (Niron et al., 2024; Timmermann-Aranis et al., 2024; Liu et al., 2021). Inoculation of this bacteria into basalt containing soils, with no plants, led to an increased rate of rock weathering and therefore CDR potential (Niron et al., 2024). A commercial company has also been trialling *B. subtilis* additions to ERW field sites in the US and also claim, in a pre-print article, to have increased rates of CDR (Timmermann-Aranis et al., 2024). Another added benefit to using *B. subtilis* is that it has already been shown to increase stress resistance in certain crops (Mahapatra et al., 2022). To date the only chemical additive being investigated to increase weathering in soils is the synthetic chelator potassium ethylenediamine-*N*,*N*′-bis-2-hydroxyphenylacetic acid (K-EDDHA) (Epihov et al., 2024). Epihov et al. (2024) found that in rock and soil mesocosms without plants, the addition of K-EDDHA increased CDR rates by 2.5 times relative to the control, potentially due to direct chelation of iron from the basalt matrix and/or indirect stimulation of the native soil bacteria's siderophore synthesis genes. Together these approaches have attempted to affect weathering at a fundamental biological level by changing the native bacterial communities by adding new members or stimulating their inherent ability to weather rock. Interestingly, despite the interest in biologically stimulating weathering in soils no attempts appear

to have been made to date to characterise CA's weathering potential in bacteria in soils.

1.7 Thesis aims and research chapter outlines

This thesis aimed to take a holistic approach to investigate different biological components and groups that theoretically should have a high capacity for weathering. As alluded to in Figure 3, different techniques were used in each chapter to analyse the weathering capacity of one or more of these components described below in the chapter outlines.

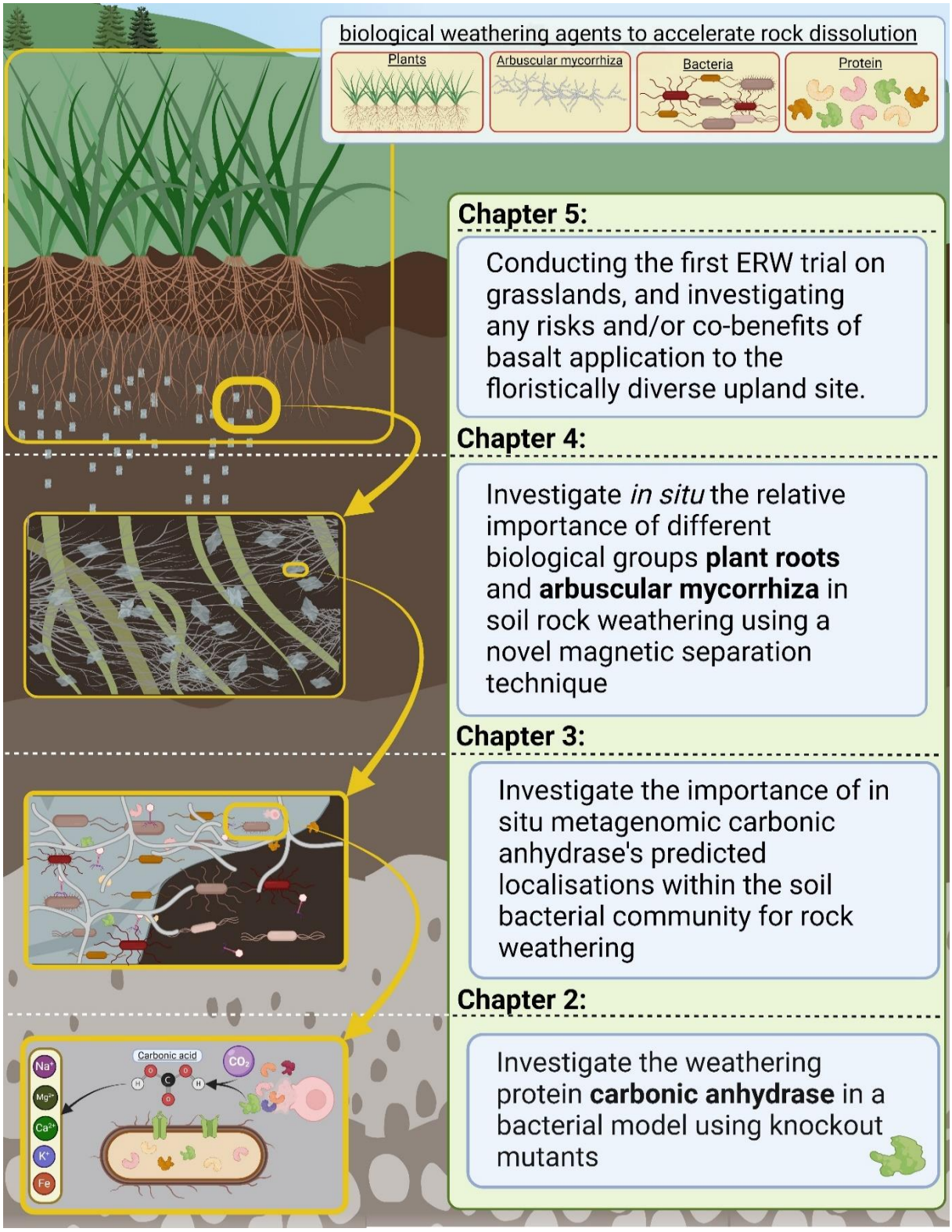

Figure 3. Thesis conceptualisation of the research across biological levels ascending in complexity from a single strain of bacteria up to a floristically diverse grassland ecosystem.

- **Chapter 2**: Past research which had exogenously added bovine (Liu and Dreybrodt, 1997; Li et al., 2007) and bacterial CAs (Xiao et al., 2015) into rock amended liquid media has identified increases in mineral solubilisation as a result of the enzyme, but no research has characterised whether *in vivo* CAs are actively responsible for weathering in bacteria. As such, initial research aimed to characterize CA's potential weathering importance in bacteria using a reverse genetics approach; this was done with the model plant growth-promoting bacterium *Burkholderia thailandensis* grown in a basalt rock-amended liquid media.
    - **Hypothesis**: When CA genes are inactivated, the weathering ability of the bacterium is compromised, regardless of the predicted cellular localisation (extracellular, cell-wall bound or cytoplasmic) of the CA gene.
- **Chapter 3**: Previous research by another group found when a soil bacterium *B. mucilaginosus* was grown in a calcium limiting media containing the calcium silicate wollastonite, after six days of growth all CA genes were significantly upregulated by the mineral, indicating CA is important for cell survival in calcium limited conditions (Xiao et al., 2015). To identify *in situ* whether there is selective pressure for CA genes within the soil bacterial community in arable field trial soils of *G. max* and *Z. mays*, amended with 50 tonnes ha^{-1} y^{-1} basalt in the U.S. Corn Belt, where increased weathering was observed, a metagenomic analysis was conducted, considering the predicted localisations of the CA enzyme.
    - **Hypotheses**: In basalt amended soils CA genes will be more abundant, perhaps due to their weathering potential. In addition, due to the previous research focussing on extracellular CA, the predicted cellular localisations of CAs extracted from soil bacteria are expected to reveal differences in magnitude of responses to basalt.

- **Chapter 4**: Extensive research has characterised the many methods of mineral dissolution used by different biological groups, as detailed in section 1.4, but to date no research has determined the relative weathering contributions of these different groups to mineral dissolution in the field. Expanding on the step-wise approach of increasing scope, the relative weathering contributions of different biological groups, plant roots and AM, was assessed *in situ* in floristically rich grassland field plots in the UK. This is the first reported field trial of ERW in grassland globally and furthermore included trialling the use of a novel magnetic separation method to retrieve basalt rock grains added to soil after incubation in the field.
    - **Hypothesis**: Roots and AM mycorrhizal hyphae will increase rates of mineral dissolution when they can access the basalt rock *in situ*, highlighting the importance of plants and AM in ERW in grasslands.
- **Chapter 5**: Building on the investigation in Chapter 4, of the relative roles of the different biological components in the grassland system on mineral dissolution, this final experimental chapter set out to quantify the potential CDR of ERW at the field-plot scale. This included analysis of hay production, and potential risks of surface-applied basalt dust to floristic diversity and to toxic metal accumulation in hay.
    - **Hypothesis**: Basalt application onto grasslands will produce signals of rock weathering in the soil and could therefore support ERW with a potential for CDR. Furthermore, rock amendment increases the yield and nutritional quality of the forage while not affecting the floristic diversity or heavy metal concentration in plants in the short term.

Chapter 2: The role of carbonic anhydrase in rock weathering and pH regulation by the soil bacterium *Burkholderia thailandensis* E264

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Abstract:

Enhanced rock weathering (ERW) is increasingly recognized as a way to sequester atmospheric carbon dioxide (CO_2) to slow global warming, but its effectiveness needs to be optimized. Carbonic anhydrase (CA), an enzyme capable of accelerating rock weathering both *in vitro* and in soil, offers a valuable target due to its ability to convert CO_2 into carbonic acid. This conversion promotes rock dissolution, enabling immediate CO_2 absorption through cation release and charge-balance mechanisms. Studies have shown that bacteria grown in axenic, rock-amended media increase CA gene expression, but the influence of bacterial CA on rock dissolution rates remains unclear. To investigate this, we used a reverse genetics approach with the phosphate-solubilizing bacterium *Burkholderia thailandensis* E264. We examined three CA-inactivated mutants alongside the wildtype, growing them in minimal media with basalt rock dust (0-10% w/v) at an initial pH of 6.0. After 7 days, we measured weathering potential through elemental concentrations, pH, and dissolved inorganic carbon. In 1% basalt medium, inactivation of the CA1 gene (BTH_I1052) significantly reduced base cation weathering by 41% compared to the wildtype, whereas inactivation of CA2 (BTH_I0345) and CA3 (BTH_I1199) had no significant effect. In the highly buffered, 10% basalt medium, CA1 had a minor role in weathering, and both CA2 and CA3 had no effect. These findings suggest that CA genes in *B. thailandensis* operate differently and that CA1's effect is pH-dependent. Surprisingly, CA1 was predicted to be localized intracellularly, raising questions about how intracellular CAs might influence mineral dissolution, potentially through acidity export or abiontic enzyme activity after cell lysis.

2.1. Introduction

Rock weathering is a geochemical process by which rocks physically and chemically break down due to the action of the atmosphere, water, and/or organisms, driving long-term carbon sequestration within the inorganic carbon cycle, that regulates Earths atmospheric CO_2 concentration and climate over geological timescales (Berner, 2004; Taylor et al., 2009). When carbonic acid reacts with calcium or magnesium silicates it results in carbon dioxide (CO_2) sequestration into bicarbonate or minerals such as calcium carbonate. This naturally occurring process is currently too slow to balance additional anthropogenic CO_2 emissions (Hartmann et al., 2009). Carbonic acid formation, a product of CO_2 dissolution in water, can be catalysed by carbonic anhydrase (CA) (Figure 1). This weak acid can then react with calcium or magnesium silicates to draw down 2 moles of CO_2 in the form of bicarbonate.

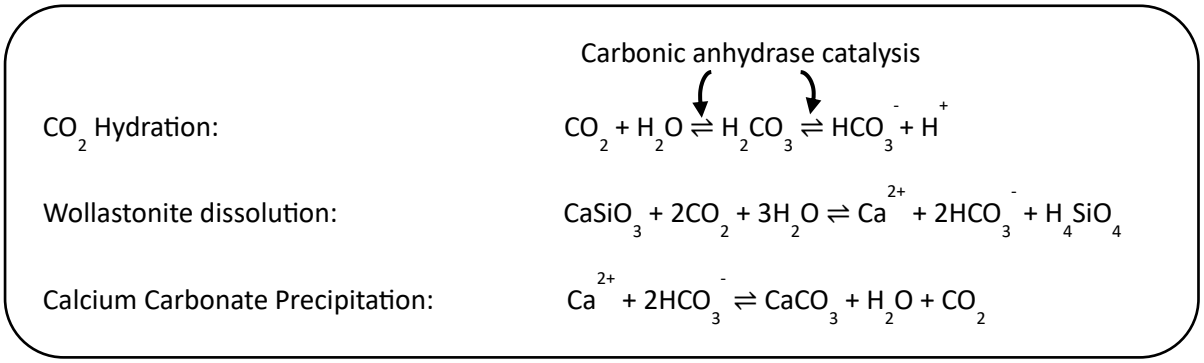

Figure 1. Wollastonite weathering reactions, with CA activity highlighted.

Enhanced rock weathering (ERW) is a land-based CO_2 removal (CDR) strategy, involving the spreading of crushed rock onto agricultural soils to artificially increase rates of calcium and magnesium silicate weathering (Schuiling and Krijgsman, 2006; Moosdorf et al., 2011; Hartmann et al., 2013). Global-scale modelling suggests that if ERW is rolled out over ~50% of croplands for major nations of the world it could result in a net sequestration of 0.5 – 2.0 Gt CO_2 yr^{-1} by 2050 (Beerling et al., 2020). In addition to the benefits of CDR, numerous short-term field trials reviewed by Abdalqadir et

al. (2024), along with the first long-term ERW field trial conducted by Beerling et al. (2024), have shown that rock amendments to soils significantly increased soil pH, inorganic nutrient concentrations, and crop yields.

Finding methods to accelerate rates of ERW within agricultural soils is important to optimize CDR rates (Epihov et al., 2024) and poses unique challenges due to the complexity of the soil-mineral-enriched environment. However, Ribeiro et al. (2020) reviewed numerous bacterially driven biological weathering mechanisms which could potentially be artificially enhanced. The catalysis of the formation and dissolution of carbonic acid in water by CA (Figure 1) provides a potentially important target for such enhancement. Soil respiration, deriving from microbiota, plant roots, and fauna leads to high concentrations of CO_2 within the soil solution, which naturally drives the formation of carbonic acid with CA estimated to increase the rate of carbonic acid formation in the soil by 10 – 300 times (Wingate et al., 2009). Consequently, there is considerable interest in understanding its potential role in accelerating ERW with bacteria (Li et al., 2007, 2009; Dhami et al., 2014; Xiao et al., 2015; Shen et al., 2017; Wang et al., 2018; Jaya et al., 2019; Nathan and Ammini, 2019) and fungi (Liu and Dreybrodt, 1997; Han et al., 2010; Xiao et al., 2016; Sun and Lian, 2019).

Current research into CA generally focuses on the human α-CA isoforms or pathogenic organisms (Nocentini and Supuran, 2019). This research provides useful information regarding the catalytic efficiencies of different CAs, with β-CA's varying up to 10-fold between different organisms (Table 1). Similar variations are also noted between the 16 different α-CA isoforms present in mammals (Pinard et al., 2015). These variations in catalytic efficiencies are likely due to structural differences between members within both the α and the β-CA classes. This indicates that there is unlikely to be a single CA activity profile in different soils, but instead large variations depending on which microorganisms are prevalent in the soil.

Table 1. β-CA catalytic potentials in bacteria (■), fungi (■), archaea (■), eukaryotes (■), and plants (■). (N/A not available)

Organism	Enzyme	Kcat (s^{-1})	Kcat per Km ($FF^{-1}s^{-1}$)	pH	Source
Pseudomonas aeruginosa	psCA1	1.8×10^5	7.5×10^7	8.3	(Lotlikar et al., 2019)
Helicobacter pylori	hpβCA	7.1×10^5	4.8×10^7	8.3	(Nishimori et al., 2007)
Escherichia coli	CynT2	5.3×10^5	4.1×10^7	8.3	(Del Prete et al., 2020)
Vibrio cholerae	VchCAβ	3.3×10^5	4.1×10^7	8.3	(Ferraroni et al., 2015)
Mycobacterium tuberculosis	mtCA 1	3.9×10^5	3.7×10^7	8.3	(Minakuchi et al., 2009)
Burkholderia pseudomallei	BpsβCA	1.6×10^5	3.4×10^7	8.3	(Angeli et al., 2020)
Saccharomyces cerevisiae	scCA	9.4×10^5	9.8×10^7	8.3	(Isik et al., 2008)
Cryptococcus neoformans	Can2	3.9×10^5	4.3×10^7	8.3	(Innocenti et al., 2008)
Candida albicans	Nce103	8.0×10^5	9.7×10^7	8.3	
Methanobacterium thermo-autotrophicum	Cab	1.7×10^4	5.9×10^6	8.5	(Smith and Ferry, 1999)
Leishmania donovani chagasi	LdcCA	9.4×10^5	5.9×10^7	8.4	(Syrjä et al., 2013)
Pisum sativum	N/A	1×10^6	1.8×10^9	N/A	(Kimber, 2000)
Flaveria bidentis	FbiCA 1	1.2×10^5	7.5×10^6	8.4	(Monti et al., 2013)

The first evidence showing CA's potential role in rock weathering was presented by Liu and Dreybrodt (1997), in which they noted that the addition of bovine α-CA increased the rate of limestone dissolution in an *in vitro* system. Subsequently, it was shown that bacteria can induce weathering within an axenic soil medium containing limestone, as their addition increased the concentration of calcium in the soil leachate (Li et al., 2005). When a known CA inhibitor, acetazolamide, was added to a bacterial or crude enzyme mixture there was less calcium released from the limestone over the course of 9 hours (W Li et al., 2007). More recent research has examined the genetic regulation of CA and attempts have been made to purify the enzyme (Xiao et al., 2015; Supuran, 2013). Xiao et al. (2015) presented promising results showing that all 5 CA encoding genes within *Bacillus mucilaginosus* were significantly upregulated following six days of growth in a wollastonite amended medium. They also showed that a purified bacterial CA protein (PCA4) when incubated at 0.039% CO_2 increased the rate of wollastonite dissolution by 4.78 x 10^{-4} mg g^{-1} min^{-1}. This evidence strongly suggests that CA plays a role in the weathering of rocks, but another *in vitro* study suggests a more complex dynamic as CA inhibited rock weathering (Di Lorenzo et al., 2018). This contradiction might be due to differences in experimental conditions since Di Lorenzo et al. (2018) ran their experiment with a pH fixed to 4; whereas Xiao et al. (2015) began the reaction at a pH of approximately 8 and allowed it to change. Through measuring ^{18}O enriched CO_2 flux of soils ranging from pH 4.5 – 8.5 with bovine α-CA added to the soils Sauze et al. (2018) demonstrated that soil pH is the primary determinant of soils CO_2 – H_2O isotopic exchange rate. Their results indicate that at a low soil pH, even with addition of CA, rates of CO_2 hydration did not change compared to the native control; whereas, at a higher soil pH CA addition increased the rate of CO_2 hydration by 20 reactions sec^{-1} (Sauze et al., 2018). Since calcium and magnesium-rich rock dusts tend to increase soil alkalinity, this suggests that CA may be an important facilitator of ERW. Research into fungal CA in *Aspergillus nidulans* has further indicated its active role in mineral weathering;

when grown in a wollastonite amended minimal medium with the CA gene *can*A inactivated, there was a loss of soluble calcium in the medium, whereas overexpression of *can*A resulted in an increase in soluble calcium (Sun and Lian, 2019). All the aforementioned literature has generated commercial interest, leading to a partnership between the two companies, Veolia and FabricNano, which has initiated field trials by adding a CA inoculum to soils, both freely and immobilized on rock surfaces (Peplow, 2024).

The cellular localization of the CA may also play a role in its effectiveness as a rock weathering enzyme; Figure 2 summarises multiple ways CA could affect its extracellular environment. Experiments with bovine α-CA added have assumed that the enzyme is most active extracellularly. In addition, four sequences obtained for the CAs examined by Xiao et al., (2015) were also estimated as having extracellular localisations through testing with PSORTb v. 3.0.3 (Yu et al., 2010).

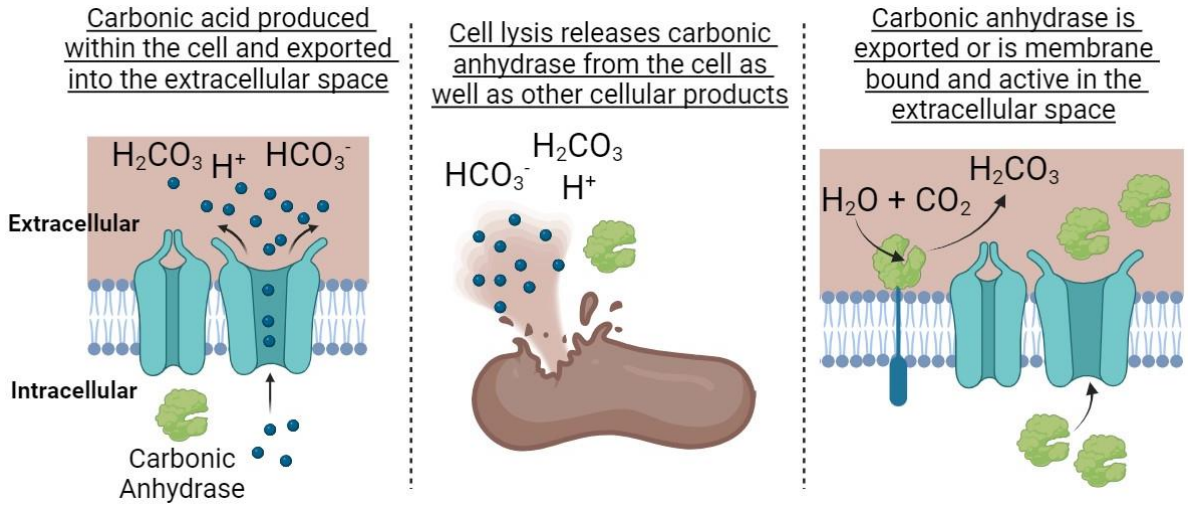

Figure 2. Potential CA methods of extracellular chemical change. Created in BioRender.com

More recent *in vitro* ERW research has used mutants from the transposon-mutant library of the soil β-proteobacterium *Burkholderia thailandensis* E264, and has seen that CA was significantly correlated with respiration gene relative abundance in basalt amended tropical soils (Epihov,

2018). *B. thailandensis*, a gram negative, aerobic, motile, and non-fermenting bacterium, is a widespread soil saprophyte in Australasia (Zakharova et al., 2022). It is also commonly used as a model organism to study the highly pathogenic *Burkholderia pseudomallei*, which has resulted in it having a well characterised genome along with the aforementioned mutant collection (Gallagher et al., 2013). *B. thailandensis* is also considered to be a plant growth promoting bacterium as when it was applied to rice growing in acid sulfate soils, along with a basalt and magnesium limestone, it reduced the rhizotoxicity of aluminium and increased solubilised phosphate and yields (Panhwar et al., 2015). Experiments have confirmed its plant growth promoting potential through identifying not only phosphate solubilisation but also nitrogen fixation genes (Sivaji et al., 2016). It can also inhibit the growth of the commercial crop pathogen *Rhizoctonia solani* (Sivaji et al., 2016), and can degrade the insecticide Fipronil (Cappelini et al., 2018). Its potential importance as a plant growth promoting bacterium in cropping systems and its well-characterised genome makes it a suitable candidate for the present study.

Our study aims to characterise whether CA's encoded within a soil bacterium play a role in weathering and therefore carbon dioxide removal. To assess this a reverse genetics approach was used through growing the wildtype model soil bacterium *B. thailandensis* and its transposon inactivated CA mutants in media amended with different concentrations of basalt rock to assess the role of bacterial CA in total rock dissolution and pH regulation when grown in media carrying different buffering capacities.

2.2. Materials & Methods

2.2.1 Mineral

Basalt, commonly used in ERW trials, was sourced from The Hillhouse Quarry Group in Troon, Ayrshire, Scotland. This basalt has a fast weathering rate (Lewis et al., 2021) and consists of 34.6% plagioclase, 32.7% augite, 14.7% forsterite, 5.2% chlorite-smectite, 4.5% spinel, 7.0% analcime, and 0.5% ilmenite (Lewis et al., 2021). As detailed further by Lewis et al. (2021), this basalt has relatively high concentrations of key carbon removal related elements, calcium (6.72%) and magnesium (5.86%), compared to the other basalts tested, making it an ideal candidate for measuring weathering.

The rock was first dry sieved to sizes of 75 - 90 µm and < 53 µm, the 75 - 90 µm fraction underwent additional washing with 0.01 M HCl to remove exchangeable bases (Yu et al., 2016) followed by rinsing with distilled water until all visible dust particles were removed (Epihov, 2018). Both fractions were dried at 100 °C overnight, with 0.35 g of the 75 - 90 µm (1% w/v basalt medium) or 3.5 g of the < 53 µm size fraction (10% w/v basalt medium) placed in autoclavable 50 mL falcon tubes. These tubes were loosely sealed, placed in an autoclavable bag, and wet autoclaved at 121 °C for 45 min twice, with at least 24 hours at room temperature between autoclave cycles. The washed basalt fines (75–90 µm) were used to create the minimally buffered medium, while finer particles (<53 µm) were used for the highly buffered medium, detailed further in Section 2.2.3.

2.2.2 Bacteria preparation

The wildtype (WT) strain E264 and a knockout (KO) library of the soil bacterium *Burkholderia thailandensis* were acquired from the Manoil lab at the University of Washington, U.S (Gallagher et al., 2013). The KO library included three sequence confirmed transposon inactivated mutants of three predicted CA genes. The specific loci of the three putative CA genes were BTH_I1052, BTH_I0345, and BTH_I1199, referred to in the following text as CA1, CA2,

and CA3, respectively. Colonies of both WT and KO organisms were grown from - 80 °C stocks on streak plates incubated overnight at 37 °C on Luria Bertani (LB) agar (Bertani, 1951). Single colonies from each respective organism were used to inoculate 30 mL LB broths, which were grown aerobically with orbital shaking at 250 rpm (New Brunswick Innova® 44/44R) overnight at 37 °C along with a non-inoculated control. Following growth, optical density at 600 nm (Geneva Bio, Jenway) were taken and broths with higher values were diluted using sterile LB broth to match the lowest absorbance. All tubes were then centrifuged at 3234 x *g* for 10 minutes and supernatants were aseptically removed prior to the pellets being resuspended in 10 mL of 0.85% sterile isotonic saline. This washing step was repeated two more times, with the final pellet being resuspended in 12 mL of 0.85% sterile saline before inoculation into the media, described in section 2.2.3.

2.2.3 Media preparation, inoculation, and growth

The minimal media, composed of 10 mM D-glucose, 1 mM sodium chloride, 0.020 mM magnesium sulfate heptahydrate, 0.050 mM dipotassium phosphate, and 1 mM ammonium nitrate, was prepared in 1 L of distilled ultra-pure water. Using the previously weighed and autoclaved rock described in section 2.2.1, three replicates with concentrations of 0, 1, and 10% w/v basalt were created for all four organisms described in section 2.2.2, alongside with six non-inoculated controls. The three concentrations of basalt were designed to create media of increasing buffering potential to characterise CA gene relevance to solution pH and weathering. This process involved adding 34.9, 34.775, and 33.65 mL of sterilized minimal media to tubes containing 0, 0.35, and 3.5 g of basalt rock, respectively.

The bacterial suspensions were thoroughly vortexed before adding 100 µL of each relevant organism or non-inoculated control to their respective 50 mL Falcon tubes, containing either 0, 1 or 10% w/v basalt. Following inoculation, a colony forming units (CFU) dilution series was performed on

LB agar and counted after overnight incubation aerobically at 37 °C for each organism and the control to determine initial numbers of bacteria inoculated.

Three of the non-inoculated controls of each basalt concentration were sampled to determine initial pH of the media. The remaining samples were incubated aerobically with 120 rpm shaking at 37 °C for 170 hours. CFU dilution series and counts were performed post-inoculation at time points: 3, 6, 9, 21, 24, 27, 48, and 170 hours. Total growth was determined using CFU counts because optical density has been found to be an unreliable method of growth with *B. thailandensis* due to its secreted metabolites (Martinez and Déziel, 2020). During the CFU counts morphology was assessed to ensure there were no outside contamination of the media; however, cross contamination between mutants was not assessed, but assumed to have been controlled by the aseptic procedures.

2.2.4 Sample analysis and CO_2 absorption estimate

At 170 hours, all tubes were centrifuged at 3234 x *g* for 20 minutes at 4 °C. The resulting supernatants were filter sterilised to 0.2 µm and transferred into 50 mL Falcon tubes. These samples were kept on ice, with aliquots removed for further analyses. For ICP-MS analysis, 1.2 mL of each sample was diluted with a mixture of 10.452 mL ultra-pure water and 0.348 mL of 69% nitric acid, resulting in a 1/10 diluted sample with 2% nitric acid. Subsequently, all tubes were sent for ICP-MS analysis with the Agricultural and Environmental Sciences Department at the University of Nottingham. Total inorganic carbon analysis was conducted by removing 9 mL of the filtered sample and placing in a sample test tube tightly sealed with polyethylene cling film to prevent CO_2 degassing (Sun and Li, 2017) until all samples could be run on the TOC-L (Shimadzu). The final pH of all experimental and control tubes was measured using the Bante930 Benchtop pH/Ion Meter equipped with an P11 glass electrode calibrated to pH's 4, 7, and 10.

Potential CDR was estimated using a modified technique of charge balancing base cations calcium and magnesium with bicarbonate ions, as described previously in soils (Hartmann et al., 2013; Kanzaki et al., 2023; Beerling et al., 2024). Briefly, the net molar change of cations calcium and magnesium in the media due to basalt amendment was totalled and using a 2:1 molar ratio of HCO_3^{-1} to cations, the CO_2 sequestration in g L^{-1} was estimated.

2.2.5 Statistical analysis

GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, Massachusetts US, www.graphpad.com) was used for plotting all figures. Normality and homoscedasticity were assessed visually using residual and Q-Q plots available within R studio (R Core Team, 2024). Using the Mass package in R (Venables and Ripley, 2002) data with non-normal distribution of residuals underwent boxcox analysis to identify the optimal lambda transformation to normalise the residuals (Box and Cox, 1964). Using Core R packages both two-way and three-way ANOVAs and Tukey multiple comparisons statistical tests were also performed in R. A result was considered significant if the *p* value was < 0.05.

2.3. Results

2.3.1 Total growth, final pH, and dissolved inorganic carbon.

Prior to inoculation, the average initial pH values of the 0, 1 and 10% w/v basalt media were 6.55, 6.51, and 8.71, respectively. The higher initial pH of the 10% w/v media was expected due to the presence of < 53 µm rock grains, which likely increased the concentration of dissolved base cations in the solution. However, after 170 hours of incubation at 37 °C, the average pH of the uninoculated control samples containing 10% w/v basalt fell to 7.52. Bacterial growth in the basalt-free medium caused consistent acidification, whereas in the 1% w/v basalt media, acidification was less pronounced and showed more variability between replicates and organisms. For example, the

KO CA1 appeared distinctive as it maintained a higher average pH of 7.1 compared to the wildtype which had a pH of 5.1. Post-hoc Tukey's tests confirmed a significantly higher pH for this mutant compared to the other organisms (Figure 3a).

The dissolved inorganic carbon found in the media post incubation (Figure 3b), showed no significant pairwise differences between organisms at any basalt concentration. The total growth over the course of the experiment, (Figure 3c) expressed as $\log_{10}$(area under the growth curve), showed a significant increase in the growth of the CA1 KO when grown with 1% w/v basalt relative to the other organisms, but there were no differences between organisms in the 0% and 10% basalt treatments. Importantly, no bacterial growth was seen in any of the abiotic controls.

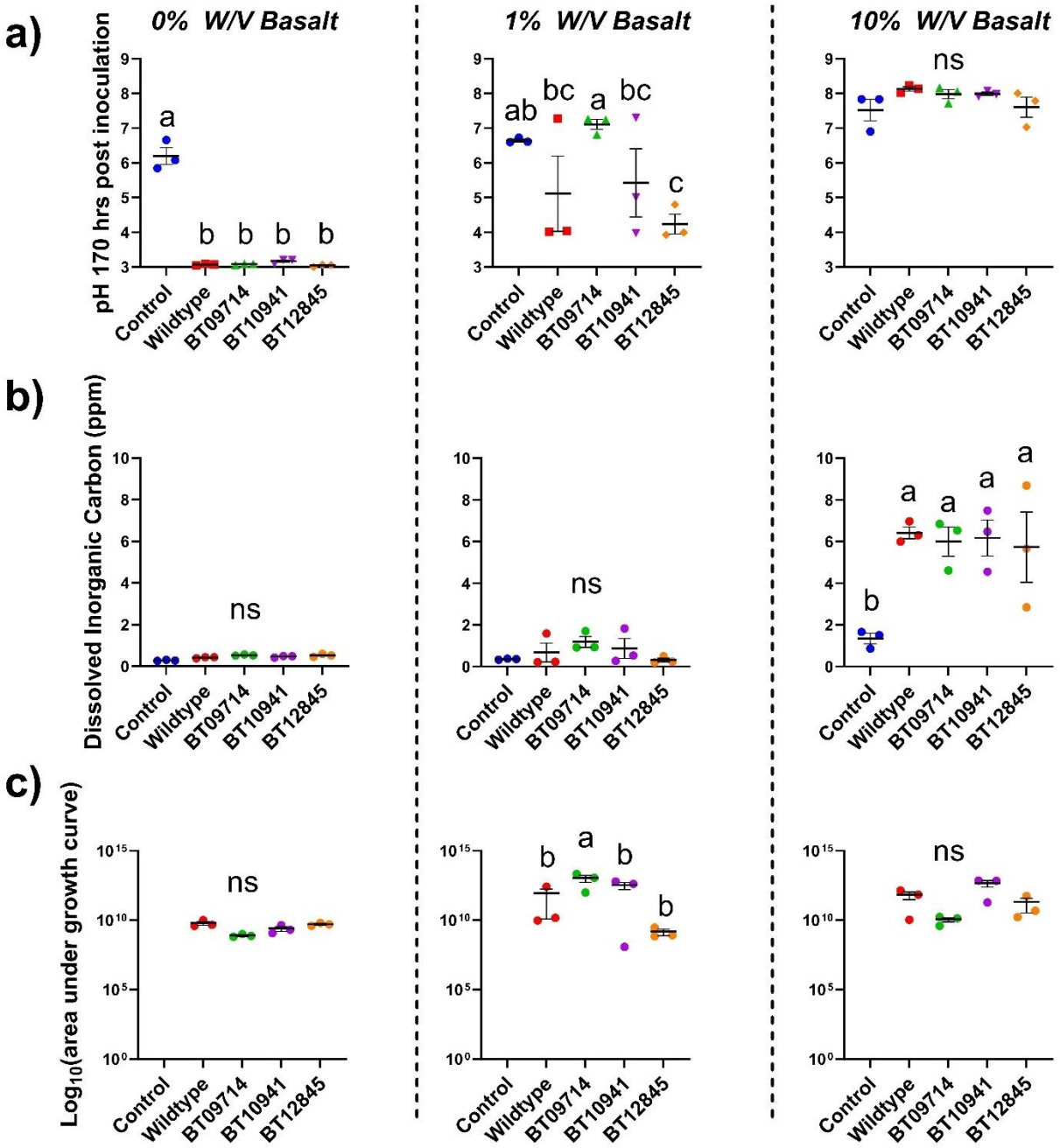

Figure 3. Effects of basalt treatments and *B. thailandensis* growth 170 hours post inoculation compared to abiotic controls on mean (± 1 standard error of mean, SEM) (a) pH, (b) dissolved inorganic carbon, and (c) total area under growth curve. ANOVA post hoc Tukey's test significance differences are denoted by letter codes, means sharing the same letter are not significantly different ($p > 0.05$) and "ns" denotes no significant pairwise differences.

2.3.2 Rock dissolution and bacterial enhanced element release into solution

When grown without basalt, elemental concentrations relative to the control (Table 1) remained consistently low for all organisms, except for the CA2 mutant, which showed significant increases in sodium, potassium, and rubidium. As seen in Table 1, inoculation of *B. thailandensis* into basalt amended medias in most cases increased the total dissolved elemental concentrations (DEC), indicating that bacteria increased weathering rates in the media. Interestingly, in the 1% w/v basalt media the KO CA1 behaved differently to the other organisms, with no elements significantly increasing relative to the control. However, in both the 1% and 10% w/v media the CA1 mutant had significantly reduced concentrations of phosphorus. Along with those reductions in the 10% media the CA1 mutant also had significant increases in the concentrations of calcium, magnesium, strontium, and rubidium but these significant increases were also observed with the other KOs and the wildtype.

Table 1. Average ICP-MS derived DEC 170 hours post inoculation of *B. thailandensis* wildtype or CA mutants in 0, 1, or 10 % W/V basalt amended media. Averaged DEC difference to Abiotic Control with significant difference (*p* values < 0.05) denoted by **an asterisk (*) presented in adjacent columns.**

0%									
Unit	Element	Wildtype		CA1		CA2		CA3	
		Concentration	Difference to control	Concentration	Difference to control	Concentration	Difference to control	Concentration	Difference to control
mg L^{-1}	Sodium	4.919	0.363	3.39	-1.16	10.1	5.55 *	5.03	0.475
	Potassium	0.633	0.0439	0.399	-0.19	1.90	1.31 *	0.675	0.0867
	Calcium	0.0479	-0.0174	0.0400	-0.0253	0.752	0.686	0.0654	0.000122
	Magnesium	0.0868	0.00751	0.0541	-0.0251	0.188	0.108	0.0923	0.013
	Phosphorus	0.196	-0.0259	0.0765	-0.145	0.328	0.107	0.161	-0.0609
	Titanium	3.88E-05	-2.5E-05	3.26E-05	-3.1E-05	0.000304	0.00024	5.95E-05	-4.3E-06
	Silicon	0.0273	0.000286	0.0292	0.00233	0.0366	0.00964	0.0255	-0.00144
µg L^{-1}	Aluminium	12.6	-0.979	9.84	-3.76	254	241	15.5	1.88
	Iron	24.1	-4.2	23.1	-5.26	38.2	9.89	30.9	2.58
	Strontium	0.300	-0.619	0.280	-0.639	2.19	1.27	0.902	-0.0168
	Rubidium	0.0553	0.00238	0.0452	-0.00772	0.483	0.43 *	0.0542	0.00124
	Nickel	0.232	-2.51	0.140	-2.6 *	2.70	-0.0348	2.91	0.174

1%									
Unit	Element	Wildtype		CA1		CA2		CA3	
		Concentration	Difference to control	Concentration	Difference to control	Concentration	Difference to control	Concentration	Difference to control
mg L^{-1}	Sodium	6.9	1.65	4.6	-0.654	8.03	-0.654 *	7.43	2.18
	Potassium	0.716	0.0606	0.444	-0.212	0.846	-0.212	0.799	0.144
	Calcium	1.41	1.11	0.401	0.103	1.41	0.103	1.75	1.46 *
	Magnesium	0.85	0.62 *	0.385	0.155	0.841	0.155 *	1	0.774 *
	Phosphorus	0.0796	-0.166 *	0.019	-0.227 *	0.0632	-0.227 *	0.0906	-0.155 *
	Titanium	0.00112	0.00072	0.000233	-0.00017	0.00113	-0.00017	0.0015	0.0011
	Silicon	3.46	3.23 *	0.825	0.598	3.72	0.598 *	4.96	4.73 *
µg L^{-1}	Aluminium	2440	2390 *	271	223	2210	223 *	3010	2960 *
	Iron	1070	974 *	93.5	2.01	1000	2.01	1420	1320 *
	Strontium	20.6	18.4 *	4.09	1.91	22.4	1.91 *	27.9	25.7
	Rubidium	0.804	0.497	0.518	0.211	0.874	0.211 *	0.914	0.607 *
	Nickel	5.41	1.85	1.12	-2.43	7.17	-2.43	10.6	7.01 *

10%									
Unit	Element	Wildtype		CA1		CA2		CA3	
		Concentration	Difference to control	Concentration	Difference to control	Concentration	Difference to control	Concentration	Difference to control
mg L^{-1}	Sodium	10.7	0.142	10.1	-0.449	10.5	-0.0766	10.8	0.237
	Potassium	1.49	0.406	1.72	0.633	1.7	0.62	1.81	0.73
	Calcium	11	8.5 *	12.9	10.4 *	12	9.48 *	13.8	11.3 *
	Magnesium	1.87	1.26 *	1.56	0.937 *	1.67	1.05 *	1.94	1.32 *
	Phosphorus	0.0726	-0.103	0.0514	-0.124 *	0.0495	-0.126 *	0.0487	-0.127 *
	Titanium	0.00933	0.00518 *	0.00566	0.00152	0.00633	0.00219	0.00666	0.00251
	Silicon	1.78	0.669	1.53	0.413	1.49	0.371	1.8	0.687
µg L^{-1}	Aluminium	644	112	269	-263	548	17	475	-56.3
	Iron	1040	441	426	-177	900	297	653	49.6
	Strontium	98.3	69.9 *	115	86.5 *	104	75.3 *	125	96.9 *
	Rubidium	2.22	1.49 *	2.82	2.09 *	2.56	1.83 *	3.14	2.41 *
	Nickel	6	0.491	3.49	-2.02	7.59	2.08	8.15	2.64

Supplemental Table 1 shows all measured elements that could be statistically evaluated using a two-way ANOVA and post-hoc Tukey's test. As expected in the 0% w/v media, DECs tended to be lower than in the basalt amended media. Furthermore, when the KO CA2 was grown in the 0% w/v media it resulted in significantly greater DECs of sodium, selenium, copper, and rubidium relative to the other organisms. This trend was observed for other elements however it was not significant.

When grown in 1% w/v media, a pattern was seen across almost all the elements, excluding vanadium. Specifically, CA1 consistently had a lower mean DEC relative to the other KOs and the wildtype. This was significantly different from all other organisms for strontium (80.12% less, $p < 0.05$) relative to the wildtype. For almost all the rest of the elements measured, this trend of CA1 giving lower concentrations showed varying significant differences compared to the other organisms.

In the 10% basalt media *B. thailandensis* colonisation significantly increased cation concentrations of calcium, magnesium, and potassium (Supplemental Figure 1 a, b, d), with no significant differences noted between organisms. Excluding caesium, all other elements showed no significant differences between organisms; however, a pattern was seen in DEC for the elements titanium, vanadium, aluminium, cobalt, nickel, silver, caesium, iron, and barium suggesting that these dissolved elements may be released at lower rates due to CA inactivation.

Interestingly, some elements, such as phosphorus, vanadium, and selenium, appeared to decrease because of *B. thailandensis* growth. Of those elements, phosphorus was the only one to see significant reductions relative to the control of 0.127 ($p < 0.05$), 0.124 ($p < 0.05$), and 0.126 ($p < 0.05$) mg L^{-1} when the *B. thailandensis* KO organisms CA1, CA2, and CA3 were grown in the 10% w/v media, respectively.

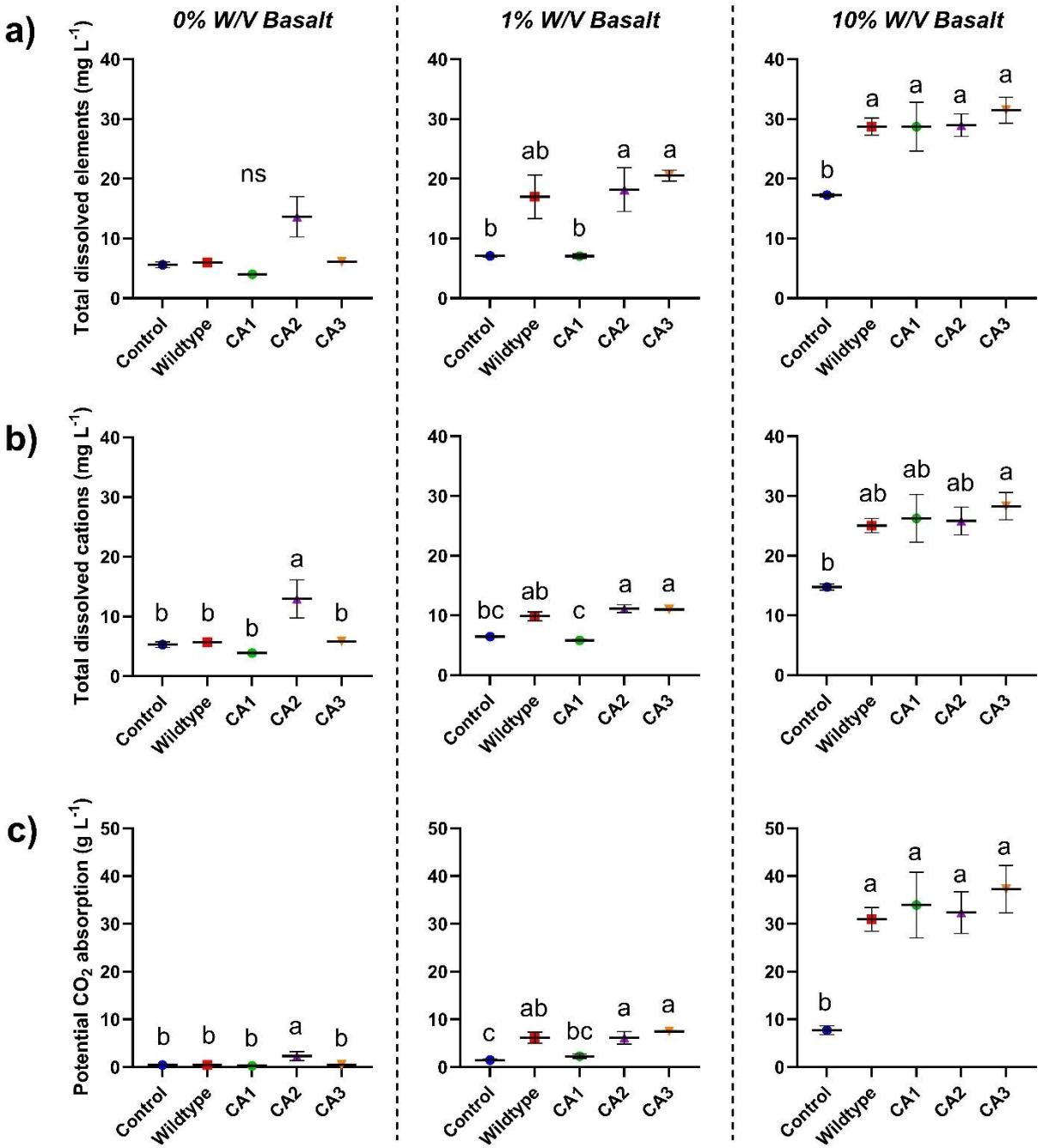

Figure 4. Effects of basalt amendment and *B. thailandensis* wildtype and mutant growth, along with abiotic controls 170 hours post-inoculation, on mean concentrations (± 1 standard error) of (a) total dissolved elements (b) total dissolved cations (calcium, magnesium, sodium, and potassium), and (c) potential CO_2 absorption. Two-way ANOVA post hoc Tukey's test significance differences denoted by letters codes within basalt treatments, where means sharing the same letter are not significantly different ($p > 0.05$) and "ns" denotes no significant pairwise differences.

When all measured elemental concentrations presented in Supplemental Figure 1 were summed, averaged across replicates, and analysed using a two-way ANOVA with post-hoc Tukey's test (Figure 4a), similar patterns were observed to those in Table 1 and Supplemental Figure 1. Specifically, the total averaged DEC in the 0% w/v media showed a greater mean DEC when the CA2 KO was grown, but when elements were totalled this became non-significant. In the 1% w/v media, the CA1 KO organism had a significant reduction in DECs relative to the other KOs, but not to the control or the wildtype. No significant pairwise differences were observed in total DEC's between organisms grown in the 10% w/v media, but all organisms differed significantly from the control, again highlighting the importance of bacteria in rock dissolution. When the cations calcium, magnesium, sodium, and potassium were totalled (Figure 4b, Supplemental Figure 1), the distribution of means was similar to that in Figure 4a at all basalt concentrations. However, in this case, the CA2 KO had significantly higher concentrations than both the control and the other organisms for these elements when grown in 0% w/v basalt, while the CA1 KO, when grown in 1% w/v basalt, had significantly reduced dissolved cation concentrations relative to all other organisms, including the wildtype. In the 10% w/v basalt media, total dissolved cations appeared to increase relative to the control, but only the CA3 KO was significantly different. Estimated potential CO_2 absorption (Figure 4c), calculated as described in Section 2.2.4, revealed a distribution of means similar to those in Figures 5a and 5b. However, as in Figure 4b, the CA2 KO, when grown in 0% w/v media, was significantly different from the control. Additionally, when grown in 1% w/v media, the CA1 KO was significantly reduced relative to the other KOs but not from the wildtype; in this case, though, the wildtype was different from the control.

2.4. Discussion

We show that in the weakly buffered 1% w/v basalt media, the inactivation of the CA1 gene appeared to play more of a role in rock

weathering; as it released significantly fewer total cations into the solution, despite achieving more cell growth than the other organisms. This trend of reduced DECs with the KO CA1 was noted for all measured elements. When totalled across elements and averaged, the inactivation of the CA1 gene resulted in significantly lower element concentrations in solution relative to the CA2 (61.2% less, $p < 0.05$) and CA3 (65.6% less, $p < 0.05$) mutants and a lower, albeit non-significant, reduction relative to the wildtype (58.4% less, $p > 0.05$). This diminished weathering potential occurred along with it carrying a final pH average of 7.11, which was significantly higher than the other organisms. The elevated pH seen with the CA1 mutant is likely responsible for the decreased rate of elemental release from the rock matrix. When the CA1 gene was functional, all organisms, including the WT, CA2, and CA3, overcame the rock's buffering capacity and acidified the 1% w/v basalt media to pH 5.11, 5.43, and 4.24, respectively. Interestingly, in Figure 3b there were no significant differences in dissolved inorganic carbon seen between organisms, suggesting that the loss in weathering potential was not due to the reduction in carbonic acid catalysis. However, as pH determines carbonate speciation in solution (Wang et al., 2013), with dissolved CO_2 and carbonic acid predominant at a pH < 6 , bicarbonate between pH 6 and 10, and carbonate at pH > 10, this explains the lack of difference observed. As the pH values of the wildtype, CA2, and CA3 media were all < 6, they likely lost dissolved inorganic carbon as gaseous CO_2 during the experiment. In contrast, the CA1 mutant, which had a pH of 7.11, likely retained inorganic carbon in the dissolved bicarbonate form. Therefore, CA1 inactivation likely resulted in reduced rates of carbonic acid formation, thereby decreasing rates of weathering, and preventing the bacteria from overcoming the buffering capacity of the 1% w/v basalt. These effects of pH buffering caused by the basalt concentration are summarized in Figure 6, which sets out hypotheses to explain the findings.

When grown in the 10% basalt media the DECs (Supplemental Figure 1) showed no organism specific significant differences for almost all the measured elements. However, there were non-significant reductions in the elements Ti, V, Al, Co, Ni, Ag, Cs, Fe, and Ba in the CA1 mutant compared to WT. Through performing a three-way ANOVA of the DEC using some of the above elements: there was a significant reduction in the average elemental release compared to the WT and the CA3 mutant. This potential reduction in weathering is not observed when base cation DECs are summed (Figure 5b), suggesting that the inactivation of the CA1 and CA2 genes affects rock weathering differently in the 1% w/v media compared to other conditions, possibly indicating that the CA genes influence cellular processes or behave differently under varying conditions. A further potential explanation for the difference in trends seen between the 1% and 10% w/v basalt media is that *B. thailandensis* produces rhamnolipids. Rhamnolipids, a glycolipidic biosurfactant, which can extract rare earth elements, functions more favourably at a higher pH (Castro et al., 2023).

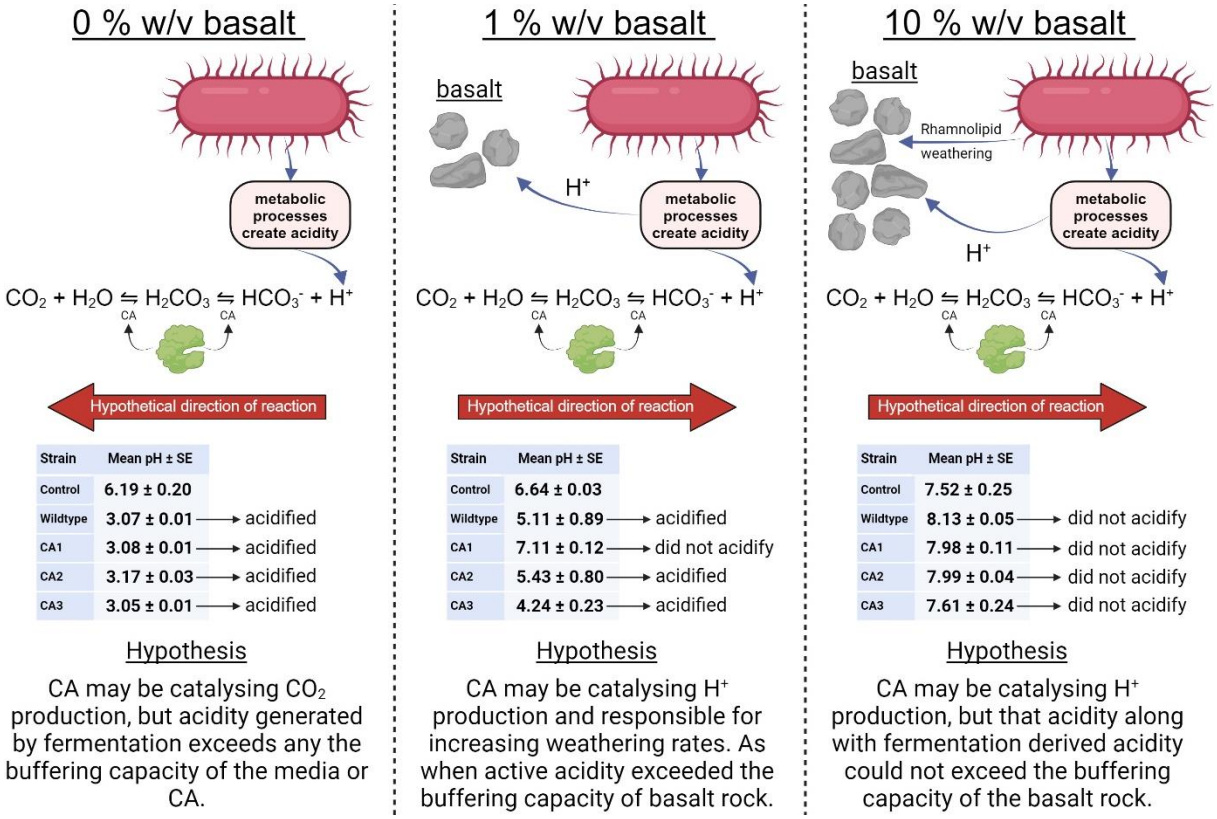

Figure 6. Conceptualisation of CA derived weathering at the three basalt concentrations of 0, 1, and 10%. Created in Biorender.com

Sun and Lian (2019) showed that the two CA genes within *A. nidulans* exhibited distinct physiological functions, with only *canB* being important for acclimation at low ambient CO_2 concentrations. This is also the case in *B. thailandensis*, which carries three β-CA's, as the mutant organisms responded differently to basalt amendment. Both the CA2 and CA3 CA genes did not appear to provide redundancy for the CA1 CA gene. These results introduce another layer of complexity to our understanding of CA's catalytic potential within soils, as not only do CAs between organisms have differing catalytic potentials, but also genes within the same organism carry functional differences. Furthermore through using the cellular localisation tool PSORTb v. 3.0.3 (Yu et al., 2010) all three *B. thailandensis* CA encoding genes were predicted to be intracellularly localised suggesting that intracellular CA's may play an important role in rock weathering, contradicting previous research that emphasized the importance of extracellular CA. Furthermore, these results may also run contrary to Sauze

et al. (2018), who found that bovine CA becomes more efficient at converting CO_2 to carbonic acid with increasing soil pH at least to a soil pH of 8.5. Our results suggest that CA played a greater role in cation dissolution when *B. thailandensis* was grown in a mildly buffered media (low basalt w/v) compared to highly buffered media (high basalt w/v) with a greater initial pH. One explanation for the variation seen between replicates seen here is that some of the mutations may have regained CA functionality over the course of the 170 hour incubation as evidence suggests bacteria in stationary phase can produce beneficial or restorative mutations (Cairns et al., 1988).

There was a significant reduction in dissolved phosphorus because of *B. thailandensis* inoculation. *B. thailandensis* is known to carry phosphate solubilising genes (Sivaji et al., 2016) and has been seen in soils to mobilise phosphate (Panhwar et al., 2015). One explanation for the loss in dissolved phosphorus is that as it is required for DNA, RNA, and phospholipid production, and is likely to be assimilated into the bacteria. As no bacterial lysis step was performed the biomass phosphorus was likely lost in the form of pelletised cellular debris during the centrifugation and filtration steps following incubation.

Three E264 B. thailandensis mutants with knockouts of the genes gluconate kinase (*gnt*K), glucose dehydrogenase (*gdh*FAD), or siderophore synthesis (*pch*D) had their dunite weathering capacity previously assessed relative to the wildtype, as reported in the literature (Epihov, 2018). As seen in Figure 7 below, relative to the wildtype none of the KO's reduced magnesium, silicon, or iron solubilisation/weathering rates by more than 50%. As expected, the inactivation of the siderophore synthesis gene *pch*D resulted in the greatest reduction in iron levels. In contrast, the inactivation of the *gnt*K gene led to an increase in weathering, potentially due to impairments in the pentose phosphate pathway, which could cause acid buildup (Epihov, 2018). As seen in Figure 7 below, the CA1 inactivation appeared to result in a greater loss of rock weathering potential compared to

all other mutants previously tested across the three elements. Again, highlighting the potential importance of CA to bacterial derived rock weathering.

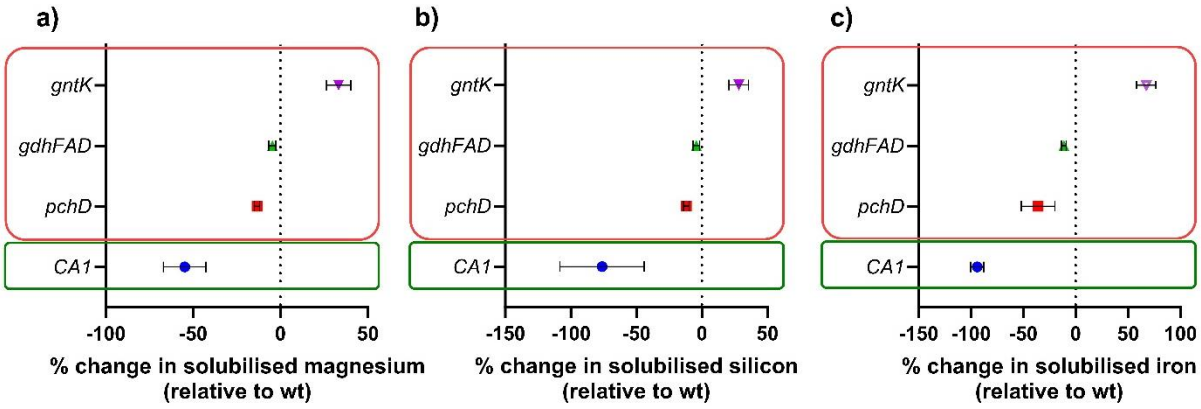

Figure 7. Percent change of solubilised magnesium (a), silicon (b), and iron (c) relative to the wildtype as a result of inactivation of *B. thailandensis* genes gluconate kinase (*gnt*K), glucose dehydrogenase (*gdh*FAD), and siderophore (*pch*D) when incubated with dunite rock, as noted in red and extracted from Epihov (2018), and the CA1 mutant (BTH_I1052) when incubated with basalt rock as seen in green.

2.5. Conclusion

Our reverse genetic approach indicates that *B. thailandensis* CA influences basalt dissolution rates *in vitro* under mild and highly buffered conditions. However, only one of the three CAs encoded in *B. thailandensis* consistently affected pH and DECs, indicating that bacterial CAs belowground may not all be relevant for rock weathering. The observed reduction in rock weathering upon inactivation of the BTH_I1052 (CA1) CA gene is likely attributable to the KO maintaining a higher pH relative to the WT and other mutants. The increase in solution acidity and rock weathering products following incubation with the other organisms may have been a direct result of carbonic acid formation as concentrations of dissolved inorganic carbon were likely affected by the pH of the solution. Furthermore, when *B. thailandensis* was grown in the highly buffered media, at a pH > 7.5, the BTH_I1052 (CA1) and the BTH_I0345 (CA2) genes do appear to play a role in

rock dissolution as the mean DEC was significantly lower than the wildtype and BTH_I1199 (CA3) KO when the solution concentrations of the elements titanium, vanadium, cobalt, nickel, silver, and caesium were averaged. These reductions in weathering were not however noted in cations. Interestingly, CA did not appear to be more functional at the higher pH as larger differences were seen between the wildtype and the KO when grown in the 1% w/v media, indicating pH and buffering capacity as important regulators for the utility of bacterial CAs for ERW-CDR. We conclude that despite its predicted intracellular localization CAs encoded for in bacteria can affect weathering rates and should be considered in the future as a potential target for biologically accelerated ERW in soils.

2.6. CRediT authorship contribution statement

Derek S Bell: Conceptualisation, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation. Writing – original draft, Writing-review & editing, Visualization, Project administration; **Jonathan R. Leake:** Conceptualisation, Methodology, Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition; **David J Beerling**: Validation, Writing – review & editing; **Jurriaan Ton:** Conceptualisation, Writing – review & editing, **Dimitar Z. Epihov** Conceptualisation, Methodology, Validation, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

2.7. Declaration of Interests

D.J.B. has a minority equity stake in Future Forest/Undo and is a member of the Advisory Board of The Carbon Community, a UK carbon removal charity, and the Scientific Advisory Council of the non-profit Carbon Technology Research Foundation. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

2.8. Funding sources

This work was supported by a BBSRC White Rose DTP studentship (BB/T007222/1) awarded to DSB and supervised by JRL and DZE.

2.9. Data statement

Data will be uploaded prior to journal submission.

2.10. Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT, an AI language model, to assist with grammar and refining content. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

2.11. Supplementary figures

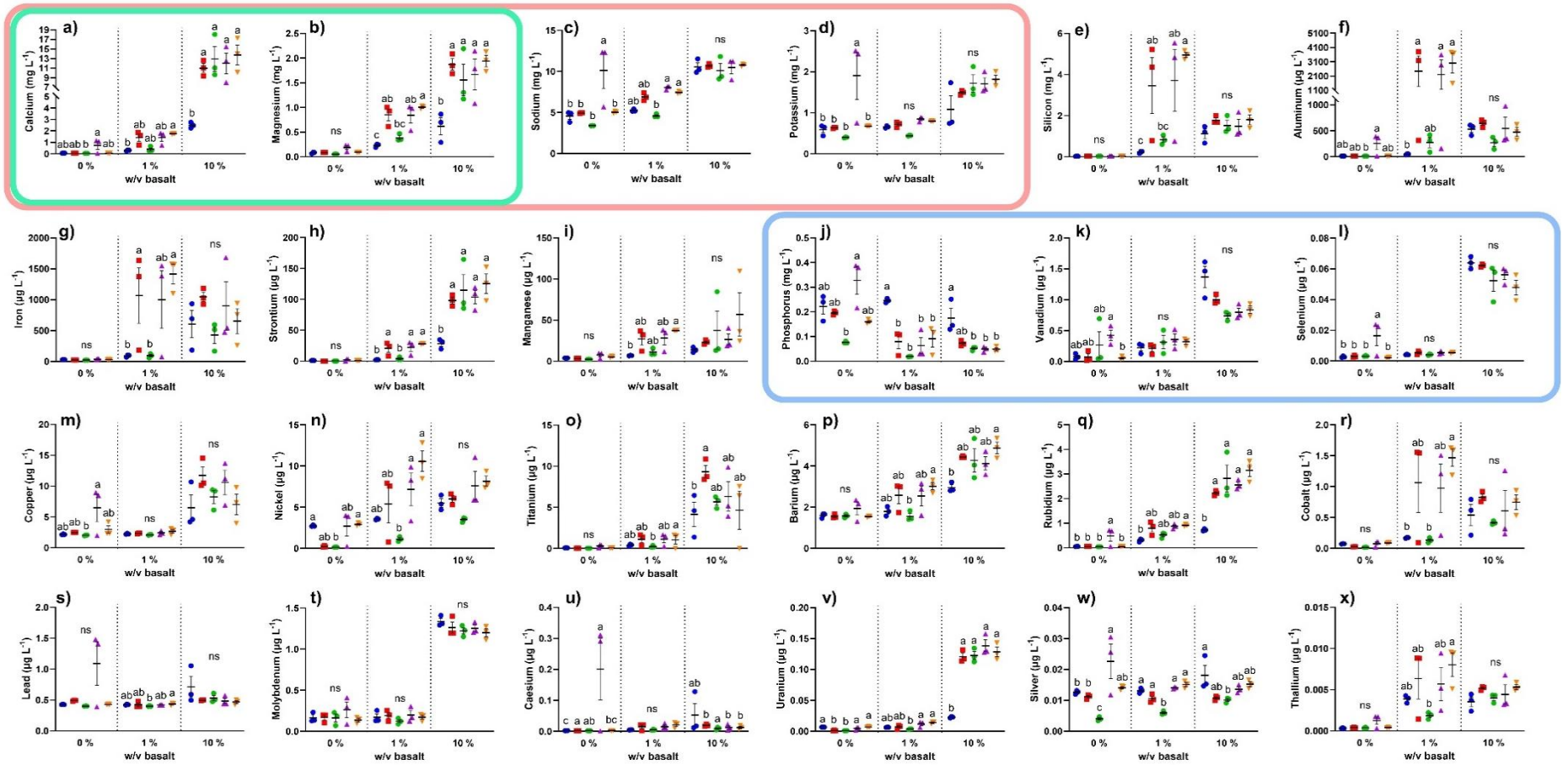

Figure 1. Effects of basalt treatments and *B. thailandensis* Organisms (●) Control, (■) Wildtype, (●) CA1, (▲) CA2, and (▼) CA3 after 170 hours post inoculation compared to non-inoculated controls on mean (± 1 standard error) dissolved elemental concentrations of (a) calcium, (b) magnesium, (c) sodium, (d) potassium, (e) silicon, (f) aluminium, (g) iron, (h) strontium, (i) manganese, (j) phosphorus, (k) vanadium, (l) selenium, (m) copper, (n) nickel, (o) titanium, (p) barium, (q) rubidium, (r) cobalt, (s) lead, (t) molybdenum, (u) caesium, (v) uranium, (w) silver, and (x) thallium. With dissolved cation ANOVA post hoc Tukey's test significance differences denoted by letters codes within basalt treatments, where means sharing the same letter are not significantly different ($p > 0.05$). Base cations, calcium and magnesium cations used for calculating potential CDR, and reduced DEC with bacterial inoculation are highlighted by pink, green, and blue frames, respectively.

Supplemental Table 1. Two-way ANOVA results of *B. thailandensis* growth media after 170 hours following growth.

Element	Two-way ANOVA effects	df	F value	P value
Sodium	w/v basalt	(1, 35)	142.31	< 0.0001
	Organism	(4, 35)	12.42	< 0.0001
	Interaction	(4, 35)	5.93	< 0.005
Magnesium	w/v basalt	(1, 35)	85.96	< 0.0001
	Organism	(4, 35)	4.28	< 0.05
	Interaction	(4, 35)	1.04	> 0.05
Calcium	w/v basalt	(1, 35)	273.64	< 0.001
	Organism	(4, 35)	6.37	< 0.0001
	Interaction	(4, 35)	3.26	< 0.05
Silicon	w/v basalt	(1, 35)	13.60	< 0.001
	Organism	(4, 35)	0.77	> 0.05
	Interaction	(4, 35)	0.10	> 0.05
Aluminum	w/v basalt	(1, 35)	9.24	< 0.01
	Organism	(4, 35)	1.68	> 0.05
	Interaction	(4, 35)	0.62	> 0.05
Manganese	w/v basalt	(1, 35)	22.35	< 0.0001
	Organism	(4, 35)	2.80	< 0.05
	Interaction	(4, 35)	0.42	> 0.05
Iron	w/v basalt	(1, 35)	24.39	< 0.0001
	Organism	(4, 35)	1.39	> 0.05
	Interaction	(4, 35)	0.20	> 0.05
Cobalt	w/v basalt	(1, 35)	16.92	< 0.001
	Organism	(4, 35)	2.08	> 0.05
	Interaction	(4, 35)	0.58	> 0.05
Strontium	w/v basalt	(1, 35)	190.30	< 0.0001
	Organism	(4, 35)	4.77	< 0.01
	Interaction	(4, 35)	1.86	> 0.05
Molybdenum	w/v basalt	(1, 35)	975.42	< 0.0001
	Organism	(4, 35)	1.00	> 0.05
	Interaction	(4, 35)	0.52	> 0.05
Silver	w/v basalt	(1, 35)	7.39	< 0.05
	Organism	(4, 35)	36.09	< 0.0001
	Interaction	(4, 35)	8.12	< 0.0001
Caesium	w/v basalt	(1, 35)	0.89	> 0.05
	Organism	(4, 35)	9.19	< 0.001
	Interaction	(4, 35)	3.08	< 0.05
Phosphorus	w/v basalt	(1, 35)	8.64	< 0.001
	Organism	(4, 35)	7.03	< 0.01
	Interaction	(4, 35)	1.21	> 0.05

Potassium	w/v basalt	(1, 35)	79.50	< 0.0001
	Organism	(4, 35)	8.22	< 0.001
	Interaction	(4, 35)	4.27	< 0.01
Titanium	w/v basalt	(1, 35)	242.69	< 0.0001
	Organism	(4, 35)	6.37	< 0.05
	Interaction	(4, 35)	1.41	> 0.05
Vanadium	w/v basalt	(1, 35)	148.583	< 0.0001
	Organism	(4, 35)	1.52	> 0.05
	Interaction	(4, 35)	4.97	< 0.01
Lead	w/v basalt	(1, 35)	5.21	< 0.05
	Organism	(4, 35)	1.27	> 0.05
	Interaction	(4, 35)	0.11	> 0.05
Nickel	w/v basalt	(1, 35)	17.74	< 0.001
	Organism	(4, 35)	6.99	< 0.001
	Interaction	(4, 35)	0.65	> 0.05
Copper	w/v basalt	(1, 35)	108.07	< 0.0001
	Organism	(4, 35)	2.66	< 0.05
	Interaction	(4, 35)	1.22	> 0.05
Uranium	w/v basalt	(1, 35)	819.37	< 0.0001
	Organism	(4, 35)	13.74	< 0.0001
	Interaction	(4, 35)	23.96	< 0.0001
Selenium	w/v basalt	(1, 35)	518.85	< 0.0001
	Organism	(4, 35)	3.38	< 0.05
	Interaction	(4, 35)	2.35	> 0.05
Rubidium	w/v basalt	(1, 35)	154.87	< 0.0001
	Organism	(4, 35)	5.87	< 0.01
	Interaction	(4, 35)	3.14	< 0.05
Barium	w/v basalt	(1, 35)	135.667	< 0.0001
	Organism	(4, 35)	3.94	< 0.01
	Interaction	(4, 35)	1.43	> 0.05
Thallium	w/v basalt	(1, 35)	12.68	< 0.01
	Organism	(4, 35)	0.80	> 0.05
	Interaction	(4, 35)	0.19	> 0.05

Chapter 3: Effect of rock dust additions on abundance of carbonic anhydrase genes in US corn belt soil- a metagenomic analysis

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Abstract:

Enhanced weathering (EW) is receiving attention as a carbon dioxide removal (CDR) technique due to its favourable CDR rates and cost, with the potential for further enhancement through the stimulation of biological weathering in soils. Carbonic anhydrase (CA), an enzyme encoded for in soil bacteria and fungi, catalyses carbonic acid formation and has been shown to increase rock dissolution rates when added to mineral amended soils and liquids. To investigate the *in situ* genetic abundance of CA genes and their response to rock dust applications, soil was collected from basalt-amended (50 tonnes ha^{-1} $year^{-1}$ from 2016 to 2019) and control plots at the University of Illinois Energy Farm. DNA metagenomic samples were extracted from topsoil during the 2019 *Glycine max* (soybean) and 2020 *Zea mays* (maize) growing seasons, and a CA database was created to identify CA genes while a PSORTb analysis was performed to estimate their cellular localisations. PERMANOVA community analysis of CA genes and the marker gene *rpoA* showed no significant effect of basalt amendment on either community. Grouping CA genes by predicted cellular localisation also revealed no significant effect on their abundance. Suggesting there was no selective pressure for CA genes in mineral-amended soil, regardless of their predicted localisation. However, the high abundance of CA genes, ~9.6 times more than the universal single-copy gene *rpoA*, suggests an important role in cellular functions. It is also possible that CA genes could be more transcriptionally or enzymatically active in rock-amended soils, which was beyond the scope of this study.

3.1. Introduction

Globally, enhanced rock weathering (ERW), where calcium and magnesium silicate rock dusts are applied to agricultural soils, is being tested as a natural carbon dioxide removal (CDR) strategy with the potential for large-scale implementation (The Royal Society, 2018). Global-scale geochemical modelling estimates suggest that if ERW is rapidly deployed, it could sequester carbon dioxide (CO_2) at a rate of 0.5 to 2 gigatonnes (Gt) per year by 2050 (Beerling et al., 2020). This means that in the last 50 years of the century alone, ERW could contribute between 25 and 100 Gt of CDR, which would be significant in relation to the 1000 Gt removal required by the end of the century limit global warming to 1.5 °C (The Royal Society, 2018; Smith et al., 2023). China, India, and the United States (US) were identified as having the greatest potential for CDR through ERW, with the US specifically estimated of having the potential to contribute between 0.11 and 0.42 Gt of CDR per year by 2050 (Beerling et al., 2020). A more recent techno-economic model by Zhang et al. (2023) has estimated that if ERW is implemented across croplands in the Midwestern US it has the potential to remove 225 to 1020 kg of atmospheric CO_2 per tonne of rock dust spread, with an estimated cost of US$ 45 to 472 per tonne of net CO_2e absorbed. This agricultural region majorly contributes to global grains, producing over 30% of the world soy (*Glycine max*) and maize (*Zea mays*) yields (FAO, 2017).

Although ERW CDR potential and costs are currently comparable with some other nature based strategies, such as the creation and addition of biochar to soils, or bioenergy with carbon capture and storage- BECCS (Zhang et al., 2023), further enhancing the rates of ERW would increase its benefits. Though the field is very nascent, research has commenced identifying ways to accelerate ERW in soils. For instance, Niron et al. (2024) in a mesocosm experiment demonstrated that when soil amended with a calcium feldspar mineral was inoculated with the plant growth promoting bacteria *Bacillus subtilis,* it significantly increased the CDR of the soil by 1.4

tonnes CO_2 ha^{-1} to a total of 3.7 tonnes CO_2 ha^{-1}. It is important to note the absence of plants in this study, so its relevance to cropped soils is unclear as *B. subtilis* inoculation into soils supporting plants may produce different results. However, a recent study in pre-print, funded and conducted by Andes Ag, Inc., measured CO_2 found in the soil using an acid treatment to release accumulated carbonate-carbon, and found that inoculating soybean fields with *B. subtilis* increased soil inorganic carbon drawdown by approximately 2.0 tonnes C ha^{-1} $year^{-1}$ attributed to the enhanced weathering of feldspar (Timmermann-Aranis et al., 2024).

As well as identifying soil microorganisms that may increase rates of weathering in the soil, researchers are also identifying cellular products that could be responsible for these increases or could be used to chemically increase rates of weathering. For example, recent research has shown that siderophore biosynthesis within microbial communities living on basalt rock grains in an ERW field trial was significantly upregulated (Epihov et al., 2024). It was further shown, when the synthetic iron chelator potassium-ethylenediamine-*N*,*N*′-bis-2-hydroxyphenylacetic acid (K-EDDHA) was incubated in soils amended with basalt but no plants, its soil concentrations were significantly correlated to the soil exchangeable calcium. The K-EDDHA either increased weathering through direct interactions with the mineral and/or by stimulating siderophore biosynthesis by the soil microorganisms due to induced iron limitation (Epihov et al., 2024). The previous studies that have tested methods to increase biological and biochemical driven weathering in soils have either added biological amendments to directly increase rates of rock weathering or they have applied additives to indirectly increase rates of weathering through stimulating the native soil microbial community (Figure 1).

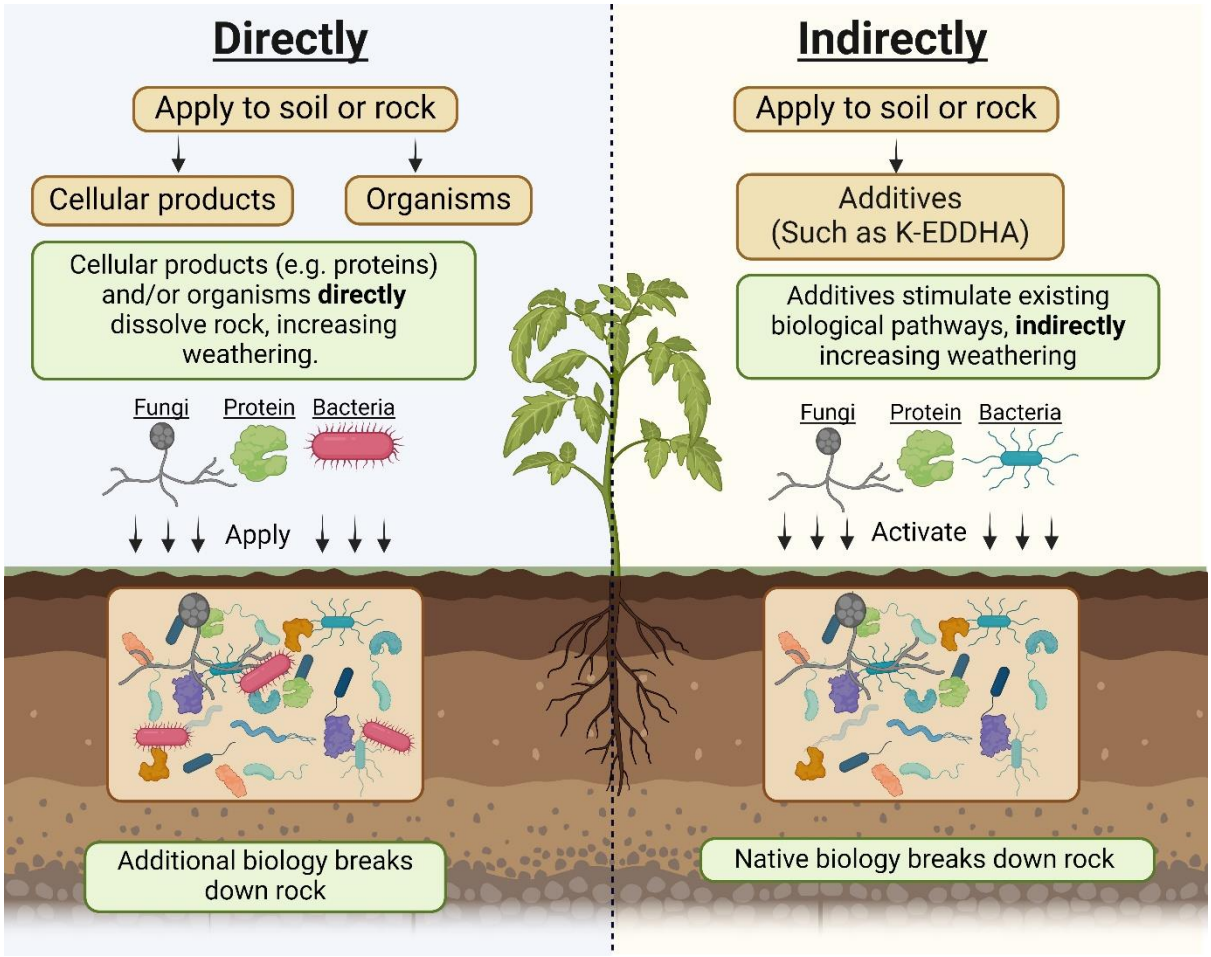

Figure 1. Concept figure demonstrating the direct and indirect methods of biological acceleration of rock weathering in soils. Created in biorender.com.

Another cellular product which may help accelerate mineral weathering and CDR is carbonic anhydrase (CA). CA is capable of catalysing the formation of carbonic acid from CO_2 dissolved in water. This is useful in industry as it may aid in preventing CO_2 emissions from flue gas, with research recently focusing on immobilising the enzyme to stabilise it and prevent degradation (Russo et al., 2022; Liu et al., 2018). Outside of industry, CA has been a focus of weathering research for more than a decade. Early research demonstrated that when bacterial derived CA was incubated with wollastonite the dissolution rate increased by 4.78×10^{-4} mg calcium g^{-1} wollastonite min^{-1} (Xiao et al., 2015). Furthermore, when proteins were extracted from bacteria and added to a column containing crushed

limestone the addition of a known CA inhibitor acetazolamide resulted in a significant reduction in calcium leachate (W Li et al., 2007). In soils, CA is estimated to increase carbonic acid production by approximately 10 to 300 fold (Wingate et al., 2009), which if harnessed could vastly improve ERW CDR potential, as carbonic acid is the only acid that would result in the immediate absorption of CO_2. Other weathering processes would not immediately result in CDR, they may however increase CDR through alternative means such as increasing the surface area to volume ratio available for weathering or preventing the formation of crusts on the surface of the rock, which could inhibit weathering. For instance, siderophores in the environment can dissolve the oxidised form of iron Fe^{3+} (Xie et al., 2024), which in the critical zone of soils where oxygen is present would normally tend to form hydrated iron oxide (ochre) gels coating mineral surfaces following the dissolution from any of iron-bearing minerals.

In soils, little is known about CA and whether it is utilised by soil microorganisms to degrade rock. *In vitro* research has indicated it has a role, at least in microbial functioning, as when *Bacillus mucilaginosus* was grown in a wollastonite amended medium that was calcium deficient all five of its CA encoding genes exhibited significantly increased expression levels relative to a free calcium rich media following 6 days of growth (Xiao et al., 2015). While research with bovine CA applied to alkaline soils showed it increased CO_2 hydration and likely carbonic acid formation (Sauze et al., 2018). The possible industrial-scale use of CA to accelerate ERW and CDR is being pioneered in field trials funded by two companies, Veolia and Fabricnano, using a novel rock immobilised CA (Peplow, 2024). Theoretically this should potentially stabilise CA in soils, similarly to the aforementioned industrial flue gas CA research, while also allowing it to directly act on the rock in the environment that surrounds the rock named the mineralosphere (Uroz et al., 2015), which is alkaline with rocks such as

basalt. To date, no published research has investigated whether CA's found in soil microorganisms play a meaningful role in *in situ* rock weathering. Furthermore, it remains unknown whether certain taxonomic groups of soil microorganisms are more or less significant in CA driven weathering processes, or if CA plays a significant natural role in mineral weathering in soils.

To date, insufficient attention has been given to the multiple potential cellular localisation sites for CA, as highlighted by Sauze et al. (2018). Currently CA weathering research has focused almost exclusively on extracellular forms of CA. However, as detailed by Chapter 2, which reported effects of CA knockout mutants on mineral weathering *in vitro* with *Burkholderia thailandensis*, intracellular or membrane bound CA's could also potentially influence weathering (see Figure 2). Extracellular carbonic anhydrase (CA) activity in soils could arise from enzymes actively secreted by living cells, or from intracellular and cell wall-bound enzymes released through cell death or lysis. Both extracellular enzymes and those released by lysis could adhere to soil colloids, where they may be stabilized, protected from degradation, and retain their activity (Nannipieri, 2006). Additionally, products of cytosolic CA catalysis could be actively excreted from cells into the soil. Our previous work found that when a CA of *B. thailandensis* that was predicted to be cytosolic was inactivated in a liquid broth containing basalt grains there appeared to be a significant reduction in weathering, possibly due to acid generation within the bacteria being inhibited. This along with the work to date on extracellular CAs leaves unanswered questions regarding the relative importance of soil microbial CAs that are intracellular, cell wall associated, and extracellular, in rock weathering in soils.

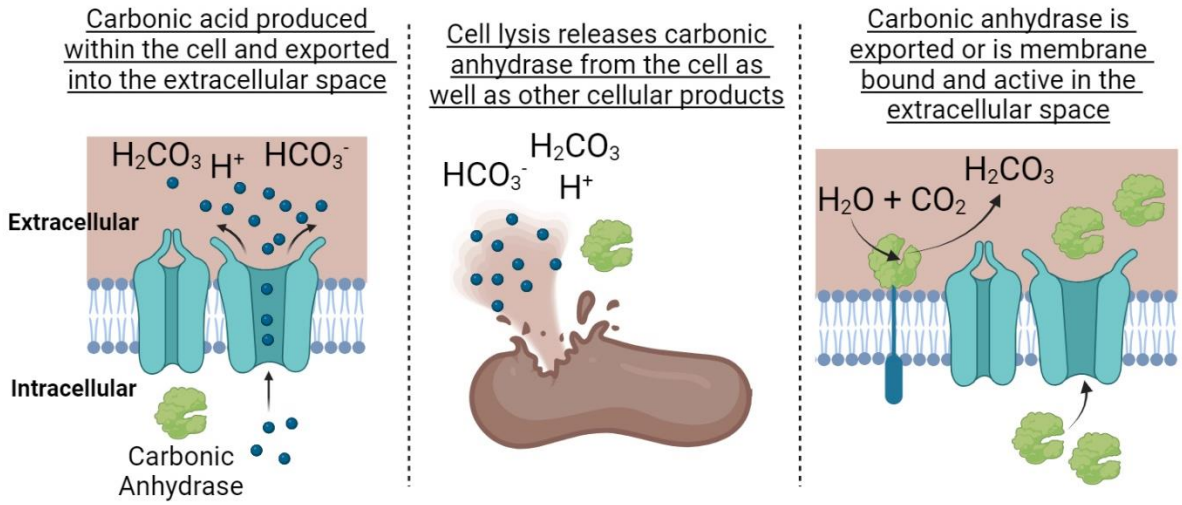

Figure 2. Potential mechanisms of CA catalysis of extracellular chemical change. Created in Biorender.com and taken from Chapter 2.

The aim of the present study was to assess whether in-field metagenomic measurements support *in vitro* and mesocosm results suggesting that CA plays a role in rock weathering. Additionally, we sought to determine whether CA genes with different predicted cellular or extracellular localisations respond differently in basalt-amended soils, and to identify specific taxonomic groups containing CA that show significant differences between basalt-amended and control plots.

3.2. Methods

3.2.1. Field site and experimental set up (conducted by the Leverhulme Centre for Climate Change Mitigation (LC3M))

Soil was collected from both basalt amended and control plots at the University of Illinois Energy Farm (40°3′46″N, 88°11′46″W). Rock amended plots had Blue ridge basalt, analysed in detail by Lewis et al. (2021), applied at a rate of 50 t ha^{-1} to a large 3.8 ha plot (known as plot 5) as well as two subplots in each of four separate plots (see Figure 3). Control, non-basalt amended, subplots were also prepared within all four plots with an additional non-amended large 3.8 ha control plot prepared alongside the large basalt-treated plot. All plots have continually been growing a soybean (*Glycine max* L.) and maize (*Zea mays* L.) rotation in a 1 season: 2 seasons ratio, with rock amendment beginning in November 2016 and continuing annually until 2019, totalling to 200 t of basalt being applied to treated plots in that time. In addition, both control and basalt amended plots received 28% urea ammonium nitrate at 202 kg N ha^{-1} immediately prior to maize, but not soybean planting.

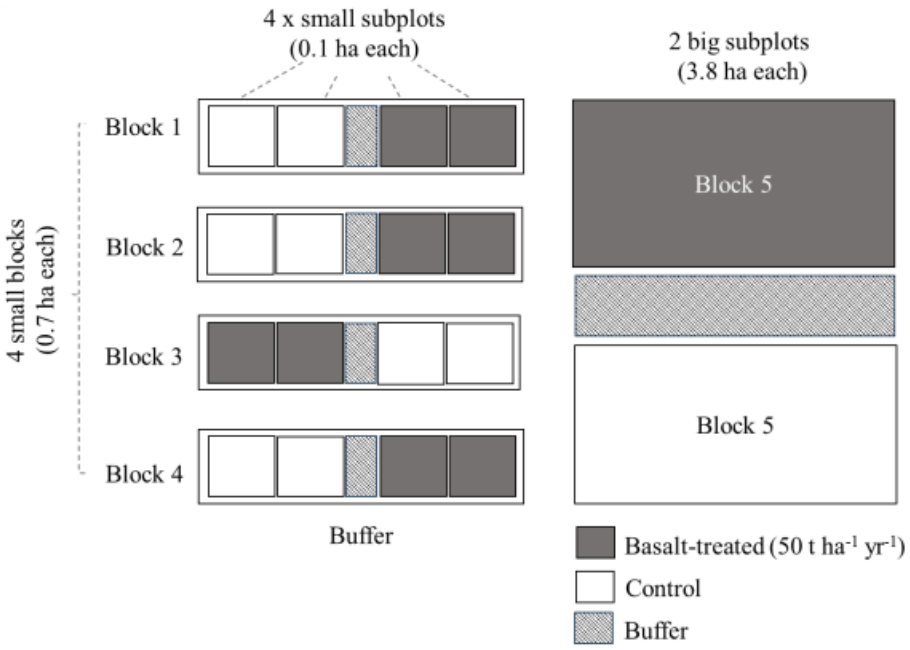

Figure 3. The design of the field plots at the Energy Farm site in the US (from Beerling et al., 2024).

The basalt dust was applied using a tractor-powered lime spreader, followed by chisel ploughing to a ~18 cm depth within a day of application. Full details of the other measurements made on the crop performance and soil chemical changes in the basalt versus control plots are described in Beerling et al. (2024), and Kantola et al. (2022, 2023).

3.2.2. DNA sampling and extraction (Performed by Dimitar Epihov in tandem with collaborators at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign)

Soil metagenomic samples were extracted from the upper 10 cm depth of the soils on 25/07/2019 during the soybean year and 19/08/2020 during the maize year. Soil was collected from bulk soil taken from uprooted root balls lifted using a shovel and spade, with the equipment being rinsed and wiped with dH_2O and 70% ethanol in-between collected samples to avoid cross-contamination. Both years, samples were randomly collected from control and basalt-treated subplots. As detailed in Table 1, during the soybean year metagenomic analysis was performed for 1-3 samples per plot. In the maize year this was increased to 3 replicates from both basalt and control subplots in blocks 1 through 4, with 4 replicates collected from both control and basalt-treated 3.8-ha blocks. Following soil collection, they were stored in 50 mL sterile tubes and either snap-frozen in liquid N_2 or placed on dry ice in the field. Upon return to the lab, samples were stored at -80^{o}C prior to their extraction for total DNA. Total DNA was extracted using the Qiagen's DNeasy PowerSoil Pro Kits and instructions therein with slight modifications. Namely, samples were shaken with the PowerSoil beads for homogenization at 2,700 rpm for 30 min on a horizontal shaking plate instead of using the PowerSoil vortex adaptor. Total DNA was handled to the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign for sequencing. Samples were quantitatively and qualitatively assessed using a Qubit instrument, and libraries passing internal quality

checks prepared using the Illumina TruSeq kits and sequenced as 2 x 150 bp paired reads on a 1 x S4 lane of the Illumina's NovaSeq sequencer.

Table 1. Metagenomic sampling distribution across both years from plots 1 to 5 (C is control, B is basalt-treated as shown in Fig. 3)

Year/crop	Block/plot									
	1		2		3		4		5	
	C	B	C	B	C	B	C	B	C	B
2019 (soybean)	3	3	1	1	2	2	1	2	1	0
2020 (maize)	3	3	3	3	3	3	3	3	4	4

3.2.3. CA and *rpoA* alignment and relative abundance

Bacterial carbonic anhydrase and *rpoA* genes were extracted from AnnoTree (Mendler et al., 2019) a database containing all sequenced prokaryotic genomes to date available in the Genome Taxonomy Database GTDB database annotated against the Kyoto Encyclopedia of Genes and Genome database-KEGG (Kanehisa et al., 2023; Kanehisa, 2000, 2019), Pfam (Mistry et al., 2021), and Tigrfam (Li et al., 2021) databases. The DNA-directed RNA polymerase alpha chain encoded by the *rpoA* gene (KEGG Orthologue or KO number K03040 in KEGG) is a universal single-copy gene (Wang et al., 2022) and as such all its sequences were acquired to provide community taxonomic identification and to normalise CA's relative abundance to account for the different sequencing depths seen between samples, as previously described in Epihov et al. (2024). As both CAs and *rpoA* genes were obtained from multiple databases duplicate reads were removed based on the gene sequence. The gene information, such as the taxonomy, was then used to create the unique FASTA title line for each sequence. Within Galaxy (www.usegalaxy.eu) (Afgan et al., 2018), an online open-source data analysis platform and processing server, all FASTA sequences were used to make a DIAMOND reference database, which was

subsequently used in the DIAMOND alignment tool (Buchfink et al., 2015). All metagenomes were searched using DIAMOND against our created CA and *rpoA* containing reference databases and the raw DNA sequencing reads were used to identify potential CA genes found in the soil. Alignments were scored using the BLOSUM62 matrix, with only the lowest expectation value (E-value) with a maximum cut off 1×10^{-8} being retained for each raw sequence read. Subsequently, gene abundance of CA and *rpoA* was determined by dividing the alignment length by the total estimated gene length, thereby, accounting for differences in gene size. For each sample, this fraction of CA gene length was then divided by the combined total of *rpoA* genes found within the sample to then normalise the data across runs as mentioned above. Multiple raw sequence alignments to the same gene then had their normalised CA gene coverage lengths combined, using the gene sequence, to provide a total estimate of genes found in the soil relative to the universal single-copy gene *rpoA*. Once localisation information was included in the results as described below CA relative abundance to *rpoA* was assessed across different localisations and taxonomic levels by totalling the CA's relative abundance counts across these respective levels in each sample.

3.2.4. PSORTb predicted CA localisations

Bacterial CA genes, acquired as detailed above, were separated into Gram-positive or Gram-negative groups, with unspecified groups being separated based on their closest known related phylum's gram state. Once selected the two FASTA formatted databases were analysed with PSORTb version 3.0.3 (Yu et al., 2010) through a docker system (Merkel, 2014) operated within a Linux Ubuntu virtual machine. PSORTb v.3.0 includes several analytical modules, each targeting specific biological characteristics that indicate subcellular localisation using gene sequences. Both gram positive and negative organism proteins can localise to the cytoplasm, cytoplasmic membrane, and extracellular space (Yu et al., 2010). However,

gram-positive organisms can also localise in the periplasm and outer membrane, while gram-negative organisms can localize in the cell wall (Yu et al., 2010). The analysis tools automatically applied by PSORTb includes: SCL-BLAST & SCL-BLASTe, Support Vector Machines, Motif & Profile Analysis, Outer Membrane Motif Analysis, ModHMM, and Signal Peptide Analysis. The outputs of these tools are then ranked based on their probability scores with a probability score above 7.5 being considered sufficient for confidently assigning a single localisation. Sequences with multiple probabilities above 4.5 (gram negative) or 5 (gram positive) are assigned a "this protein may have multiple localisation sites". It is important to note that PSORTb does have certain limitations including its inability to detect lipoprotein motifs, and the tool is only as powerful as the database that is used to train it, where it is difficult to acquire pure non-cytoplasmic sequences (Yu et al., 2010).

Subsequent results were parsed using the tidyverse package (Wickham et al., 2019) in R (R Core Team, 2024). To create a functional column format and then concatenated to create a CA database containing sequence information along with their predicted localisations and taxonomic origins. This database information was then transferred to the alignment results file by combining columns based on the gene sequence using the dplyer function in the tidyverse package (Wickham et al., 2019).

3.2.5. Statistical analysis and figures

Permutational multivariate analysis of variance (PERMANOVA), was used to analyse the data as it is one of the most flexible types of non-parametric analysis and due to its permutation design has a low false discovery rate (Thorsen et al., 2016).

To assess how community structure of *rpoA* and CA genes was affected across basalt and control treatments, years/crops, and plots for different bacterial taxonomic levels, a PERMANOVA analysis was performed

(Anderson, 2017), with NMDS figures created to visualize the variance. The tidyverse package (Wickham et al., 2019) was used for data manipulation, while the vegan package (Oksanen et al., 2024) was used for PERMANOVA and NMDS coordinate generation. The ggplot2 (Wickham, 2016) in combination with ggforce (Pedersen, 2024) packages were used to create the NMDS visualizations. To compute whether there were significant differences between the variables basalt treatment, year/crop, and plot, data was grouped at each taxonomic level phylum, class, order, and family within each sample, with the predicted gene coverage data prior to *rpoA* normalisation being used. Following data grouping an abundance matrix was created and a distance matrix was calculated using the Bray and Curtis (1957) dissimilarity metric. The PERMANOVA variation in the distance matrix due to variables treatment, year/crop, and plot were then statistically assessed using the adonis2 function in the vegan package. This analysis was conducted for both the *rpoA* and CA aligned results, as well as for a combined dataset of both, to assess the differences between the *rpoA* and the CA community.

To assess whether CA relative abundance to *rpoA* differed significantly between basalt and control fields, a PERMANOVA test was performed, using treatment, year/crop, and plot as variables. CAs total relative abundance in basalt amended vs control plots was assessed by totalling the CA relative abundance across each sample and performing the above statistical test. Subsequently, within each sample, data was separated based on each alignments predicted localisation (cytoplasmic, cytoplasmic membrane, extracellular, periplasmic, outer membrane, multiple localisation sites, or unknown localisations), and the treatment effects were statistically determined through using the same method above where the relative abundance was totalled across the specified groups. Additionally, within each separated localisation, taxonomic groups at the phylum, class, order, or family levels were further subdivided and statistically assessed to identify

any taxonomic groups that differed due to treatment. It is important to note that only taxonomic groups present at all field locations were included in this analysis to ensure adequate replication.

3.3. Results

PERMANOVA analysis compared the *rpoA* bacterial community structure in relation to the variables: basalt treatment, year/crop, and plot at the four taxonomic levels: phylum, class, order, and family (Figure 4). The model estimated that the basalt treatment accounted for 0.8%, 2.1%, 2.6%, and 3.0% of the variation at each respective taxonomic level and as such, at no level was the treatment effect considered statistically significant. This is shown by the 95% confidence interval ellipses seen in Figure 4, which show consistent overlap between treatment groups across all bacterial taxonomic levels, reflecting the lack of differences in response to basalt

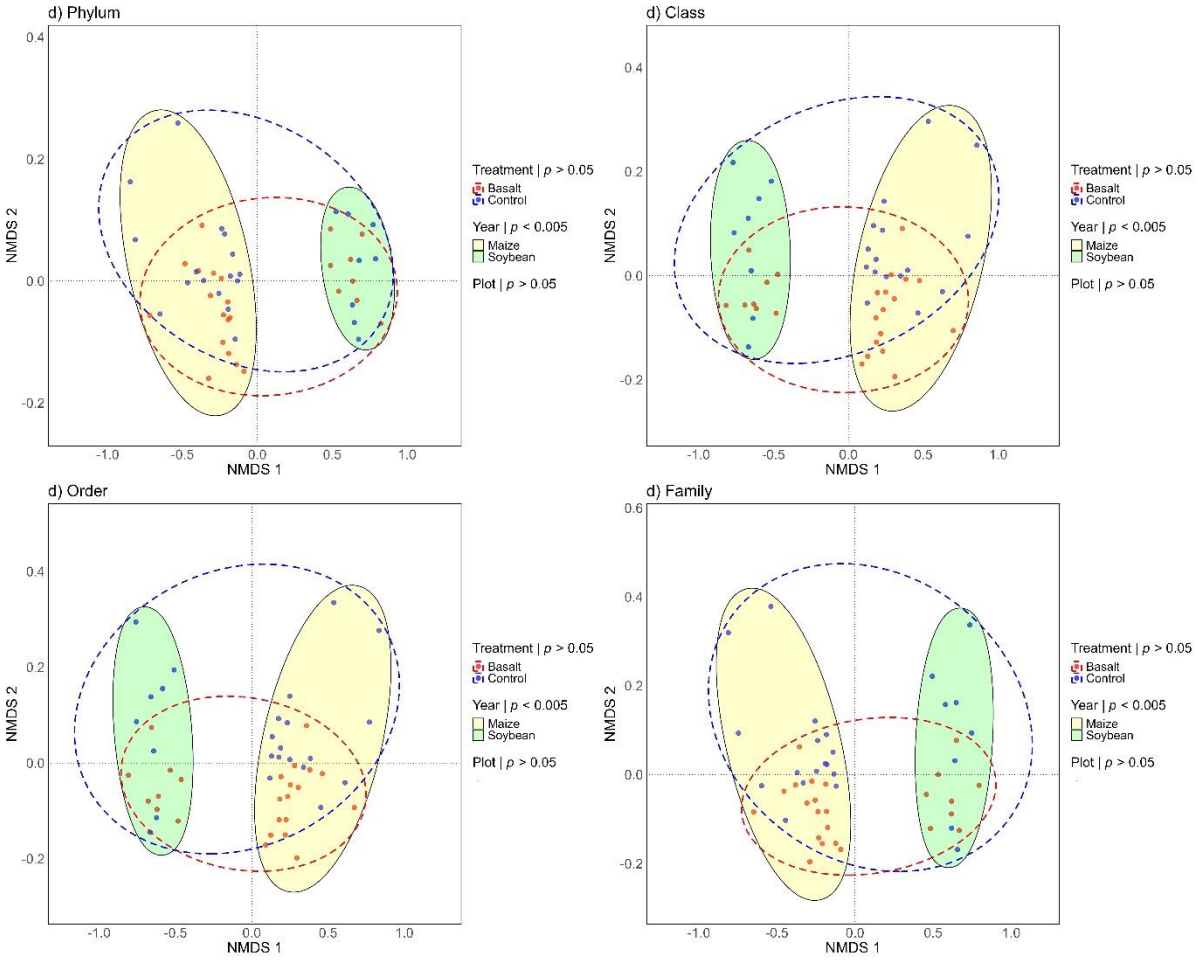

Figure 4. NMDS plots of *rpoA* gene total abundance with red and blue treatment coordinates. Solid-filled 95% confidence interval ellipses represent year and crop, with yellow for maize and green for soybean, while dashed empty ellipses indicate treatment, with red for basalt and blue for control. PERMANOVA significant differences are noted for treatment, year, and plot for each panel looking at four descending taxonomic levels: (a) phylum, (b) class, (c) order, and (d) family.

Similarly, there were no significant differences across all taxonomic levels due to the five plot locations. When looking at the effect of year on the *rpoA* community distinct 95% confidence interval ellipses (Figure 4) and PERMANOVA significant differences ($p < 0.005$) were observed across all bacterial taxonomic levels. This outcome may have been expected given that soybean is a N_2-fixing plant and maize is not, and the maize years received 240-320 kg N ha^{-1} yr^{-1} doses of 28%-UAN urea fertilizer that was not used in the years when soybean was grown.

When the same analysis was conducted on the identified bacterial CA genes, basalt treatment again did not significantly affect the CA community composition (Figure 5). The PERMANOVA model estimated that the basalt treatment accounted for only 1.7%, 1.8%, 1.9%, and 2.0% of the variation at the phylum, class, order, and family taxonomic levels, respectively. Across each taxonomic level, similar to the *rpoA* analysis, year again significantly affected ($p < 0.005$) the CA community composition accounting for 23.7%, 23.6%, 23.4%, and 23.1%, of the variance seen at each descending taxonomic level. Interestingly, despite these significant differences, the ellipses by year overlap and as can be seen in Figure 5 the samples from the maize year appear to be separated into two distinct groups. Although there were significant effects of sampling plot ($p < 0.05$) on the CA community composition it did not appear to explain the differences seen in the maize year when tested (not shown).

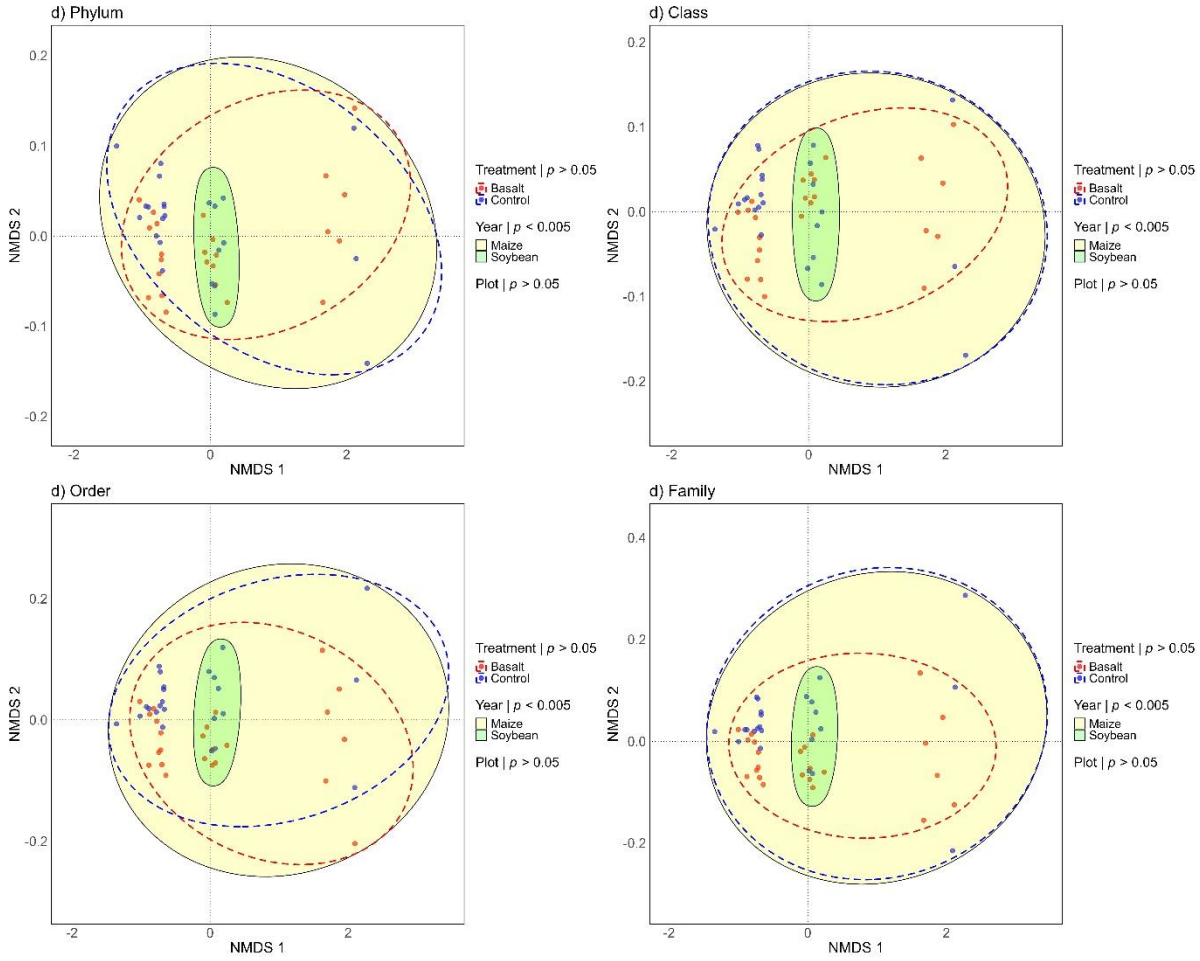

Figure 5. NMDS plots of CA gene total abundance with red and blue treatment coordinates. Solid-filled 95% confidence interval ellipses represent year and crop, with yellow for maize and green for soybean, while dashed empty ellipses indicate treatment, with red for basalt and blue for control. PERMANOVA significant differences are noted for treatment, year, and plot for each panel looking at four descending taxonomic levels: (a) phylum, (b) class, (c) order, and (d) family.

When CA community composition was compared to the *rpoA* communities they appeared to be significantly different ($p < 0.005$) regardless of taxonomic level, as evidenced by their distinct 95% confidence interval ellipses (Figure 6). When both gene datasets were tested together, basalt treatment did not appear play a role in their combined community composition at any taxonomic level, while the year when sampled did appear to significantly affect ($p < 0.005$) the combined community composition.

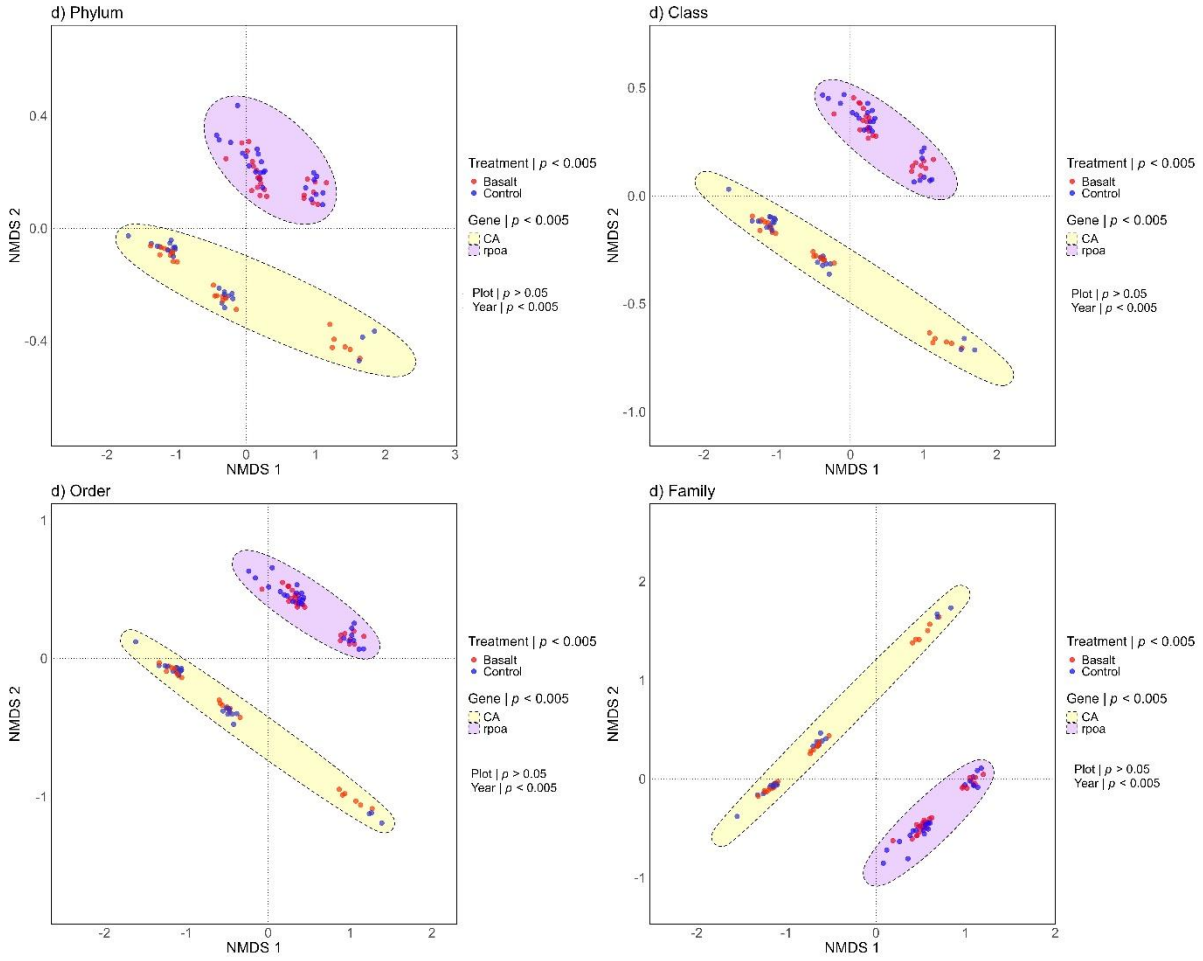

Figure 6. NMDS plots of combined CA and *rpoA* gene total abundance with red and blue treatment coordinates along with dashed-filled 95% confidence interval ellipses representing the corresponding gene, with yellow for CA and purple *rpoA*. PERMANOVA significant differences are noted for treatment, gene, plot, and year for each panel looking at four descending taxonomic levels: (a) phylum, (b) class, (c) order, and (d) family.

Through comparing the total CAs identified in both basalt amended and control plots across both years there appeared to be no significant differences in CA's relative abundance to *rpoA* (Figure 7a). When CA's were further separated based on their PSORTb predicted localisations and their relative abundances re-assessed there were still no significant differences seen between basalt and control plots. Figure 7 (b through h) illustrates the mean relative abundance of predicted localisations from both basalt-treated and control soils. Among the identified CA genes, those with cytoplasmic

predicted localisations exhibited the highest abundance, followed by genes with unknown localisations with a further progressive decrease in abundance from cytoplasmic membrane, periplasmic, extracellular, and outer membrane predicted localisations, spanning 5 orders of magnitude of abundance across all categories.

When compared to the distribution of localisations found within the CA database (Figure 8a), cytoplasmic, unknown, cytoplasmic membrane, multiple localisation sites, periplasmic, extracellular, and outer membrane predicted localisations accounted for 82, 10, 4, 3, 0.9, 0.6, and 0.00002% of the genes acquired from Annotree (Figure 8a).

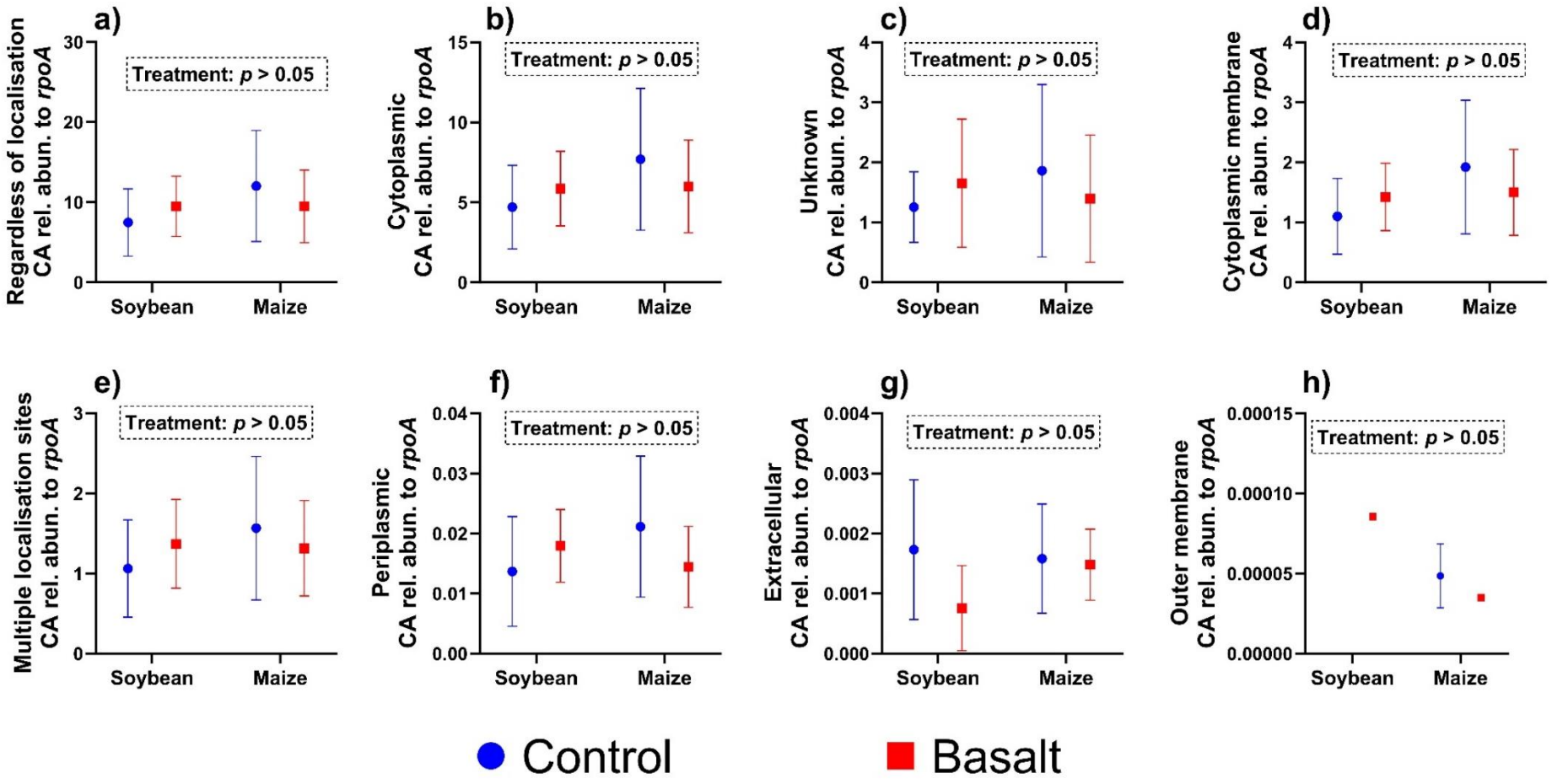

Figure 7. CA relative abundance to the marker gene *rpoA* from both soybean and maize years with mean ± SE of control (blue) and basalt amended (red) soils. For (a) total CA identified, and in descending abundance of predicted localisations, (b) cytoplasmic, (c) unknown, (d) cytoplasmic membrane, (e) multiple localisation sites, (f) periplasmic, (g) extracellular, and (h) outer membrane.

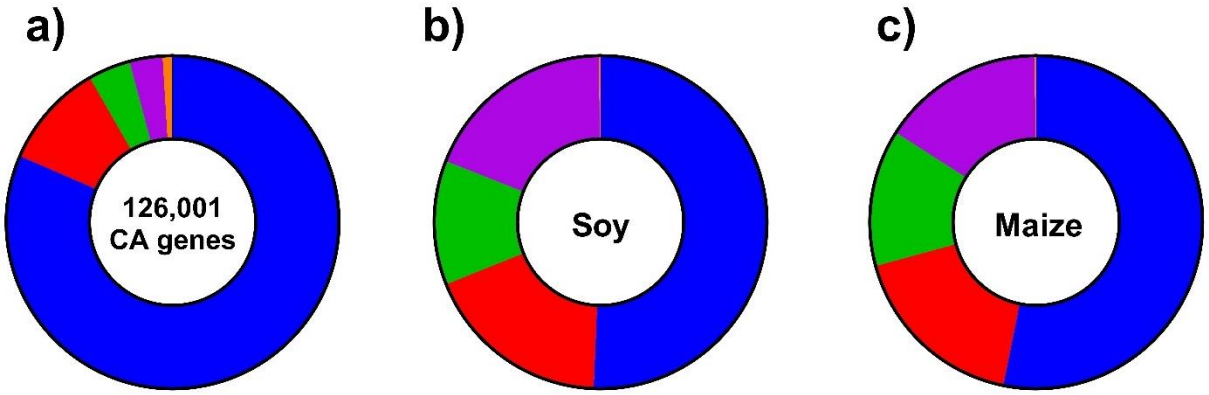

Figure 8. Distribution of CA gene predicted localisations ■ cytoplasmic, ■ unknown, ■ cytoplasmic membrane, ■ multiple localisation sites, ■ periplasmic, ■ extracellular, and ■ outer membrane in the CA database (a) and the combined basalt and control mean distribution of predicted localisations from both the (b) soybean and (c) the maize year.

When control and basalt amended plots percent localisations were averaged for both the soybean (Figure 8b) and the maize (Figure 8c) year the distribution of measured genes did not perfectly match the database. For instance, CAs predicted to have multiple localisation sites exceeded the CA database value of 3% and ended up accounting for 18.8% and 15.8% of the CA's identified in the soybean and maize year, respectively. Indicating that the CAs within the Annotree database, which takes genes from many different environmental sources, may not accurately represent CAs found only within soils.

The small to non-existent standard error seen in the relative abundance of predicted outer membrane localised CAs (Figure 7h) highlights that not all plots had similar proportions of CA localisations. In this case, outer membrane localised CA only appeared in plots 1, 4 and 5, this is most likely due to outer membrane localised CAs only being identified twice in the CA database, but this could also be an indication that they are exceedingly rare in nature. When by plot contributions to CA's relative abundance are analysed it can be seen that in the maize year, control subplots in plot 2 had few CA genes identified regardless of their predicted localisations and despite the increased replication seen in the maize year.

Plot 2's control and basalt amended sub plots in the previous soybean year, though only representing 1 replicate, had similar total CAs identified in basalt amended plot in the maize year (Figure 9. Importantly plot 5, the 3.8 ha basalt amended field, was not sampled during the soybean year which is why it is not seen to contribute.)

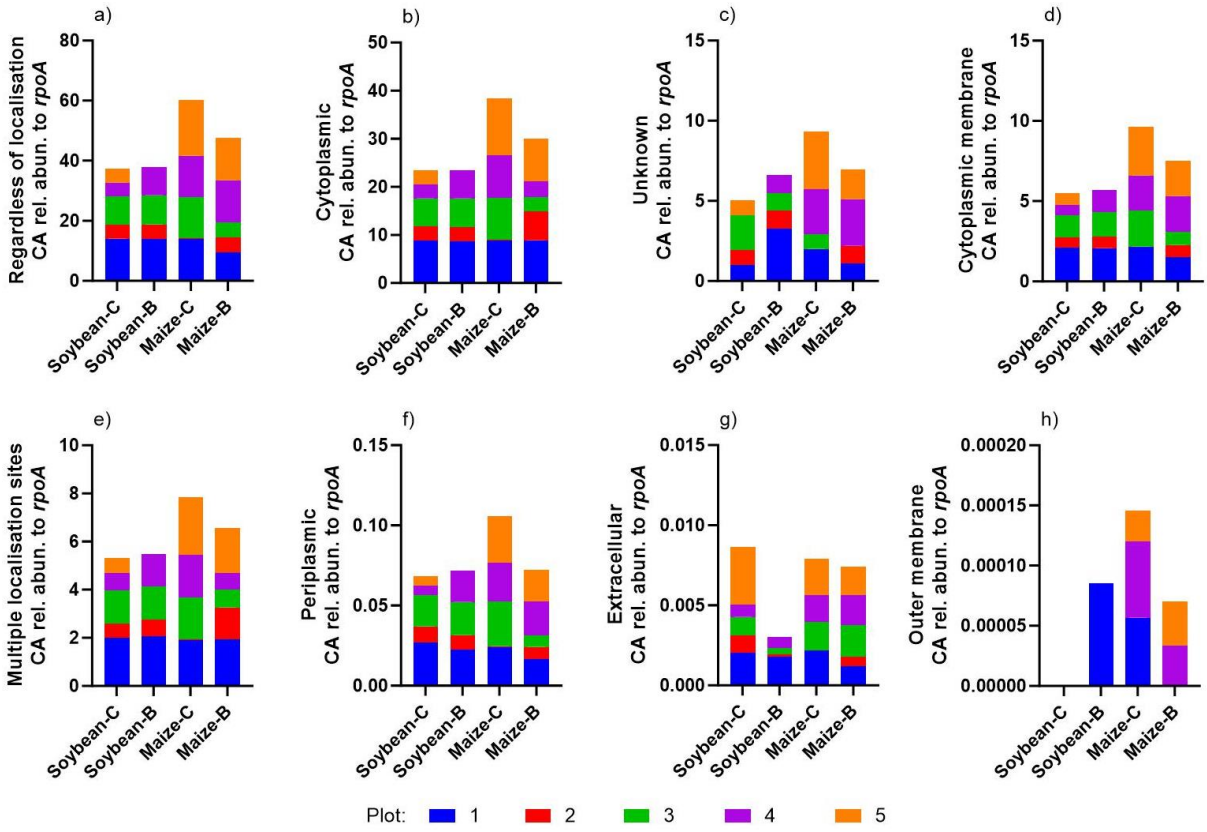

Figure 9. Average plot contribution of CA for soybean and maize, control (-C) and basalt-treated (-B) plots showing (a) total CA identified, and in descending abundance of predicted localisations, (b) cytoplasmic, (c) unknown, (d) cytoplasmic membrane, (e) multiple localisation sites, (f) periplasmic, (g) extracellular, and (h) outer membrane.

No significant differences in CA's relative abundance were seen between basalt treated and control soils, regardless of the predicted localisations (Figure 7). Despite this, some significant differences in individual phyla, class, orders, and families were seen in taxonomic groups that were present across all plots and so could be independently statistically

assessed (Supplemental Table 1). Across the taxonomic levels phylum, class, order, and family a total of 2, 5, 19, and 28 respective taxa had their CA relative abundance significantly affected by basalt amendment. In the majority of instances basalt amendment appeared to significantly reduce these taxa's CA relative abundance, as only 2 of the 19 orders and 3 of the 28 families had their CA relative abundance significantly increased due to amendment. In total, when related taxa are grouped, these increases in CA abundance only accounted for 0.184% and 0.187% of the total CA's identified across all soil plots. In contrast when all the contributions of the families with significant differences were totalled basalt resulted in a loss of 0.879% and 1.258% of the CAs identified across all plots from both soybean and maize years, respectively. Though it is important to remember that taxa that were significantly affected accounted for less than 5% of the CAs identified across all plots and would likely represent an even smaller number if CAs not identified in all plots were included. Also important to remember is that though PERMANOVA has a generally low false discovery rate, there is a risk that some of the taxa are false positives.

By first segregating the CA relative abundance data by predicted localisations it allows for taxa responsible for significant changes in differently localised CAs to be identified (Table 2). When performed the decreases seen in most identified taxa in Supplemental Table 1 are also reflected in Table 2. Only 1, 4, 12, and 12 phyla, classes, orders, and families respectively, were significantly affected by basalt amendment, with the majority of those being classed as cytoplasmic with 1, 4, 8, and 9 of those being identified at each descending taxonomic level. The three other predicted localisations, cytoplasmic membrane, multiple localisation sites, and unknown, only accounted for 4 significantly affected orders and 3 significantly affected families. Only the order *Acidimicrobiales* and a non-related family *JADJPG01* appeared to be significantly increased due to basalt amendment, but these taxa only accounted for less than 0.2% and 0.7% of

the total CA genes found across all plots in both the soy and maize year, respectively. In addition, across all plots no taxa which had extracellular or periplasmic predicted CA genes were significantly affected by basalt amendment.

Table 2. Significant between treatment differences in predicted localisation grouped CA gene abundances of taxa identified in all plots, along with associated percent difference relative to the non-amended control soils and the basalt amended taxonomies' mean percent contribution to the total CA's identified in the soil community. Related taxonomic groups are denoted by superscript number and taxa which significantly increased due to basalt amendment across both years are highlighted in red.

Taxa	Predicted localisation	Treatment *p* value	Change with basalt relative to control (%)		Basalt amended share of CA (%)	
			Soy	Maize	Soy	Maize
Phylum						
Eremiobacterota[1]	Cytoplasmic	0.029	-54.2	-43.7	0.059	0.057
Class						
Eremiobacteria[1]	Cytoplasmic	0.022	-44.8	-46.2	0.0539	0.0512
Ktedonobacteria[2]		0.007	-45.0	-55.3	0.0803	0.0491
Terriglobia[3]		0.028	-12.2	-51.1	2.1567	1.1217
UBA4802[4]		0.029	-22.1	-57.7	0.0177	0.0125
Taxa	**Predicted localisation**	**Treatment *p* value**	**Change with basalt relative to control (%)**		**Basalt amended share of CA (%)**	
			Soy	**Maize**	**Soy**	**Maize**
Order						
Baltobacterales[1]	Cytoplasmic	0.015	-38.6	-46.8	0.0502	0.0492
Ktedonobacterales[2]		0.007	-45.0	-55.3	0.0797	0.0489
Terriglobales[3]		0.01	-25.9	-62.1	0.9238	0.4683
UBA7540[3]		0.003	-61.1	-58.0	0.0327	0.0175
UBA4802[4]		0.027	-22.1	-57.6	0.0175	0.0125
Micropepsales[5]		0.035	-30.2	-48.9	0.1677	0.1018

Streptosporangiales[6]		0.045	-9.5	-47.2	0.4871	0.3962
ATCC43930		0.042	-28.3	-41.9	0.0843	0.0583
Acidimicrobiales[3]	Cytoplasmic membrane	**0.042**	**103.4**	**NA**	**0.0945**	**0.1913**
Streptosporangiales[6]		0.041	-11.1	-45.8	0.5208	0.3756
Bryobacterales[3]	Multiple localisation	0.008	-31.8	-49.3	0.3273	0.2441
Streptosporangiales[6]	Unknown	0.029	-18.5	-44.2	1.2016	1.1502
Family						
Baltobacteraceae[1]	Cytoplasmic	0.027	-38.6	-46.7	0.0476	0.0488
Ktedonobacteraceae[2]		0.012	-43.8	-51.5	0.0743	0.0471
Acidobacteriaceae[3]		0.025	-33.1	-64.5	0.1558	0.0967
SbA1[3]		0.011	-14.8	-60.2	0.5034	0.2436
Gp1-AA117[3]		0.01	-45.8	-68.3	0.0876	0.0386
UBA7540[3]		0.002	-61.1	-58.0	0.0322	0.0174
Micropepsaceae[5]		0.043	-30.2	-48.9	0.1651	0.1010
Koribacteraceae[7]		0.018	-29.5	-66.9	0.0881	0.0448
JADJPG01		**0.014**	**117.5**	**174.3**	**0.0617**	**0.0583**
Streptosporangiaceae[6]	Cytoplasmic membrane	0.042	-11.1	-45.8	0.5188	0.3747
Bryobacteraceae[3]	Multiple localisations	0.006	-31.8	-49.3	0.3082	0.2450
Streptosporangiaceae[6]	Unknown	0.032	-18.7	-44.2	1.2026	1.2099

3.4. Discussion

Our study has for the first time generated a database containing 126,001 distinct CA genes and compared this to soil DNA samples from control and basalt amended plots that have been under conventional soybean-maize rotation cropping in the corn belt of the US. Despite four consecutive years of 50 t ha^{-1} $year^{-1}$ basalt applications to treatment plots, there were no significant differences in the abundance of CA genes between control and treated soils. In addition, basalt amendment did not appear to significantly impact the predicted community composition that retained CA genes, or the relative abundance of these genes across all tested taxonomic levels. The reported average increase of ~ 0.9 pH units in basalt amended soils (Beerling et al., 2024) did not result in CA gene propagation, which is interesting in the context of Sauze et al.'s (2018) work where bovine CA became more active with increasingly soil alkalinity. When general community composition was assessed using *rpoA*, a universal single copy gene (Wang et al., 2022), there was again no significant treatment differences identified. As pH is considered to be one of the primary drivers of soil microbial diversity this runs contrary to what was expected (Fierer and Jackson, 2006). As some intensive agriculture techniques are known to significantly impact soil diversity, as reviewed by Gupta et al. (2022), it is possible that the annual ploughing and bi-annual mineral fertiliser applications could have been more responsible for soil diversity outcomes. Alternatively, the sequencing depth of the *rpoA* and/or CA database may not have been comprehensive enough, leading to the potential loss of rarer species in the former case or an artificially low gene recovery rate in the latter case.

In contrast to *rpoA* the CA community appeared to have significant by-plot variation further highlighting the complexity of CAs found in soils. This variation is highlighted in Figure 9 where the control samples from plot 2 during the maize year, despite the higher replication, had very few

CAs identified relative to the other plots sampled. One consistent and expected difference seen in both the *rpoA* and CA communities was the effect of crop rotation over time (Wang et al., 2023), where regardless of the taxonomic level the effect was strongly significant ($p < 0.005$) across both communities. Interestingly, in the CA community NMDS (Figure 4) there is a clear separation of points during the maize year. Although there were significant overall differences between the sampled field plots, when plot 95% confidence interval ellipses were plotted separately over the maize year no single plot was responsible for this separation indicating an unknown variable contributing to the variance. Some of this variance in the maize year could be attributed to there being twice as many basalt-amended and control replicates relative to the soybean year, perhaps resulting in greater spatial distances between samples. When both communities *rpoA* and CA recovered data was combined and statistically assessed the community composition was significantly different between the two genes, and there was again a significant year effect, but no significant treatment or plot effect. These results could suggest two things: either the CA community is not driving the soil's total community's taxonomic abundance, or that the natural soil community is not responsible for determining CAs community composition.

Though the communities that genetically encode CA genes did not appear to significantly change due to basalt amendment, it is still possible that different groups of CA's responded differently. Previous research has noted that the many potential cellular localisations of CA's in the environment have not been considered (Sauze et al., 2018), with the majority of studies focussed on extracellular CA, including sources such as bovine CA that would not occur naturally in soils. These bovine CA studies provide a reference to the potential of CA in weathering, but they may not represent the activity potential of CA's found naturally in the soils.

Specifically, the optimal activation pH, temperature, and catalytic efficiency may not be similar to CA's found in the soil.

Amongst the unique soil bacterial CAs used to create the CA database only 0.6% were predicted to be exclusively extracellular, while 82% was predicted to be localised cytosolically. Although this is not definitive it does perhaps highlight the scarcity of these CA genes in at least the samples that contribute to the KEGG, Pfam, and Tigrfam databases. Perhaps partially due to the high abundance of cytoplasmic localised CAs in the database, regardless of the treatment, it accounted for 50.7% and 53.2% of the CA's identified in the soybean and maize year, respectively. Interestingly, despite their low abundance of less than 5% in the database, cytoplasmic membrane localised CAs contributed 12.1% and 13.3% of the CAs identified, while CAs with multiple predicted localisation sites contributing 18.8% and 15.8% to the identified CAs, regardless of treatment. Perhaps indicating that they may carry more relevance in soils than the CAs available in the database would suggest.

Though the CA localisation information is informative, when CA genes are grouped based on their predicted localisations and the effects of basalt amendment over the two years are re-assessed we see no significant differences between basalt and control plots for any of the predicted localisations. Again, indicating that CA genetic abundance may not play a significant role in rock weathering and that no one localisation is more important in that context. Importantly though if there exist certain taxa that are more relevant in a weathering context any changes to their CA genetic abundance could be obscured by other non-relevant groups. Statistical assessment of taxa containing CAs, identified in all plots, at the taxonomic levels of phylum, class, order, and family, revealed significant differences in CAs relative abundance due to treatment in some taxa. Interestingly, the majority of taxa that were significantly affected due to treatment actually decreased with basalt amendment, resulting in a total

loss of 0.879% and 1.258% at the family level of ubiquitous CAs identified across plots from both the soybean and maize years, respectively. The few non-related groups that increased significantly with basalt amendment, *Thermosynechococcales* (order), *Nevskiales* (order), *Cyclobacteriaceae* (family), and *Crocinitomicaceae* (family) only accounted for a very minor total CA increase of 0.184% and 0.187% in the soy and maize year, respectively. Though minor these groups across both years had at least 50% more CA genes relative to the same taxa in the control soils, perhaps indicating that if their populations increased in the soil it may result in more CA activity in the soils.

Although no evidence suggests that specific localisations play a greater role in rock weathering, the presence of multiple CA genes with different localisations within a group could obscure significant changes of one of the localisations. When groups are first separated by localisation and then by individual taxa, and CA relative abundances are re-assessed as done above some significant changes appear in CA's gene abundance. Similar to when localisations are not considered, the majority of taxa whose CA relative abundance was significantly affected by basalt amendment showed reductions in CA abundance compared to control soils. Only two predicted cytoplasmic CA groups increased as a result of basalt application, *Acidimicrobiales* (order) and *JADJPG01* (family) and together they only accounted for 0.156% and 0.250% of the ubiquitous CAs detected. Interestingly, no extracellular or periplasmic CA containing taxa were significantly affected, suggesting no selective pressure for these localised CAs in the soil. However, it is important to remember that none of the predicted localisations showed meaningful changes in CA relative abundance in the overall balance of total CAs in the soil.

Though no metagenomic evidence exists that there is any selective pressure to acquire or build up CA genes in the soil to facilitate rock weathering, it may not prevent it from playing a significant role. Its high

abundance in the soil, being on average 9.6 times more prevalent than the single-copy *rpoA* gene, suggests that selective pressure may not be occurring because it is already abundant. As CA is found to be so prevalent it indicates that it may play a role in cellular functioning, but it is important to remember that genetic abundance is not always linked to genetic expression. Future work should include further transcriptional research which could identify whether CA gene expression is stimulated with basalt amendment, strengthening the argument that CA's found in the soil communities play a role in rock weathering. Additionally, although CAs were separated based on factors such as predicted localisation and taxonomy, further separation could be made by enzyme class, as previous literature suggests that different CA classes may function differently in soils (Smith and Ferry, 2000; Smith et al., 1999). Another area of research is to try to identify whether there are increases in CA abundance and expression in communities living in the mineralosphere (Uroz et al., 2015) versus the surrounding soil. Likely the organisms with the greatest weathering potential are those that colonise the rock, but no evidence exists to definitively prove that these communities are responsible for the majority of bacterially derived weathering in soils. Finally, there remains the possibility that a portion of the intracellular CAs of bacteria are released into the soil on cell senescence or lysis and that these enzymes remain active in the soil accelerating weathering without being under the direct control of the organisms. Further research is needed on the fate in soil of the enzymes coded for by the CA genes we have studied.

3.5. Conclusion

Our metagenomics study finds no evidence that there is a selective pressure to acquire or have CA genes in basalt amended soils, possibly suggesting that CAs encoded in the soil community do not play an important role in rock weathering. However, further research is needed to determine if certain classes of CA enzymes are selected for differently in

basalt-amended soils and if CA transcription is affected by this rock amendment. Previous research has generally focussed on how extracellular or exogenously added CAs affect rock weathering in rock and soil mesocosm experiments, but there have been relatively few CA genes that code for extracellular CAs found in online databases potentially indicating their scarcity in soils. When CAs in the soil bacterial community that have sequences that are predicted lead to extracellular release, were separately tested no significant differences due to rock amendment were found. The majority of CA genes were predicted to have either multiple localisation sites or be localised to the cytoplasm or cytoplasmic membrane. Importantly, when totalled, none of the predicted CA localisations were significantly affected by basalt amendment and contrary to what was expected, the majority of significant treatment effects when assessing individual taxa showed a general decrease in CA relative abundance due to the amendment.

3.6. Supplemental tables

Supplemental Table 1. Significant between treatment differences in CA gene abundance of taxa identified in all plots, along with associated percent difference relative to the non-amended control soils and the basalt amended taxonomies' mean percent contribution to the total CAs identified in the soil community. Related taxonomic groups are denoted by superscript number and taxa which significantly increased due to basalt amendment across both years are highlighted in red.

Taxa	Treatment *p* value	Change with basalt relative to control (%)		Basalt amended share of CA (%)	
		Soy	Maize	Soy	Maize
Phylum					
Bacillota[17]	0.014	-23.955	-49.4185	0.157515	0.140727
Dormibacterota	0.047	-13.6834	-22.4973	0.086839	0.092565
Class					
Ktedonobacteria[2]	0.002	-59.7661	-43.746	0.075128	0.07131

Bacilli[17]	0.009	-23.955	-49.4185	0.158924	0.141636
Terriglobia[3]	0.011	-24.978	-50.2363	1.720466	0.954192
UBA4738[12]	0.036	-2.67501	-39.9605	0.095746	0.081126
UBA6919[11]	0.042	-9.26974	-42.9526	0.032597	0.034657
Order					
ATCC43930[16]	0.037	-26.9132	-37.8232	0.089896	0.062655
Streptosporangiales[6]	0.028	-25.0791	-48.629	0.534148	0.436099
Terriglobales[3]	0.005	-32.2152	-58.4414	0.841443	0.451313
Thermosynechococcales[13]	**0.004**	**80.49442**	**77.77318**	**0.026712**	**0.027732**
Bacillales[17]	0.004	-9.7418	-55.4588	0.068157	0.053362
UBA4738[12]	0.015	-7.54276	-43.1749	0.08412	0.066985
UBA6919[11]	0.041	-9.26974	-42.9526	0.033178	0.035083
UBA7540[3]	0.015	-62.6537	-34.621	0.032164	0.026572
WQYP01[10]	0.009	-42.2626	-39.0547	0.031913	0.029435
Binatales[14]	0.022	-27.4068	-43.4934	0.039394	0.021977
Bryobacterales[3]	0.031	-23.8531	-48.7184	0.435761	0.245089
54-19	0.004	-35.6811	-60.894	0.01123	0.011715
CR04bin15[15]	0.034	-21.8267	-40.181	0.007411	0.008484
Fen-1088[8]	0.028	-25.0127	-39.4507	0.043409	0.057203
Isosphaerales[10]	0.029	-22.0553	-52.5349	0.124212	0.118808
Ktedonobacterales[2]	0.003	-59.7661	-43.746	0.076468	0.072186
Micropepsales[5]	0.013	-41.532	-53.6733	0.11734	0.069217
Nevskiales[16]	**0.046**	**63.59262**	**83.65471**	**0.045202**	**0.052366**
Paenibacillales[17]	0.01	-41.6271	-37.8153	0.023358	0.030324
Family					
Gp1-AA117[3]	0.002	-39.7614	-51.5439	0.106106	0.059303
Gp1-AA112[3]	0.006	-6.02908	-42.6031	0.047374	0.031774
Acidobacteriaceae[3]	0.002	-35.6566	-56.943	0.166306	0.114932
Acetobacteraceae[16]	0.045	-22.5487	-44.5288	0.203147	0.15623
Fen-1088[8]	0.025	-25.0127	-39.4507	0.044691	0.058577
Cyclobacteriaceae[9]	**0.012**	**87.88736**	**62.74521**	**0.097782**	**0.084611**

Cyanobacteriaceae[13]	0.033	1.150404	-37.9432	0.083247	0.081894
Crocinitomicaceae[9]	**0.006**	**137.3588**	**89.62188**	**0.014451**	**0.022268**
CR04bin15[15]	0.039	-21.8267	-40.181	0.00763	0.008688
WQYP01[10]	0.006	-42.2626	-39.0547	0.032855	0.030142
UBA7540[3]	0.017	-62.6537	-34.621	0.033114	0.02721
Bryobacteraceae[3]	0.032	-23.9542	-49.2562	0.442743	0.244913
UBA6919[11]	0.036	-9.26974	-42.9526	0.034158	0.035925
UBA4738[12]	0.004	-37.1597	-51.6997	0.042119	0.032429
UBA2294[14]	0.033	-14.0705	-44.595	0.009113	0.013636
Thermosynechococcaceae[13]	**0.005**	**80.49442**	**77.77318**	**0.027501**	**0.028398**
Binataceae[14]	0.013	-27.4068	-43.4934	0.040557	0.022505
Streptosporangiaceae[6]	0.031	-25.1332	-48.585	0.549082	0.446192
Selenomonadaceae	0.042	-15.9973	-27.8663	0.021005	0.021631
SbA1[3]	0.001	-28.5617	-56.925	0.428031	0.220792
Rhodanobacteraceae[16]	0.03	-48.3817	-53.9551	0.053482	0.062687
Micropepsaceae[5]	0.013	-41.532	-53.6733	0.120806	0.07088
Micromonosporaceae[12]	0.03	-6.1324	-45.0443	0.196012	0.219134
Ktedonobacteraceae[2]	0.023	-52.5466	-38.7506	0.06918	0.061295
Koribacteraceae[7]	0.001	-36.8951	-64.944	0.068536	0.037767
Acutalibacteraceae	0.041	-3.95238	-41.6311	0.047394	0.057192
JACPPG01[10]	0.033	-25.1157	-51.78	0.015841	0.013293
Isosphaeraceae[10]	0.02	-22.0553	-52.5349	0.127881	0.121661

3.7. CRediT authorship contribution statement

Derek S Bell: Conceptualisation, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation. Writing – original draft, Writing-review & editing, Visualization, Project administration; **Jonathan R. Leake:** Conceptualisation, Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition; **David J Beerling**: Validation,

Writing – review & editing; **Dimitar Z. Epihov** Conceptualisation, Methodology, Validation, Formal analysis, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

3.8. Declaration of Interests

D.J.B. has a minority equity stake in Future Forest/Undo and is a member of the Advisory Board of The Carbon Community, a UK carbon removal charity, and the Scientific Advisory Council of the non-profit Carbon Technology Research Foundation. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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3.10. Data statement

Associated data will be uploaded prior to publishing.

3.11. Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT, an AI language model, to assist with grammar and refining content. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

Chapter 4: Biological Simplification and Magnetic Extraction of Basalt Grains to Unravel Root and Mycorrhizal Roles in Enhanced Rock Weathering in Upland Hay Meadow Soil.

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Abstract:

Plants, invertebrates, fungi, and bacteria can accelerate rock weathering, but their relative importance in enhanced rock weathering (ERW) trials and role in CO_2 absorption remain unclear. Some studies use *in si*tu incubation of rock grains in mesh bags to quantify weathering rates, but this may limit natural interactions with soil, roots, and larger organisms. We used step-wise size exclusion columns with decreasing mesh pore sizes, called biological simplification columns, and magnetic basalt separation to simplify biotic-driven *in situ* weathering and compare mesh bags with magnetic rock reclamation. These techniques investigated the ERW potential of plants and filamentous fungi during the growing season in a biodiverse, mildly acidic upland hay meadow amended with basaltic rock dust. Magnetic separation effectively removed basalt grains, though visible soil contamination remained despite pre-screening and washing, significantly altering soil and mineral chemistry. In the field, biological simplification columns allowing filamentous fungal in-growth but excluding plant roots showed significant ($p < 0.05$) increases in soil extractable calcium (166.8 mg kg^{-1}, 8.1%), strontium (0.294 mg kg^{-1}, 8.3%), iron (0.917 mg kg^{-1}, 12.1%), and titanium (32.16 mg kg^{-1}, 8.7%) compared to columns restricting both groups. The calcium increase corresponded to potential CO_2 absorption of ~344 kg CO_2 ha^{-1} yr^{-1} in the upper 10 cm of soil. In contrast, potassium (14.0 mg kg^{-1}, 8.9%) and molybdenum (0.00424 mg kg^{-1}, 15.5%) significantly decreased in fungal in-growth columns. When plant roots colonised columns, soil potassium dropped an additional 34% (39.6 mg kg^{-1}), while magnesium, calcium, strontium, silicon, and titanium also significantly decreased, suggesting plant element uptake or weathering inhibition, possibly due to soil drying.

4.1. Introduction

The potential for enhanced rock weathering (ERW), which involves spreading ground calcium or magnesium silicate rock onto agricultural soils to remove carbon dioxide (CO_2) from the atmosphere (Hartmann et al., 2013; Beerling et al., 2018), has been studied by geochemical modelling, mesocosm, and field trials (Beerling et al., 2020, 2024; Kelland et al., 2020; Kantzas et al., 2022). A recent modelling study has estimated that if silicate rock dust was applied across arable land in the UK it could sequester between 6 – 30 megatonnes of CO_2 annually by the year 2050 (Kantzas et al., 2022), showing the potential scale of ERW contribution to meeting CO_2 removal targets. However, most geochemical models of ERW to date do not include any biological processes, although there is clear evidence that roots and their associated mycorrhizal fungi and rhizosphere bacteria play important roles in generating acids and assimilating and transporting elements released from minerals (Taylor et al., 2009; Leake and Read, 2017; Epihov et al., 2021). Recently, some geochemical models are starting to take into account biological activity when assessing rock weathering (see Vicca et al., 2022). Furthermore, whilst biogeochemical models are providing insights into the potential for ERW to contribute to global atmospheric CO_2 removal (CDR), verification of modelling results has been constrained by a lack of long term field trial data to validate them (Goll et al., 2021). The situation is complicated by the large numbers of variables that change between studies and locations including mineral type and amount, soil type and properties, climate, weather, crop type, and the biology within fields. Differences in environment and trial designs between different field studies give rise to very different outcomes, for example, Beerling et al. (2024) found soy and maize yields increased by 16 and 12% respectively with a yearly 50 tonne hectare^{-1} basalt application to soils in the US, while Haque et

al. (2020) found non-significant increases in soybean yield 20 weeks after one application with 5 tonne hectare^{-1} wollastonite.

Rock weathering, which occurs both abiotically and biotically, is a primary control of atmospheric CO_2 and the global climate over geological time (Kump et al., 1999). Its impact was seen most dramatically during the middle to late Devonian geological era with the evolutionary advances of vascular plants and their mycorrhizal fungal partners (Taylor et al., 2009) along with the development of deep-rooting trees supporting these fungi (Stein et al., 2020). These increases in terrestrial weathering led to enhanced calcium and magnesium export to the oceans (Quirk et al., 2014) locking up atmospheric CO_2 into marine carbonate minerals and changing the global atmosphere and climate (Leake and Read, 2017). The increasing biomass, rooting depth, and more advanced mycorrhizal fungal partners, together enhanced pedogenesis, increased soil CO_2 concentrations, and anchored the soil, leading to sustained hydrological interactions and intensified silicate weathering (Berner, 1997; Algeo and Scheckler, 1998; Taylor et al., 2009; Quirk et al., 2014; Quirk et al., 2015; Ibarra et al., 2019; Qie et al., 2019). The geological and evolutionary evidence emphasises the critical role of biology in rock weathering and global climate evolution (Leake and Read, 2017). Extensive research has been performed to characterise the mechanisms behind biological rock weathering, and the roles of plants (Dontsova et al., 2020), fungi (Finlay et al., 2020), bacteria (Ribeiro et al., 2020), and macro-invertebrates, such as earthworms (Vicca et al., 2022) have been reviewed exploring the different biotic mechanisms used to degrade rock.

Three widely cited ERW modelling studies by Beerling et al. (2020), Goll et al. (2021), and Kantzas et al. (2022) all focus exclusively on the application of ERW on arable land. However, grasslands offer a greater ERW potential in terms of area available as it exceeds cropland area by 5.2 million hectares in the UK (DEFRA, 2023). These grasslands, commonly found in

humid environments, are also often made up of acidic soils which would be expected to facilitate weathering. Furthermore, herbage production often requires the use of lime to maintain pH and yields (Holland et al., 2018), which could be substituted by a calcium and magnesium-rich rock dust (Beerling et al., 2018). In a mesocosm experiment with rye grass, an amendment of olivine at a rate of 204 t ha^{-1} significantly increased plant growth and elemental concentrations (ten Berge et al., 2012). Specifically, the concentrations of potassium, magnesium, silicon, and nickel increased by 16.8, 44.4, 182.6, and 402.6 %, respectively, while the overall growth improved by 15.6%, and the resulting CO_2 removal was estimated to be 2.69 t ha^{-1} (ten Berge et al., 2012). Kantola et al. (2023) demonstrated that basaltic ERW amendments to fields in the US significantly increased the perennial bioenergy grass crop miscanthus yields by 29 – 42% and resulted in a positive net ecosystem carbon balance (NECB) of 0.63 – 1.29 t C h^{-1} $year^{-1}$, indicating CO_2 sequestration. In the same study, basalt applications to maize and soybean resulted in an improved, but still net negative NECB compared to basalt-free controls, indicating that carbon was still lost and that the perennial grass system gave greater CO_2 absorption than the arable cropping (Kantola et al., 2023). Kantola et al. (2023), suggested that the benefits of miscanthus may be due to its longer growing seasons or an increased belowground biomass allowing for more intensive interactions with rock particles in the rhizosphere. The significant increase in belowground biomass relative to soy and maize was previously characterised in the same fields for the perennial crops miscanthus, switch grass (*Panicum virgatum*), and native prairie (Kantola et al., 2022). A further factor that may have improved weathering rates is the presence of more active mycorrhizal communities in the perennial grasses compared to seasonal arable crops following tillage. Wild et al. (2021) estimated that when olivine was left to incubate for 9 months in a magnesium deficient forest soil, mycorrhizal colonisation was responsible for 16% of the weathering. Though the likely contributor to this weathering was

ectomycorrhiza and not arbuscular mycorrhizae (AM) which are dominant in grasslands. Mycorrhizae may increase rates of rock weathering through increasing the total reactive surface area compared to roots alone (Smith and Read, 2008), directly weathering the rock (Quirk et al., 2015), forming symbiotic relationships with weathering bacteria (Koele et al., 2014), or forming more water stable aggregates enhancing soil water storage and allowing for more weathering during drier periods (Wilkes et al., 2021).

Field studies of ERW are especially needed in permanent grasslands, that to date have received little attention, but are biologically distinct from croplands in at least three ways. (a) having very high densities and biomass of perennial roots (Sun et al., 2018; McNally et al., 2015), (b) high biomass, and hyphal lengths of mycorrhizal fungi compared to arable crops and soils that are frequently cultivated (Leake et al., 2004), and (c) high concentrations of soil organic matter compared to most arable and cultivated soils (Conant et al., 2017). Although plants, mycorrhizae, bacteria, and animals can all weather rock, no research has been done to characterise *in situ* the contributions of these biotic groups to ERW in grasslands. An important methodological development to resolve the relative contributions of major groups of biota to soil processes and functions has been the use size-selective screening to progressively simplify grassland soil biota by excluding major functional groups of organisms by size (Wagg et al., 2014). This showed that reductions in soil biological diversity disrupted ecosystem multifunctionality by affecting plant diversity, decomposition, nutrient retention, and nutrient cycling (Wagg et al., 2014). Building on these approaches and the hyphal in-growth cores developed by Johnson et al. (2001) to study the functioning of mycorrhizal hyphal networks in permanent grassland, we developed novel ERW mesh-walled columns containing soil and basaltic rock grains to achieve sequential exclusion of plant roots and mycorrhizal fungi (and any saprotrophic fungi that depend on living roots outside the mesh cores) to

enable their *in situ* contributions to rock weathering to be characterised. The use of use of nylon mesh barriers permeable to mycorrhizal hyphae but too small to permit in-growth of roots has been widely used to study arbuscular mycorrhizal (AM) functioning, since these fungi are obligately dependent on plant roots for their organic carbon sources. Disturbance of the growth of hyphae across the nylon mesh by rotation or raising and lowering cores at frequent intervals can be used to evaluate effects of the mycorrhiza-ingrowth and proliferation inside the mesh cores, compared to the situation where the hyphae are undisturbed.

Measuring CO_2 removal by ERW presents a number of technical challenges as it is mostly estimated by indirect approaches. These include testing for inorganic carbon or alkalinity in leachate and soil pore waters (Larkin et al., 2022), extracting and measuring weathering products in the soil (Kelland et al., 2020), and measuring elemental changes in the rock amendment (Epihov, 2018). The latter poses a problem as once mixed in the soil the rock grains are difficult to recover and test. The burial of rock grains in nylon mesh bags in soil in the field (Quirk et al., 2014; Epihov, 2018; Bärlocher, 2020) and recovery of these months or years later allows collection of the same rock grains to study their chemical alteration. However, this method has drawbacks: the rock is not mixed in with the soil as it would if it were spread on soil, and the bag may affect how roots and larger biota, as well as water interacts with the rock. Another recently developed method, the TiCAT mass-balance approach, compares changes in mobile elements in the soil and rock mixture against immobile elements such as titanium (Reershemius et al., 2023). While promising, scaling this method may pose a challenge as specialist equipment is needed to perform hydrofluoric acid digests as well as isotopic dilutions. To address the need for a cheaper scalable solution, we trialled the use of neodymium magnets to firstly separate magnetite-rich basalt particles from ground basaltic rock, secondly to deplete magnetic minerals from the test soil, and finally to

recover the magnetic basalt particles from soils following *in situ* weathering. The solid-phase chemistry was then characterised using portable x-ray fluorescence (pXRF) to assess its efficacy compared to the mesh-bag incubation method while avoiding the use of expensive hydrofluoric acid digests requiring specialist lab facilities.

The first aim of this research was to use size-selective biological simplification method to characterise the effects of roots and filamentous fungi on ERW in a mildly acidic, floristically diverse, upland hay meadow, which has historically received periodic inputs of lime to reduce soil acidity. The second aim was to assess whether the novel magnetic rock extraction method could be employed to separate rock from soil for a time and cost-effective, scalable weathering analysis.

4.2. Methods

4.2.1. Soil and rock preparation

Soil was collected from Moss End Farm, sampling the upper 10 cm layer of the acidic (pH 6.3 in 0.01 M $CaCl_2$) upland hay meadow with sandy clay soil, located at an elevation of 320 meters above sea level in the Peak District, England (53°10'45.7"N, 1°59'36.4"W). Once collected and air-dried in a fume cabinet at room temperature until dry to the touch, the soil was then sieved to 2 mm and all visible plant roots and seeds were removed. This soil underwent four repeated magnetic screenings to remove magnetic particles using a novel neodymium magnet separation method described in section 2.2 below.

Prior to magnetic separation, some soil was wet-sieved to collect particles sized 120 – 500 µm and washed with distilled water to remove dust and organic material, leaving the sand-sized mineral grains native to the Moss End Farm field site. Basalt from the Hillhouse quarry site, characterised by Lewis et al. (2021), was also wet-sieved to 120 – 500 µm. Following wet-sieving both basalt and the native sand fractions of the soil

were dried in an oven at 105 °C for 3 hours. Once dry basalt and native sand fractions were screened for magnetism, with only the basalt showing magnetism with approximately 30% of the measured fraction being magnetic. The magnetic basalt fraction then underwent three repetitions of the magnetic selection, described below in section 2.2, to collect and purify the magnetic rock particles.

4.2.2. Neodymium magnet separation of soil and basalt particles

Magnet separation involved using a bespoke wooden board (42 x 36 cm) with 50 embedded 50 mm x 10 mm x 1.5 mm N35 neodymium magnets arranged in a 5 x 10 array (Spider Magnetics Ltd.), covered with aluminium foil. In a fume cabinet dry soil or rock particles were spread over the board. The non-magnetic particles slid off the board on tilting and were collected. Magnetic particles were held on the foil surface and then manually brushed off into a separate container. Basalt rock was screened to select the magnetic fraction, while soil was screened to remove the magnetic fraction.

The same method was later applied after the field incubation experiments to the mesh-walled columns containing loosely distributed basalt and native sand to separate the magnetic fraction for subsequent pXRF analysis described in section 4.2.5 below.

4.2.3. Biological simplification columns

We coined the term biological simplification to describe the step-wise size exclusion columns designed and presented in Figure 1, which were prepared based on the mycorrhiza ingrowth / exclusion columns of Johnson et al. (2001). These columns were 85 mm long with an inner and outer diameter of 13 and 16 mm, respectively. Nylon mesh windows covered the bottom and approximately 75% of the sidewalls to allow water flow.

As detailed in Table 1, for this experiment two of the biological simplification treatment columns, like Johnson et al. (2001), consisted of columns with a 35 µm pore size mesh that excluded plant roots. One of these

treatment columns was lifted (Blanke et al., 2012) weekly to sever fungal in-growth (ff-, p-), while the other remained static to allow filamentous hyphal in-growth and proliferation (ff+, p-). The third treatment column, which also remained static, used a mesh size of 120 µm to allow fine root and mycorrhizal fungal in-growth, as well as allowing small invertebrate colonisation (ff+, p+). This size was chosen based on previous fine root diameter estimates (De Baets et al., 2020) and supported by a recent study by Erktan et al. (2023) who found that the average fine root diameter in managed grasses was 100 ± 60 µm wide.

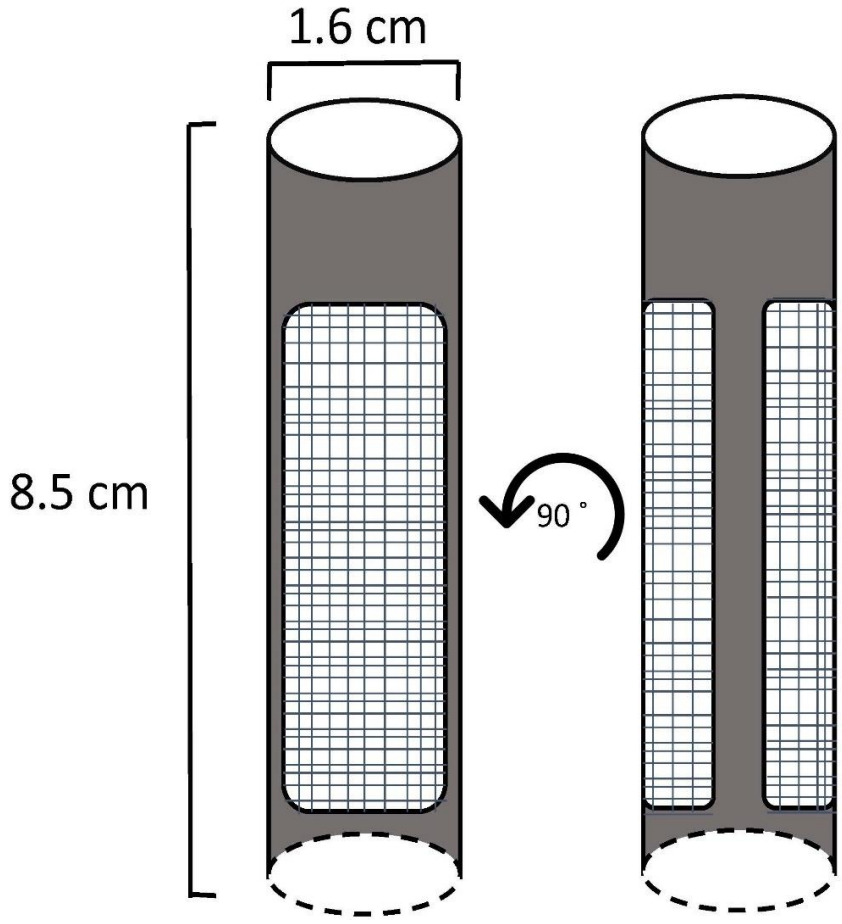

Figure 1. Diagram of biological simplification column design

The biological simplification columns were filled with 10 g of magnet-screened soil mixed with 0.66 g of either loose or 120 µm nylon mesh-bagged magnetic basalt or non-magnetic native sand, simulating a 50 t ha^{-1} application rate of basalt or sand (Kantola et al., 2023). Once filled, a coarse

permeable foam was cut to fill the top to prevent contaminants from falling into the top of the column while also allowing water to flow through. Ten control columns for each combination of loose or bagged native sand or magnetic basalt were set aside at room temperature and ambient humidity for the duration of the field trial.

Table 1. Experimental design of rock grain substrate, soil incubation method (bagged vs loose), and biological simplification treatments.

Rock substrate	Bagged vs loose	Mesh size (µm) + severing treatment	Treatment: - Mycorrhizae and plant roots allowed (ff+, p+) - Mycorrhizae allowed but plant roots excluded (ff+, p-) - Mycorrhizae and plant roots excluded (ff-, p-) - Control: lab incubated without plants or mycorrhiza
Non-magnetic native sand	Bagged	120	ff+ p+
		35	ff+ p-
		35 + severing	ff- p-
		120	Control (lab incubated)
	Loose	120	ff+ p+
		35	ff+ p-
		35 + severing	ff- p-
		120	Control (lab incubated)
Magnetic basalt	Bagged	120	ff+ p+
		35	ff+ p-
		35 + severing	ff- p-
		120	Control (lab incubated)
	Loose	120	ff+ p+
		35	ff+ p-
		35 + severing	ff- p-
		120	Control (lab incubated)

4.2.4. Field experiment design

Five separate 1.7 by 1.4 m plots were prepared on March 17th, 2022. Within each plot, a grid of 24 soil cores 16 mm wide and of 75 mm deep were removed, in a 4 x 6 row grid with 300 mm between each core. Each row of 6 cores was replaced with mesh-walled cores with random allocation of biological simplification columns of the three treatment types, these cores containing soil mixed with either native sand from the field site or magnetic basalt. Half of the rows in each plot had loosely distributed rock, while the other half had bagged rock substrate. The biological simplification columns were incubated in the field for six months and removed on September 1st, 2022.

4.2.5. Rock and soil elemental analysis

On removal, the soil columns were air-dried at room temperature in a fume cabinet until completely dry to the touch. Treatment and control columns containing bagged rock substrate then had the bags removed, while columns containing loosely distributed rock underwent three magnetic screenings to isolate magnetic particles. The pooled magnetic particles from each core were further screened three more times to purify the sample of any non-magnetic contamination. Both the bagged rock and the magnetic fraction underwent a wash in 15 mL analytical grade pure ethanol for one hour in a glass beaker placed in a 45 KHz, 80 W ultrasonic bath of 3 litre capacity containing water running at full power to remove soil contaminants from the rock matrix (Rigopoulos et al., 2018). Ethanol was subsequently removed by oven drying at 80 °C overnight. The dry samples were then pressed into pellets using 10 tonnes per square inch pressure with a manual hydraulic press. Both sides of the resulting rock pellets elemental concentrations were assessed by using a P-XRF instrument (Nitron XL3t900 GOLDD analyser: Thermo Scientific Winchester, UK) under a helium atmosphere. The pXRF was calibrated using Si-spiked synthetic methyl cellulose (Sigma-Aldrich, product no. 274429) and validated using Certified Reference Materials of NCS DC73349 ‘Bush branches and leaves’. Elemental percent concentrations as oxides were calculated from the raw elemental data by applying element-to-oxide conversion factors to major elements silicon, magnesium, calcium, and potassium, while trace elements were not converted following format provided in GeoRem for basalt (Jochum et al., 2005). To calculate the 100% nominal oxide basis the converted oxide values were then summed, and the relative percent contribution of each elemental oxide concentration to the total measured oxide content was determined. Initial variation between reads of the same pellet were both propagated and non-propagated to assess quality of the pXRF data.

Soil exchangeable cation concentration was determined using a modified ammonium acetate extraction (Carter and Gregorich, 2008). Briefly, soil was mixed at a 1:10 ratio of soil to 1 M ammonium acetate at pH 7, vortexed, and then shaken at 250 rpm on an orbital shaker for 2 hours at room temperature. After incubation, the sample was centrifuged at 4700 x *g* for 5 min with the resulting supernatant being diluted to 1/10 and acidified to 2% nitric acid. Samples were left overnight at room temperature and then filter sterilised to 0.2 µm before being analysed by inductively coupled plasma mass spectrometry (ICP-MS) at the University of Nottingham Agricultural and Environmental Sciences department.

4.2.6. Statistical analysis

R statistical and graphing software was used for all statistical analysis (R Core Team, 2024). One-way ANOVAs with Tukey's tests were performed on both soil and rock reclaimed from the control columns. Statistical analysis of biological simplification column soil and rock for both bagged and loose basalt columns following field incubation were analysed using three-way ANOVA's with Tukey's tests for all elements with treatment, bagged vs loose, and plot being the variables. Prior to statistical tests normality and homoscedasticity were assessed visually in R with any non-normally distributed residuals undergoing Box Cox transformation (Box and Cox, 1964; Venables and Ripley, 2002), and then being re-assessed to confirm normality and homoscedasticity. Any outliers exceeding ± 100% of the mean were removed from the data prior to statistical analysis, with significance being determined as any p value that was < 0.05. GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, Massachusetts US, www.graphpad.com) was used for plotting all figures.

4.3. Results

4.3.1. Analysis of loose vs bagged rock particles in control soil columns

Compared to the laboratory-incubated native sand bagged control columns, which acted as a non-magnetically sampled control, there was a consistent pattern of reduced concentrations of ammonium acetate-extractable soil elements in the lab incubated soils which underwent magnetic screening. For instance, in the native sand and basalt loose control column soils there were significant reductions seen in 23 and 15 of the 29 elements measured, respectively. In contrast, no significant differences were observed in any soil elements between the bagged native sand and bagged basalt columns.

Large differences were also seen in the chemical composition between fresh rock grains and reclaimed rock grains from the control columns. Importantly, during pXRF scanning each pellet had both sides scanned resulting in two technical replicates. Averaging these replicates and propagating their initial variation into the final statistical test resulted in no significant differences, despite a mean difference of 133% across all elements when measured with the pXRF. When this initial variation within the substrate is ignored some significant differences in percent oxide concentrations are seen. The native sand is non-magnetic so the significant differences in silver ($p < 0.01$), lead ($p < 0.005$), and tin ($p < 0.05$) are the result of magnetic soil particles, which remained in the soil despite pre-screening to remove magnetic particles. Similarly, the reclaimed rock from the basalt loose columns visually had magnetic soil contamination, as despite it undergoing an additional wash step to remove non-magnetic soil particles it still had significant differences in the concentrations of magnesium oxide ($p < 0.001$) and silicon dioxide ($p < 0.001$) compared to fresh basalt.

4.3.2. Biological simplification

4.3.2.1. Soil elemental concentrations

Using a three way ANOVA and post hoc Tukey's tests, to analyse the effects of bagged versus loose rock particles, biological simplification treatment, and plot, some significant differences in ammonium acetate extractable soil concentrations were noted between lab incubated control and field incubated soil columns containing soil mixed with either basalt or native sand. In basalt containing columns, all measured elements, with the exception of boron ($p > 0.05$), were either significantly increased or decreased as a result of biological simplification (Figure 3, supplemental Figure 1). Whereas, when native sand was in the columns and incubated in the field its concentrations of soil magnesium, calcium, strontium, titanium, molybdenum, and silicon were all not significantly affected by treatment ($p > 0.05$) (supplemental Figure 2).

Regardless of the rock (basalt or native sand) in the column, soil concentrations of sodium and sulphur significantly increased relative to the lab incubated controls regardless of the biological simplification treatment (Figure 3, Supplemental Figure 1 and 2). Furthermore, significant reductions ($p < 0.05$) relative to the lab incubated controls were seen across biological simplification treatments for elements aluminium, manganese, zinc, arsenic, selenium, silver, cadmium, lead, and uranium (Supplemental Figure 1 and 2). However, for these elements there were no significant pairwise differences seen between the biological simplification treatments in either the native sand or basalt containing columns. When the columns contained native sand and soil, only potassium (ff+p+ vs ff-p-: $p < 0.05$), copper (ff+p+ vs ff-p-: $p < 0.001$ and ff+p+ vs ff+p-: $p < 0.05$), and nickel (ff+p+ vs ff-p-: $p < 0.05$) showed significant pairwise differences between biological simplification treatments (Figure 2).

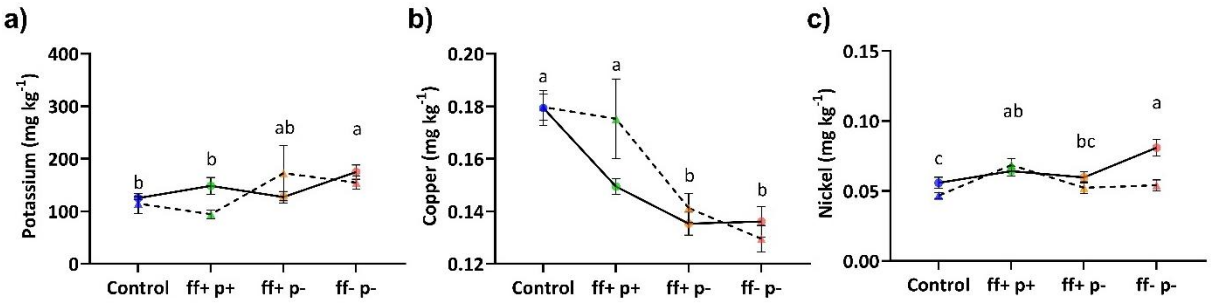

Figure 2. Significant between treatment post-hoc Tukey's test comparisons, signified with letter codes, of soil elemental concentrations (mg kg^{-1} soil) from bagged native sand containing columns mean (● with connecting solid black line) and loose native sand containing columns mean (▲ with connecting dashed black line) for elements potassium (a), copper (b), and nickel (c).

Among the soil extractable elements with significant three-way ANOVA treatment effects in basalt-containing columns, significant pairwise differences between treatments were noted for 10 elements, with these differences varying between elements (Figure 3). Plant root colonisation (ff+p+) resulted in significant reductions in soil extractable magnesium, calcium, strontium, titanium, and silicon of 19.0, 155, 0.236, 26.7, and 0.473 mg kg^{-1} soil, respectively, relative to plant root excluding columns ff+p-. When the columns exclude roots but permit mycorrhizal hyphal in-growth (ff+p-) concentrations of these elements tend to increase but fall when mycorrhizae are also prevented from proliferating in the columns (ff-p-). This reduction however is only statistically significant for calcium ($p < 0.05$), strontium ($p < 0.005$), and titanium ($p < 0.01$), where reductions of 166.8, 0.294, and 32.16 mg kg^{-1} soil, respectively, are seen. Potassium concentrations within the soil acted differently, as with increasing biological simplification there was increasing concentrations of potassium within the soil. When plant roots were restricted from accessing the column (ff+p-) there was a significant ($p < 0.01$) increase of 39.6 mg kg^{-1} soil and subsequently when mycorrhizae were prevented from proliferating in the columns (ff-p-) there a further significant increase ($p < 0.05$) of 14.0 mg kg^{-1} soil. Iron concentrations progressively and significantly reduced relative to

the control soils with the increasing biotic exclusions. Within treatments there were significant reductions of 1009 and 917 µg kg $^{-1}$ soil relative to ff+p+ ($p < 0.01$) and ff+p- ($p < 0.05$), respectively, when both plants and mycorrhizae (ff-p-) were restricted from the column. Concentrations of molybdenum were significantly ($p < 0.005$) reduced by 4.24 µg kg $^{-1}$ soil when mycorrhizae colonised the column (ff+p-) relative to when it was restricted (ff-p-), which might be linked to the need for molybdenum in N-fixing root nodules in legumes like clover that are heavily mycorrhizal. There was a significant reduction in soil phosphorus with soil incubation for all treatment conditions, with a further significant reduction of 0.368 mg kg^{-1} soil between plant root permitted (ff+p+) and plant and mycorrhizae restricting columns (ff-p-).

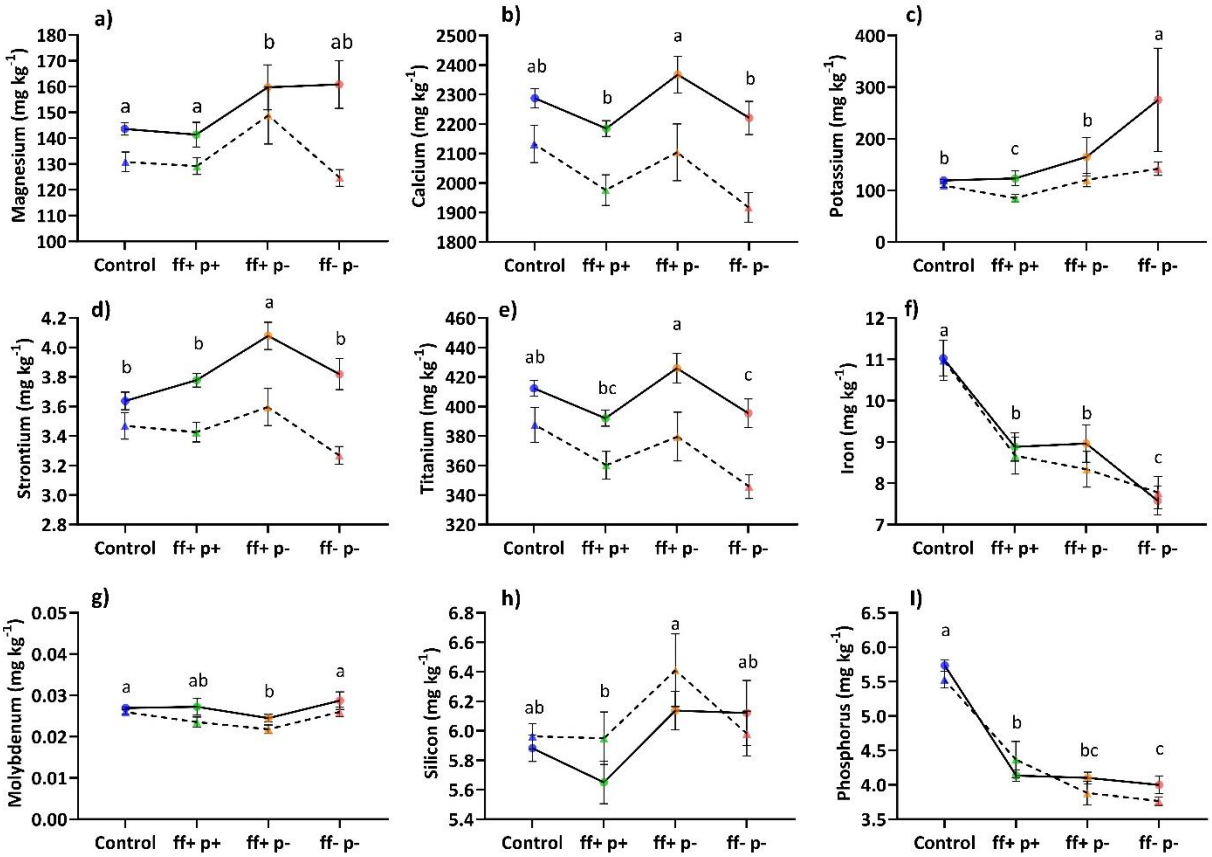

Figure 3. Significant between treatment post-hoc Tukey's test comparisons, signified with letter codes, of soil elemental concentrations (mg kg^{-1} soil) from bagged basalt containing columns mean (● with

connecting solid black line) and loose basalt containing columns mean (▲ with connecting dashed black line) for elements magnesium (a), calcium (b), potassium (c), strontium (d), titanium (e), iron (f), molybdenum (g), silicon (h), and phosphorus (I).

As seen in Figure 3, for almost all the elements, excluding silicon, when basalt was loosely distributed in the soil and subsequently magnetically separated the measured values were consistently lower than when basalt was bagged. With other measured elements (supplemental Figure 1) sulphur, zinc, aluminium, arsenic, selenium, silver, cadmium, lead, and uranium magnetic separation resulted in reduced mean soil concentrations relative to soils in bagged columns. Boron, sodium, nickel, manganese, and phosphorus had similar concentration regardless of magnetic separation, while copper and barium appeared in higher concentrations when soils were magnetically separated. When comparing the pairwise differences between biological simplification columns from bagged and magnetically separated basalt columns separately, Tukey test results showed significant differences only for sodium, phosphorus, titanium, zinc, barium, and thallium.

4.3.2.2. Rock elemental concentrations measured by pXRF

As seen in Figure 4. Of all the elements measured with pXRF no pairwise significant differences were seen between any of the biological simplification columns or the controls regardless of whether initial variation seen in the two sides of the sample pellets were propagated through.

Differences in the mineral oxide concentrations of magnetically reclaimed and bagged basalt are dependent upon element. For instance, there appears to be greater lead concentrations seen in magnetically reclaimed basalt while there were lower magnesium oxide concentrations when magnetically reclaimed. Tukey's test comparisons between bagged vs magnetically reclaimed basalts mineral oxide concentrations have revealed

significant differences between the experimental methods with minerals magnesium oxide ($p < 0.0001$), antimony ($p < 0.0001$), tin ($p < 0.005$), calcium oxide ($p < 0.005$), potassium oxide ($p < 0.0001$), and lead ($p < 0.0001$) all being significantly different.

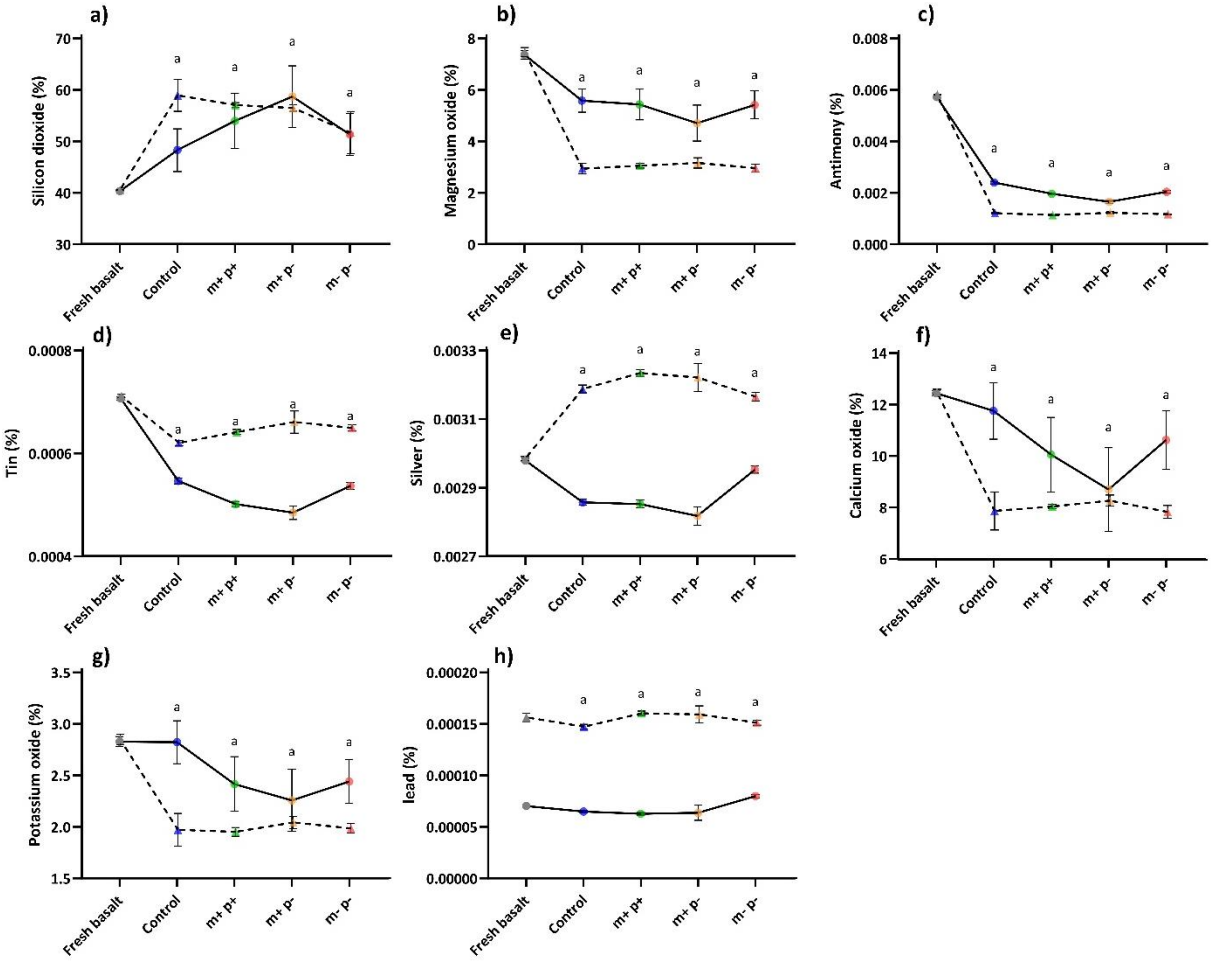

Figure 4. Percent oxide concentrations from bagged (● with connecting solid black line) and magnetically reclaimed basalt (▲ with connecting dashed black line) for minerals silicon dioxide (a), magnesium (b), antimony (c), tin (d), silver (e), calcium oxide (f), potassium oxide (g), and lead (h) with Tukey's test comparisons between treatments denoted with letter codes.

4.4. Discussion

4.4.1. Magnetic separation

The novel magnetic separation of basalt rock from soils has the potential to improve validation of rock weathering, but also experienced limitations in the present work. Previous literature has highlighted that soils developed on basalt parent materials gain their magnetism primarily from the minerals magnetite, maghemite, or hematite (Lu et al., 2008). Lewis et al. (2021) assessed six different basalt minerals potential for ERW, including the Hillhouse basalt used in this experiment, and found that all contained iron oxides to different extents. Our results confirm that at least for Hillhouse basalt, a proportion of the rock is magnetic and can be reliably separated from the soil after mixing and incubation in the field. However, magnetic screening resulted in some problems. When comparing the elemental analyses of native sand and basalt containing control columns, which were not incubated in the field, magnetic screening of the soil resulted in a significant loss of almost all measured elements, excluding boron, copper, silicon, lithium, vanadium, and chromium. In the native sand containing columns calcium and magnesium, which are known to be important in estimating ERW's CDR potential, significantly decreased by 19 and 27% respectively as a result of magnetic screening. When incubated in the field this problem persisted as the concentrations of elements magnesium, sulphur, potassium, calcium, titanium, aluminium, zinc, copper, arsenic, selenium, strontium, molybdenum, silver, cadmium, barium, lead, and uranium were significantly different between magnetically screened soils and non-screened soils. Though the control results indicate that most of these differences are likely due to magnetic screening it is possible that some of the differences are the result of the rock weathering differently as it was loosely distributed in the soil. Despite the differences between methods, the concentration trends for most elements were similar across the biological simplification treatments. Only for the

elements sodium, phosphorus, titanium, zinc, barium, and thallium did the Tukey's tests result in different pairwise comparisons relative to the non-screened soils. For instance, the ff-p- columns soil sodium concentrations were not significantly different to the lab incubated control when the basalt rock was loosely distributed in the soil, but it was significantly different when the basalt was bagged. Generally, the results do suggest that the magnetic screening consistently affected the soils, allowing for comparisons between the biological simplification treatments; however, the final values should be viewed as relative, not absolute.

Though technically the pXRF results were non-significant due to error propagation from variation within pellets of the unreacted control samples, differences between the pXRF elemental analysis of native sand loose and bagged rock columns were seen consistently. This confirmed that despite pre-screening the soils, magnetic soil particles were still present as an increased concentration of 413% lead was seen in the loose particles recovered from the soil relative to those that were bagged. These magnetic soil particles were also seen when the magnetic basalt was reclaimed from the columns as lead also increased by 105% relative to fresh basalt.

In this experiment, despite pre-screening the soil for magnetic particles, contamination issues affected both the soil and rock chemistry results. One solution for this is to ensure this technique is only used on soils with an undetectable initial magnetism. However, literature has noted that both magnetite and hematite are generally considered fast weathering relative to other minerals found within basalt (Lewis et al., 2021). As such, magnetic rock extraction may only be suitable for a finite period of time as soils weather the iron oxides and become themselves more magnetic, as can be seen in soils with basalt parent materials (Lu et al., 2008). The timeline of when soil magnetism could become a problem with this method would require more research, but it may well exceed any meaningful timeline for ERW. In addition to the soil contamination problem, it may be difficult to

equate the magnetic fractions weathering rate to the non-magnetic fraction weathering rate. As basalt can be composed of many different minerals with different weathering rates, at a minimum an in-depth rock analysis would be required of the magnetic fraction to inform predictions of the total weathering rate in the field. Despite the mentioned challenges, magnetic separation of basalt from soil could still be a viable method in certain situations, especially with additional adjustments. For example, conducting soil analysis before magnetic screening could offer a more accurate assessment of soil chemistry compared to the mesh bag method. Another way to improve pXRF results would be to involve a further separation, such as by particle size or density, to remove contaminating soil prior to analysis.

4.4.2. pXRF viability

The control and biological simplification column results also highlighted issues arising from using pXRF as a means of measuring basalt rock dissolution. As previously mentioned, reclaimed rock was pelletized prior to pXRF analysis, with both sides of the pellet scanned to create technical replicates. When the technical replicates were averaged and the initial variation was propagated through, the error derived from that initial variation was large and led to any significant differences between treatments being lost. These results indicate that despite the basalt already being magnetically screened, which likely reduced mineral complexity, there was still enough variability within or between basalt grains to make any reliable statistical assessment difficult. These problems could be overcome through increased technical replication, but such replication would have to ensure that the pXRF scans avoided re-scanning the same areas. Alternatively, Epihov et al. (2021) used a more pure mineral dunite, and further milled it to a fine powder to homogenise the rock prior to pXRF scanning. Milling samples into a finer powder prior to making pellets would be worth investigating in future. Alternatively, samples could be melted and

glassified prior to XRF, but this requires specialist facilities and incurs additional costs and time (Nakayama and Wagatsuma, 2017).

4.4.3. Biological simplification and rock weathering

The biological simplification columns, designed to control which biological groups (plant roots and mycorrhiza, mycorrhiza without roots, and neither roots or mycorrhiza) could access the soil and rock, showed significant differences in how the soils changed with sequential biological simplification of the basalt-containing columns. The differences due to biological simplification in the soil concentrations of magnesium, calcium, potassium, strontium, titanium, iron, molybdenum, silicon, and phosphorus, varied depending on the specific element. The concentrations of magnesium, calcium, potassium, strontium, titanium, and silicon within the soil all appeared to be significantly reduced when plant roots were able to access the columns (ff+p+). Indicating either enhanced nutrient removal from the column or weathering inhibition, possibly by soil drying.

Potassium and molybdenum, nutrients required for plant growth, significantly increased in the soil when mycorrhizal fungi and plant roots were restricted from the column (ff-p-), indicating that filamentous fungi, potentially comprised of AM and saprophytic fungi reliant on root carbon sources, were either involved in extracting them from the soil in the columns or in restricting abiotic, or other biological derived weathering. There is limited research into AM's role in plant potassium uptake (Garcia and Zimmermann, 2014); however, Clark et al. (1999) demonstrated in a mesocosm experiment that when *Panicum virgatum* L. (Switchgrass) was grown in the presence of 6 out of 8 different AM species, shoot potassium significantly increased relative to the non-inoculated controls. Suggesting that mycorrhizal in-growth could be responsible for the reductions to soil potassium concentrations. In addition to this, despite native sand biological simplification columns having lower soil potassium concentrations, there

was a significant difference in soil potassium seen between ff+p+ and ff-p-. Supporting that plant root in-growth either removed potassium from the columns or inhibited potassium liberation from the native soils and substrate. Molybdenum, though an important nutrient in small amounts (Kaiser et al., 2005) can cause toxicity in plants (Shi et al., 2018), but mesocosm research has demonstrated that AM fungi can increase molybdenum uptake in plants *Sorghum bicolor* and *Solanum lycopersicum* while preventing toxicity (Vultaggio et al., 2024; Shi et al., 2018). In grasslands, legumes like clovers and vetches form nitrogen-fixing symbioses with rhizobium bacteria in their root nodules. The process of nitrogen fixation requires significant amounts of molybdenum, which arbuscular mycorrhizal (AM) fungi can supply to the plants (Zhou et al., 2023).

Relative to the basalt-containing controls, iron concentrations within the soils were significantly reduced when plant roots could access the columns (ff+p+), and this reduction persisted even when plant roots were restricted but mycorrhizal fungi could access the soil in the columns (ff+p-). However, there was a further significant reduction in soil iron when mycorrhizal fungal hyphae were prevented from developing in the columns (ff-p-), indicating that mycorrhizal fungal colonisation affected either iron weathering or iron accumulation in the soils. As reviewed by Rajapitamahuni et al. (2023) and Liu et al. (2023) AM's role in iron cycling is complex. Evidence has shown that AM can produce siderophores (Winkelmann, 2017), which with restriction from the column may have resulted in a loss of iron reduction from the basalt. Alternatively, the reduction may be the result of a loss of the symbiotic relationships with bacteria at or near the surface of the rock. Previous ERW research may support this as basalt application to soils does increase the bacterial communities siderophore synthesis gene expressions, which may increases weathering rates (Epihov et al., 2024).

Mycorrhizal fungal colonisation also significantly increased soil concentrations of calcium, strontium, and titanium compared to the ff-p- columns, suggesting that fungal hyphal in-growth also increased the rates of weathering or deposition of these elements within the column. There is no current consensus on AM's role in calcium mobilisation. Fu et al. (2023) found that AM significantly increased concentrations of calcium in the root tissue of apple seedlings (*Malus robusta*) and increased the plant expression of genes responsible for calcium translocation to the shoot. Whereas, McGonigle and Grant (2015) found that sterilisation of soils growing *Zea maize* to prevent AM symbiosis did not affect rates of strontium, used as a tracer for calcium, uptake within the plant. Titanium on the other hand is considered an immobile trace element in soils (Brimhall et al., 1991; Middelburg et al., 1988), and in the case of TICAT has been considered vital in tracking basalt weathering by serving as a reference element not actively depleted by biological processes (Reershemius et al., 2023). Regardless of the potential biological mobilisation and possible uptake of trace amounts into plants, no research has suggested that mycorrhizal fungi actively transports and deposits these element in soils, so the significant increase of titanium and perhaps calcium and strontium in mycorrhiza in-growth columns excluding plant roots relative to the ff-p- columns are likely due to increases in rock weathering. When plants could access the column (ff+p+) soil concentrations of titanium significantly reduced compared to when they were restricted (ff+p-), further indicating that decreases in soil elemental concentrations are more likely the result of weathering inhibition than element extraction from the columns, though this may not be the case for all elements.

The pXRF results from bagged basalt rock, though not statistically significant, suggests that when plants could access the rock the concentrations of magnesium and calcium oxides were greater than when they couldn't, supporting the suggestion seen with the soil titanium data,

that plant colonisation perhaps inhibited rates of rock weathering; this inhibition could have been driven by a reduction in water, as during the drier months of the summer plant root colonisation may have removed water from the weathering sites. The bagged basalt pXRF results also suggests that mycorrhizal fungal colonisation increased rates of weathering, as consistently across all measured elements, excluding silicon, their mean concentrations within the rock matrix were lower relative to when filamentous fungal in-growth was restricted (ff-p-). The mean relative increase in silicon dioxide concentrations may be the result of amorphous silica precipitation on the rock surface following chemical weathering (Hellmann et al., 2013).

Magnesium and calcium, important indicators of carbon dioxide removal following weathering (Reershemius et al., 2023; Kelland et al., 2020), release from the silicate minerals, are both seen to significantly increase when filamentous fungi gain access to the column. When the mean magnesium and calcium concentrations are totalled, mycorrhizal fungal colonisation may have increased weathering by 7.5% relative to un-colonised columns. Such an increase potentially highlights the importance of AM and/or root-associated saprophytic fungi in grassland ERW; however, it also reflects the potential of using fungi in other agriculture that can support it. Previous research with liverworts with and without AM fungi have shown evidence of enhanced release of Ca from basalt grains due to AM, but the amounts of weathering was low compared to that achieved by trees forming AM associations, and appeared to increase in proportion to the biomass of the plants (Quirk et al., 2015). In agricultural systems AM can be directly increased through reducing tillage, reducing mineral nutrient inputs, and careful choice of crops in rotations and in cover cropping (Austen et al., 2022). In permanent grasslands and hay meadows AM is likely to be an especially important component of the soil biota, but it is important to remember that these novel biological simplification columns

are additive in nature; specifically, mycorrhizal fungi and plants are sequentially added to bacterial, saprophytic fungi, and invertebrates. The complex interactions of abiotic and biotic-driven weathering processes could improve or reduce rates of weathering based on indirect community driven effects, and further work is needed to better resolve the contributions of these other components of the soil ecosystem in weathering.

4.5. Conclusion

In conclusion, using a novel biological simplification method in a mildly acidic floristically diverse upland grassland during the spring and summer growing season, mycorrhizal fungi were shown to play an important role in rock weathering. However, the changes seen in soil chemistry due to biological derived rock weathering varied depending on the element measured, perhaps due to their individual nutritive roles. Concentrations of molybdenum and potassium significantly decreased, while calcium, strontium, and titanium significantly increased with mycorrhizal colonisation. Further significant reductions in soil elemental concentrations of magnesium, calcium, potassium, strontium, titanium, and silicon when plant roots were allowed to colonise soil indicate a reduction in weathering potential rather than enhanced nutrient removal by the plant, as titanium, a non-mobile element, decreased with plant root colonisation. Future work should determine whether this relationship is maintained outside the growing season and in different fields and cropping scenarios.

The novel magnetic separation, though promising, had some problems. Despite attempts to pre-screen and wash the magnetically reclaimed rock grains, soil contamination continued to be an issue, along with significant changes in soil chemistry resulting from the magnetic screening. Further difficulties in pXRF analysis compounded these problems

making any conclusion unreliable. These problems could be resolved with changes to the experimental method and by only performing this on soils with little to no magnetic particles. Taken forward with further development, this method still holds promise for being potentially scalable for reclaiming rock particles and measuring weathering in a variety of scenarios.

4.6. CRediT authorship contribution statement

Derek S Bell: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation. Writing – original draft, Writing-review & editing, Visualization, Project administration; **Dimitar Z. Epihov** Conceptualization, Validation, Writing – Review & Editing, Supervision, Project administration, Funding acquisition; **David J Beerling**: Validation, Writing – review & editing, **Jonathan R. Leake:** Conceptualization, Methodology, Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition.

4.7. Declaration of Interests

D.J.B. has a minority equity stake in Future Forest/Undo and is a member of the Advisory Board of The Carbon Community, a UK carbon removal charity, and the Scientific Advisory Council of the non-profit Carbon Technology Research Foundation. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4.9. Data statement

Data will be submitted prior to publication

4.10. Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT, an AI language model, to assist with grammar and refining content. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

4.11. Acknowledgements

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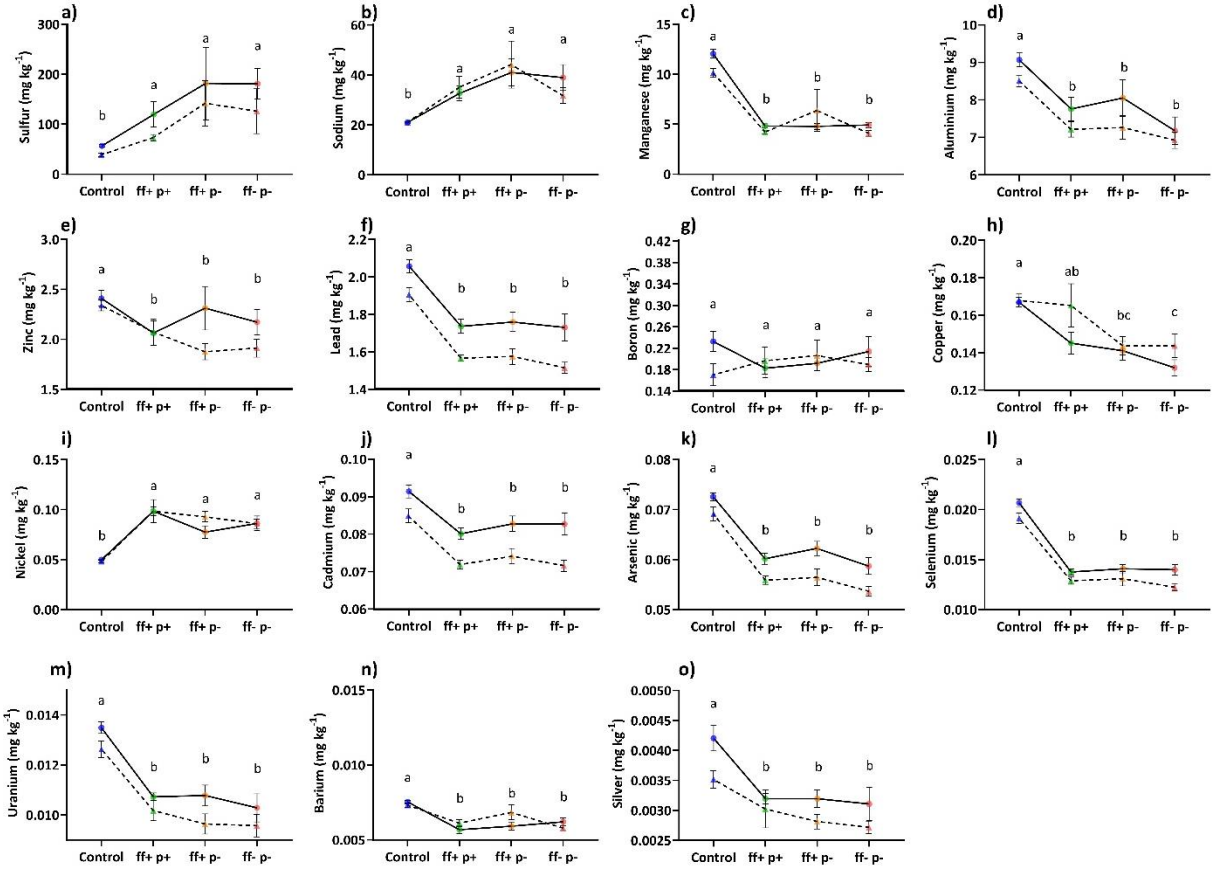
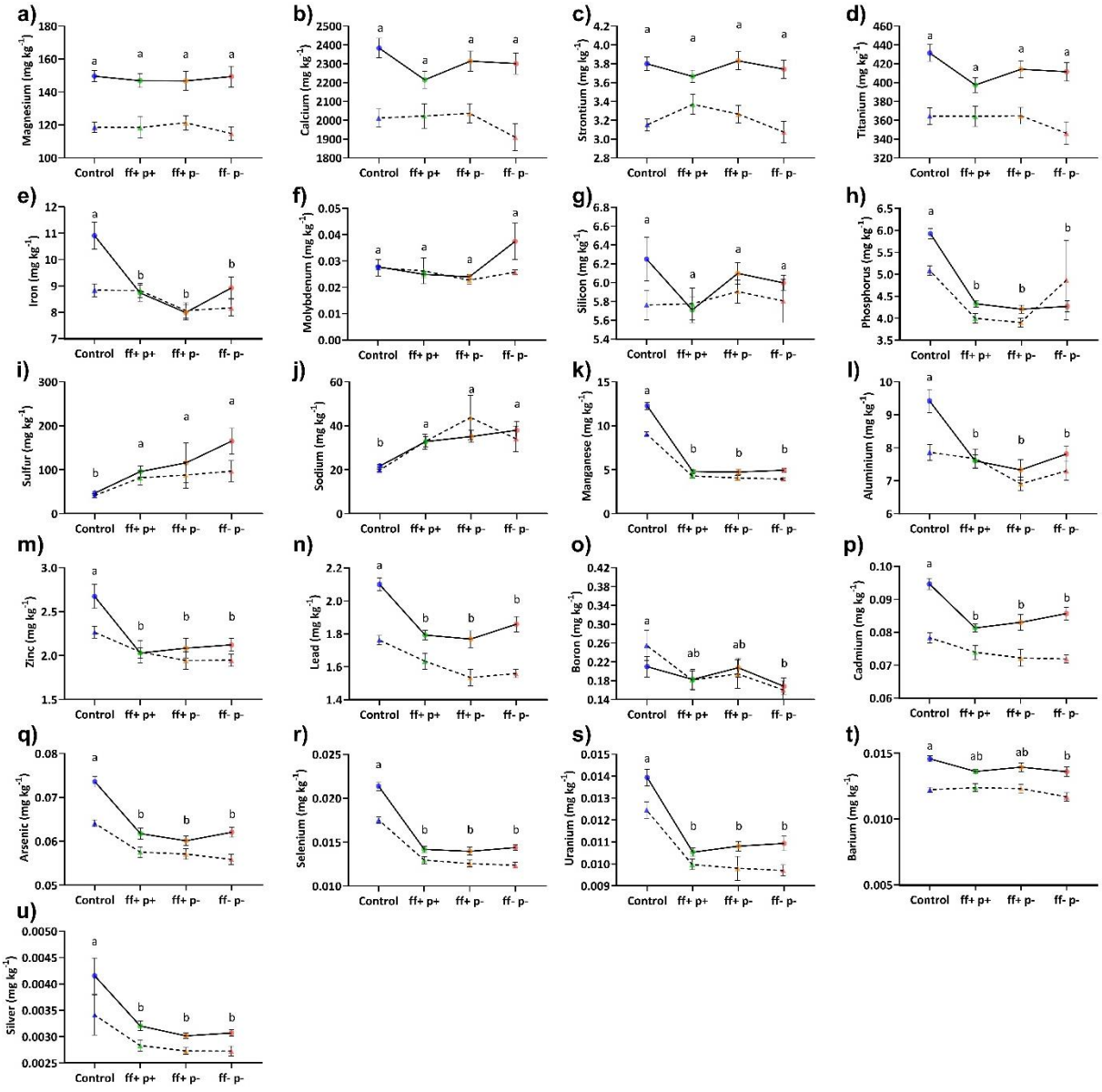
4.12. Supplementary Figures



Supplemental Figure 1. Mean soil elemental concentrations (mg kg^{-1} soil) from bagged basalt containing columns (● with connecting solid black line) and loose basalt containing columns mean (▲ with connecting dashed black line) for elements sulfur (a), sodium (b), manganese (c), aluminium (d), zinc (e), lead (f), boron (g), copper (h), nickel (i), cadmium (j), arsenic (k), selenium (l), uranium (m), barium (n), and silver (o).



Supplemental Figure 2. Mean soil elemental concentrations (mg kg^{-1} soil) from bagged native sand containing columns (● with connecting solid black line) and loose native sand containing columns mean (▲ with connecting dashed black line) for elements magnesium (a), calcium (b), strontium (c), titanium (d), iron (e), molybdenum (f), silicon (g), phosphorus (h), sulfur (i), sodium (j), manganese (k), aluminium (l), zinc (m), lead (n), boron (o), cadmium (p), arsenic (q), selenium (r), uranium (s), barium (t), and silver (u).

Chapter 5: Enhanced rock weathering in grassland: benefits and risks of basalt rock dust to soils, forage production, and floristic diversity in a slightly acidic hay meadow.

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Key words: Enhanced silicate weathering, terrestrial carbon removal, negative emission technologies, grassland biodiversity, trace metals

Abstract

Enhanced rock weathering (ERW) is increasingly recognized as a potentially significant atmospheric carbon dioxide removal (CDR) technique with agricultural co-benefits, although its efficacy in grasslands remains largely unexplored. Permanent grasslands such as hay meadows can support high floristic diversity but their agronomic and biodiversity responses to surface-applied silicate rock dusts are unknown. To address these issues, 50 t ha^{-1} basalt rock dust was spread on a floristically rich (>28 vascular plant species), mildly acidic (pH = 6.35) upland hay meadow in the UK, and its effects on soil and plant chemistry along with floristic diversity were studied. After 1 year, the basalt-amendment increased soil pH at 0 – 10 cm by 0.25 units, and significantly increased concentrations of the following elements: calcium (+13%), magnesium (+14%), sodium (+22%), and silicon (+35%). This corresponds to a potential CDR in the upper 10 cm of soil of approximately 542 kg CO_2 ha^{-1} yr^{-1}. The soil exchangeable trace elements barium, cadmium, caesium, cobalt, nickel, chromium, strontium, and vanadium were increased by the basalt additions, but only strontium significantly increased in the plant shoots, and none of the elements were close to ecotoxic thresholds. The plant nutrient magnesium had increased concentrations in plant shoots, as did sodium, and in hay analysis the phosphorus concentration was also increased. Despite these changes, the annual hay cuts showed no significant differences in sugar, protein, digestibility, or yield, so appeared not to affect the forage quality and quantity. Importantly, basalt amendment did not significantly impact Simpson or Shannon-Weiner plant diversity indexes or species richness. These findings emphasize that basalt application on floristically diverse UK grasslands may result in CDR, while providing some nutritional elements to both the plants and soil.

5.1. Introduction

Addressing the effects of rising anthropogenic carbon dioxide (CO_2) concentrations to minimize its impacts on the global climate, agriculture, and ecosystems is one of the greatest scientific challenges of the 21st Century. Almost all proposed scenarios for limiting global warming to 1.5 °C include forms of CO_2 removal (CDR) (Smith et al., 2023). It is estimated that up to 1000 gigatonnes (Gt) of CO_2 will need to be removed from the atmosphere by the end of the century to meet global temperature targets (Masson-Delmotte et al., 2022). Enhanced rock weathering (ERW), which has been gaining increasing attention, is a terrestrial CDR strategy where ground calcium and magnesium silicates are spread onto soils to accelerate carbon capture by the inorganic carbon cycle (Schuiling and Krijgsman, 2006). If ERW is rolled out globally across cultivated land, models estimate that it could sequester from 0.5 to 2 Gt of CO_2 annually by the year 2050 (Beerling et al., 2020). How ERW results in CO_2 drawdown is described in detail by Hartmann et al. (2013). Briefly, CO_2 dissolves in rainfall and the soil solution to form carbonic acid, which then reacts with calcium or magnesium silicates. This reaction releases cations from the rock, while forming bicarbonate (HCO_3^-) and by-products, effectively drawing down CO_2 from the atmosphere. The cation and bicarbonate can later react pedogenically or biogenically to form carbonates, resulting in a more stable storage of CO_2, but with a 50% loss of CO_2 back to the atmosphere. This process can be interrupted by the presence of other strong acids, such as nitric or sulphuric acid, which can dominate silicate weathering and/or react with HCO_3^- resulting in an outgassing of CO_2 to the environment (Guo et al., 2015) and a loss of CDR potential.

The potential co-benefits of ERW in agriculture are reviewed by Abdalqadir et al. (2024), who noted that research has been performed using multiple different rock substrates with each carrying different risks, co-benefits, and CDR potentials. The abundant igneous rock basalt is rich in

calcium and magnesium silicates, but less reactive than olivine-rich ultramafic rocks and mafic rocks like phonolite or trachy-andesite (Van Der Bauwhede et al., 2024; Lewis et al., 2021). Basalt is however highly suitable for ERW, as it is widely available as quarry fines from existing extractive operations for road stone and other uses, and carries low concentrations of toxic elements like nickel and chromium (Kantola et al., 2017; Beerling et al., 2018, 2020). However, concerns have been raised regarding toxicity accumulation in soils with repeated applications (Dupla et al., 2023), although soils derived from basalt parent material are generally fertile and widely used to grow crops and there are only a few records of some soils with basalt parent materials causing unacceptably high toxic element concentrations in crops (Wang et al., 2020).

In agriculture, ground basalt application increases alkalinity by raising the pH of soil (Gillman et al., 2002; Anda et al., 2015), and soil leachates (Buckingham and Henderson, 2024), and therefore can substitute for agricultural lime (Beerling et al., 2018). Basalt also is a multi-element rock that contains most of the essential elements required by plants, so its weathering can serve as a fertilizer (Lewis et al., 2021). For example, after 4 years of 50 t ha^{-1} $year^{-1}$ basalt amendments to trial plots of the widely grown *Zea mays*/*Glycine max* rotation in the US, significant increases in the concentrations of nutritionally important potassium, magnesium, manganese, phosphorus, and zinc have been found in grains, while potentially toxic trace metal concentrations remained unchanged (Beerling et al., 2024). Similarly, grain concentrations of silicon, calcium, strontium, and potassium all significantly increased with basalt amendment to *Sorghum bicolor* (Kelland et al., 2020). Other studies of basalt additions have reported yield gains or growth improvement including in the staple cereals maize – *Z. mays* (Chiaravalloti et al., 2023; Guo et al., 2023; Luchese et al., 2023; Beerling et al., 2024), wheat - *Triticum aestivum* (Guo et al., 2023), and *S. bicolor* (Kelland et al., 2020). Other crops studied include soybean – *G. max*

(Guo et al., 2023; Luchese et al., 2023; Beerling et al., 2024), potato – *Solanum tuberosum* (Vienne et al., 2022), sugar cane – *Saccharum officinarum* (D'Hotman De Villiers, 1961), and cocoa – *Theobroma cacao* (Shamshuddin et al., 2011).

To date, there have only been a few recent studies of the effects of soil basalt amendment on grasses, including *Lolium perenne* (Reynaert et al., 2023), Festulolium (Reynaert et al., 2023), and *Miscanthus* (Kantola et al., 2023). The perennial bioenergy grass *Miscanthus* had increased yields and a greater potential net ecosystem carbon draw-down with basalt additions than *Z. mays* and *G. max* in a field trial in the US (Kantola et al., 2023). A recent model predicting the UK's potential CDR using ERW found that it could remove from 6 – 30 megatonnes of CO_2 annually by the year 2050; however, this model only included annual croplands and not grasslands (Kantzas et al., 2022). Permanent grasslands cover 40% of the UK's total land area, amounting to 9.7 million hectares, compared to only 4.5 million hectares for croplands (DEFRA, 2023). Despite this, there have been no field trials characterizing ERW's potential CDR, co-benefits, or risks for semi-natural grasslands. Permanent grasslands are extremely important contributors to UK biodiversity, but given the historical adverse impacts of agriculture on UK plant and animal communities (Burns et al., 2023), it is important to establish if basalt additions to species-rich grasslands pose a threat to their floristic diversity.

Base-rich grasslands underlain by chalk or limestone support some of the most biodiverse plant communities in Europe and the UK (van Swaay, 2002; Mortimer et al., 1998), so increasing soil alkalinity in acidic grasslands could potentially enhance biodiversity and help reverse historical acidification due to acid deposition from anthropogenic pollutants (Breemen et al., 1984). Agricultural lime amendments, typically using crushed limestone, which increases pH and base calcium concentrations and improves grassland productivity and livestock weight

gain, have sometimes been reported to reduce floristic diversity, especially when combined with phosphorus and potassium fertilizers (Yu et al., 2011). However, other studies have shown no major biodiversity losses with lime use, although lime plus manure inputs can reduce diversity (Kirkham et al., 2008, 2014). Limestone reacts 3 orders of magnitude faster than calcium silicates like basalt (Lasaga, 1984; Morse and Arvidson, 2002; Bufe et al., 2021; Lewis et al., 2021), so when applied at the same mass basalt will cause a slower increase in soil pH compared to limestone perhaps reducing any potential biodiversity loss. Basalt may also further act differently to lime due to its multi-element nutrient composition, potentially delivering nutrients that improve livestock production (Yu et al., 2011).

To address the knowledge gaps in ERW deployment in temperate grasslands, a single basalt amendment (50 t ha^{-1}) was applied to a species-rich upland hay meadow. The 1-year (2 growing seasons) study aimed to achieve three objectives. Firstly, to assess whether the soils showed any signals of rock weathering such as changes in pH and elemental concentrations. Secondly, to assess whether basalt amendment significantly altered plant community composition. Thirdly, to determine if there were any benefits or risks to the farmer in terms of hay yields, plant nutrition, and toxic element accumulation.

5.2. Materials & Methods

5.2.1. Field trial design:

The field trial was established on an upland (320 m above sea level) acidic hay meadow (sandy clay) in the Peak District, England (53°10'45.7"N 1°59'36.4"W), comprising five (0.8 x 2 m) plots with basalt treated and untreated control areas. The grassland carried an average plant Shannon-Weiner index (Shannon, 1948) value of 2.82 across all plots and was historically grazed by cattle, but has for the past decade been grazed by primarily sheep and more infrequently by natural herbivores such as rabbits

and hares. Alongside rotational sheep grazing, annual hay cuts were also performed. In March 2022, Hillhouse basalt, from Troon, Scotland and previously characterised by Lewis et al. (2021), of < 4 mm particle size was spread by hand at the equivalent rate of 50 t ha^{-1} following rates used in crop trials (Kantola et al., 2023) while the control plots were left undisturbed. Sheep were excluded from the plots during each subsequent growing season from March to September of 2022 and 2023 to allow grass to grow for hay and floristic analysis to take place and to prevent irregular enrichment of the plots via sheep droppings or urine during that period.

5.2.2. Soil analysis

Prior to basalt amendment on March 17th, 2022, a soil core, extracted from the upper 10 cm layer with a 7 by 12 cm bulb planter, was obtained from randomly selected positions in all treated and control plots; subsequent soil cores were collected from each plot every six weeks throughout the growing season until September 1st, 2022. An additional set of 5 cores per plot was taken on April 12th, 2023, following more than one year since basalt amendment. After removal of plant biomass from the cores, (see below section 2.5), a portion of homogenized fresh soil was taken for pH measurement while the remainder was air-dried at room temperature in a fume cabinet until completely dry to touch. Once dried, the soils were sieved to < 2 mm, with removal of any remaining roots and stones.

5.2.3. Soil pH, exchangeable acidity, and CDR estimate

Two grams of the air-dried fine soil was added to either 10 mL of dH_2O (Carter and Gregorich, 2008), 0.01 M $CaCl_2$ (Schofield and Taylor, 1955), or 1 M KCl (Kabała et al., 2016) at a 1:5 ratio. The $CaCl_2$ extraction approximates the concentration of salts within the solution of fertile soils, reducing the effect of seasonality on pH, which has been well characterised using dH_2O (Kissel et al., 2009). KCl, with its high ionic strength, removes hydrogen and

aluminium from exchangeable sites within the soil with the aluminium then reacting with any hydroxide ions present (Kome et al., 2018).

Each soil-solution was shaken for 1 hour at 250 rpm on an orbital shaker (SH-200, Cole-Parmer). pH measurements were then taken using the Bante930 Benchtop pH/Ion Meter equipped with an P11 glass electrode calibrated to pH values 4, 7, and 10.

Potential CDR was estimated through charge balancing base cations calcium, magnesium, sodium, and potassium with bicarbonate ions, as described previously (Hartmann et al., 2013; Kanzaki et al., 2023; Beerling et al., 2024). Briefly, the net molar change of cations in the soil due to basalt amendment was totalled. Using a 2:1 molar ratio of HCO_3^{-1} to cations, the CO_2 sequestration in moles kg^{-1} was estimated. With a sampling depth of 10 cm and assuming a bulk density of 0.94 g cm^{-3}, based on data from a nearby field (UKSO, 2024), the total soil mass per hectare was estimated to be 940,000 kg ha^{-1}. This value was then multiplied by the estimated CO_2 drawdown rate (CDR) to calculate the potential CO_2 sequestration per hectare.

5.2.4. Soil element extractions

The air-dried fine soil samples from each plot and treatment were analysed for exchangeable cation concentrations, phosphorus, and soil silicon and molybdenum using the extraction methods: modified ammonium acetate (Carter and Gregorich, 2008), Olsen-P (Olsen et al., 1954; Fixen and Grove, 2018), and hot water (Beerling et al., 2024), respectively. The plant availability of basalt's most common potentially toxic trace elements arsenic, barium, cadmium, chromium, cobalt, copper, lead, nickel, vanadium, and zinc (Kabata-Pendias, 2000; Bradl, 2005; Lar and Gusikit, 2015) was also measured using the ammonium acetate extraction (Castilho and Rix, 1993; Ure et al., 1993). Briefly, 2 g of soil were mixed with 20 mL of 1 M ammonium acetate solution at pH 7 followed by

shaking at 250 rpm on an orbital shaker (SH-200, Cole-Parmer) for 2 hours. The Olsen-P plant-available phosphorus content was extracted using a modified method from Sims (2000), in which 1 g of air-dried fine soil was mixed with 0.5 M sodium bicarbonate at pH 8.5 and shaken at 250 rpm for 30 min. Finally, soil silicon and molybdenum were extracted using a hot water extraction technique described by Beerling et al. (2024). This involved boiling 10 g of air-dried fine soil in 25 mL ultra-high purity H_2O at 98 °C in a hot water bath (stirring water bath, Cleaver Scientific Ltd.) for 15 min, followed by brief vortexing. All extractions were subsequently centrifuged at 4700 x g for 5 min with their supernatant being acidified to 2% nitric acid before being sent for inductively coupled plasma mass spectrometry (ICP-MS) at the University of Nottingham Agricultural and Environmental Sciences department.

Total carbon and nitrogen analysis was also performed on air-dried fine soils taken pre-basalt amendment and one year post amendment. These soils, which carried minimal carbonate content, were ball-milled (Pulverisette, Fritsch) and dried at 105°C for 24 hours, and ~30 mg was then transferred into tin boats for analysis of total C percentage, total N percentage, and C:N ratios determined by dry combustion in a CN analyser (Vario EL Cube, Hanau, Germany) using acetanilide (4–7 mg) as a standard.

5.2.5. Plant biomass

During the 2022 growing season, subsets of above and below ground biomass were taken with each soil core. Samples then had their above ground biomass cut at the soil level with the plant matter being rinsed with H_2O to prevent soil contamination. Roots were extracted from the fresh soil samples manually. Following separation roots were washed thoroughly with H_2O to remove soil particles. Both above and below ground biomass were then dried at 60 °C for 24 hours prior to being weighed.

Above ground biomass was further measured for the entire plot three times: the first being a hay cut on July 4th, 2022, the second being an aftergrowth cut on September 1st, 2022, the third and final being a hay cut on July 9th, 2023. The hay was cut at ~ 2 cm above the soil level and allowed to dry on the field for 2 days with regular turning of the grass to aid initial drying. Plot hay cuts were then milled and homogenised (SM 100, Retsch) and dried for 24 hours at 60 °C prior to weighing.

Subsets of the homogenised samples were then powdered (A10 basic, IKA), with these undergoing portable X-ray fluorescence spectroscopy (P-XRF), ICP-MS, and digestibility analysis. P-XRF was performed using the method described in Reidinger et al. (2012), using a P-XRF instrument (Nitron XL3t900 GOLDD analyser: Thermo Scientific Winchester, UK) held in a test stand (SmartStand, Thermo Scientific, Winchester, UK). Powdered material was pressed at 10 tonnes per square inch into pellets using a manual hydraulic press with a 13 mm die (Specac, Orpington, UK). The P-XRF machine was calibrated using Si-spiked synthetic methyl cellulose (Sigma-Aldrich, product no. 274429) and validated using Certified Reference Materials of NCS DC73349 'Bush branches and leaves' obtained from China National Analysis Centre for Iron and Steel. To avoid signal loss by air absorption, the analyses were performed under a helium atmosphere (Reidinger et al., 2012). Sulphur and silicon were measured using pXRF only, while phosphorus, calcium, and potassium (% dry weight) were analysed by both pXRF and ICP-MS. ICP-MS was also used to measure additional elements not analysed by pXRF. Prior to ICP-MS 0.2 g of sample was digested using an Anton Paar Multiwave microwave-assisted nitric acid and hydrogen peroxide digestion, provided by the University of Nottingham Agricultural and Environmental Sciences department. The digestibility analysis, conducted by Sciantec, was a Hay/Haylage Analysis (FN04); the analysis included assessments of crude protein, oil-b, ash, neutral

detergent fibre, acid detergent fibre, sugar, d-values, metabolised energy, and digestible energy.

5.2.6. Plant diversity measures

Plant diversity per plot was assessed in June 2022 and 2023; percent frequency for three 0.25 m^2 quadrats, with 25 internal squares, was performed on each plot. The three quadrat readings together accounted for 47% of each plot area. Using visual identification methods, percent frequency was calculated by checking each smaller square of the quadrat for the presence or absence of each plant species. The total number of squares containing a particular species was divided by the total number of squares and multiplied by 100 to obtain percent frequency of each plant species in one quadrat. Species richness (Magurran, 2013) and Shannon-wiener biodiversity index (Shannon, 1948) was calculated based on the percent frequencies.

The Principal Component Analysis (PCA), based on the covariance matrix, using GraphPad Prism 10.0.0 for Windows (GraphPad Software, Boston, Massachusetts US, www.graphpad.com), was calculated with the percent frequency values. The results were visualized using a biplot.

5.2.7. Statistical analyses

Statistical analysis was conducted using SAS/STAT software, version 9.4 (SAS Institute Inc), and R studio (R Core Team, 2024) with the lme4 package (Bates et al., 2015). The effects of basalt and time on the soil and plant parameters were assessed using linear mixed effects models using the procedure PROC MIXED (Bolker et al., 2009). Basalt application (treated vs untreated) and time (referring here to the successional sampling campaigns) were set as fixed effects. The plot, within which a basalt-treated area was compared to an untreated area as well as the area itself were set as a random effect to ensure that the independence assumption was respected (Breslow and Clayton, 1993). The limited sample size did not provide

sufficient degrees of freedom to account for the effect of time as a repeated measure in our statistical model. Time was therefore systematically initially included as an interaction with basalt in the fixed effects and only maintained if the interaction was significant at the 0.05 *p* value threshold of the type 3 test of fixed effects. To ensure accuracy, the denominator degrees of freedom for the tests of fixed effects was computed using Satterthwaite approximation (Satterthwaite, 1946). Finally, when required, a Box Cox data transformation was conducted until the normality of residuals and the homoscedasticity assumptions were met (Box and Cox, 1964). For each assessed variable, the type 3 test for fixed effects *p* value for basalt was included in the text and figures.

GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, Massachusetts US, www.graphpad.com) was used for plotting all figures and performing the independent two tailed t-tests on pretreatment basalt and control plots as well as analysing soil percent carbon and nitrogen one year post application. Significance being determined as any *p* value that was < 0.05 for both the Satterthwaite approximation and the independent two tailed t test results.

5.3. Results

5.3.1. Soil pH

Basalt amendment significantly increased soil alkalinity relative to the control (Figure 1) increasing the mean pH over five time points from April 2022 when measured with dH_2O by 0.21 units ($p < 0.001$), with 0.01 M $CaCl_2$ by 0.18 units ($p < 0.001$), and with 1 M KCl by 0.10 ($p < 0.05$). It is important to note that the specific increase in pOH may vary slightly due to the non-linear behaviour of the Nernst equation within the neutral range; however, an error of 0.01 is generally considered reasonable within this range (Cheng and Zhu, 2005).

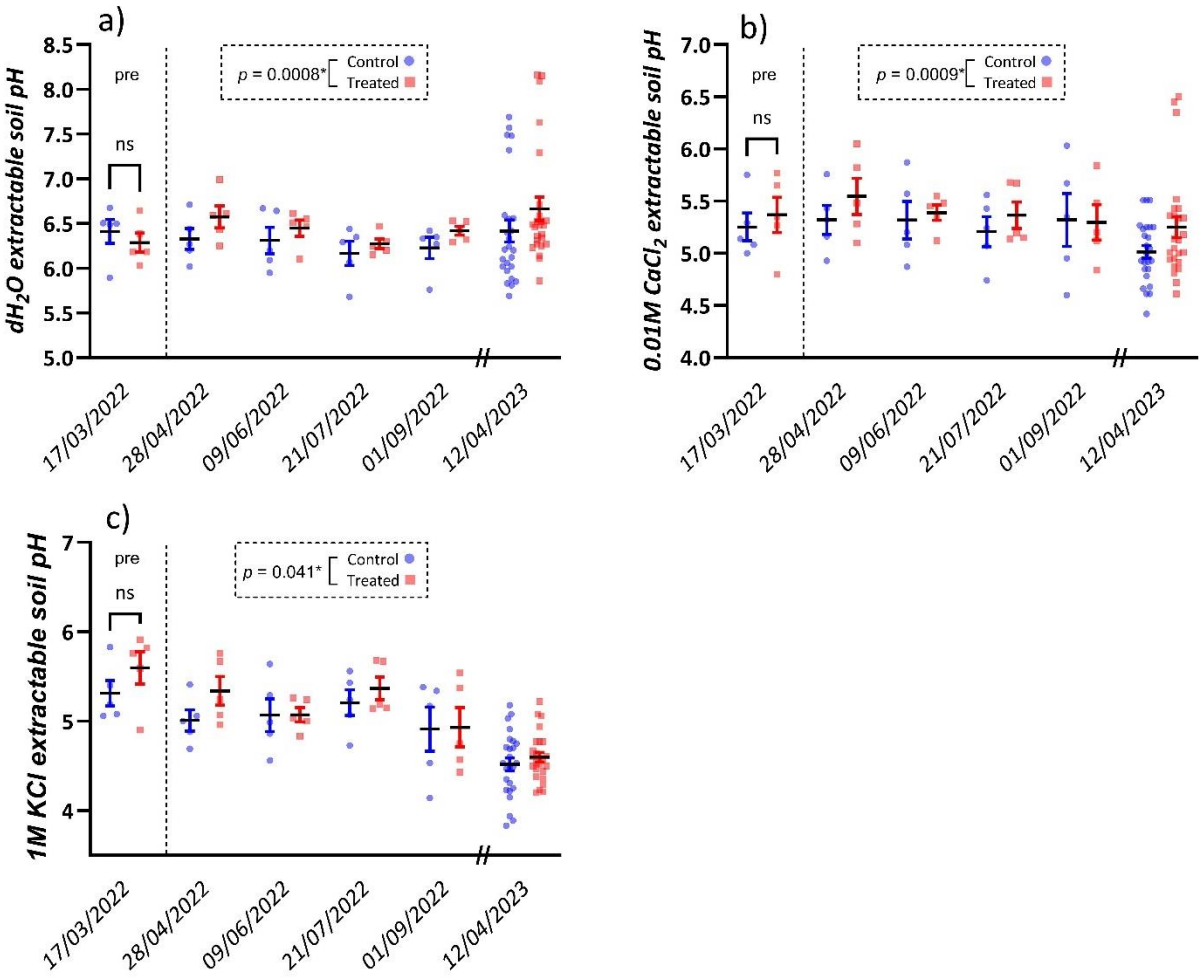

Figure 1. Soil pH measured with 1:5 dH_2O (a), 0.01 M $CaCl_2$ (b), and 1 M KCl (c) over time from both control (●) and basalt amended (■) plots. Individual measurements and standard error depicted in treatment colours while mean depicted in black. Data to the left of the vertical dashed line represent pre-basalt application values with T test significant differences between treatments displayed above. Data to the right of the vertical dashed line represent post-basalt application values, used for subsequent mixed linear effects modelling. The mixed linear effects models treatment *p* value displayed in the dashed box with a significant *p* value denoted by *.

5.3.2. Soil elemental concentrations

Basalt application resulted in significant increases in the soil extractable concentrations of calcium (+230.2 mg kg^{-1}, $p < 0.01$), magnesium (+27.1 mg kg^{-1}, $p < 0.0001$), sodium (+4.869 mg kg^{-1}, $p < 0.05$) and silicon (+4.869 mg kg^{-1}, $p < 0.0001$) over one year (Figure 2). Conversely, basalt

significantly decreased soil extractable potassium concentrations by 20.55 mg kg^{-1} ($p < 0.001$), while phosphorus concentrations remained unaffected.

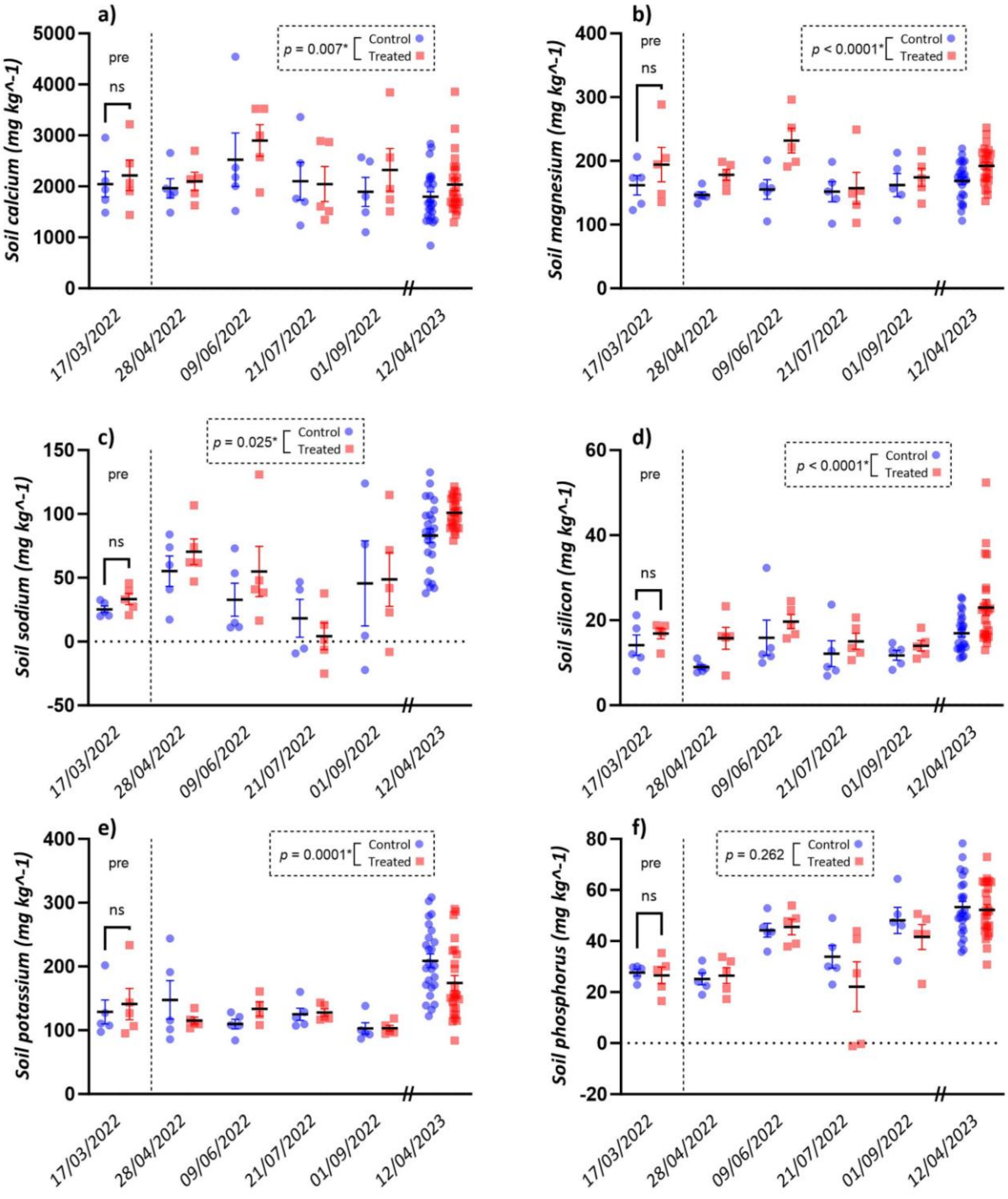

Figure 2. Soil extractable Ca (a), Mg (b), Na (c), Si (d), K (e), and P (f) over time from both control (●) and basalt amended (■) plots. Individual data and standard error depicted in treatment colours while mean in black. Data to the left of the vertical dashed line represent pre-basalt values with T test differences between treatments displayed above. Data to the right of the

vertical dashed line represent post-basalt application values, used for subsequent mixed linear effects modelling with treatment *p* value shown.

Using the calculation mentioned in section 2.3 total moles of cations was estimated to be 0.0065 mol kg^{-1} soil equivalent to an estimated maximum sequestration potential of 0.013 mol CO_2 kg^{-1} soil. When calculated for the upper 10 cm across 1 hectare an initial estimated maximum CDR potential per hectare was 541.518 kg ha^{-1}.

The addition of basalt to soil significantly increased the ammonium acetate extractable concentrations in the soil of some of the most common toxic trace elements in the rock (Figure 3): barium (+10.1 %, $p < 0.01$), cadmium (+11.1 %, $p < 0.01$), caesium (+23.4%, $p = 0.01$), cobalt (+ 53.5 %, $p < 0.001$), nickel (+ 789.6 %, $p < 0.001$), chromium (+187.2 %, $p < 0.0001$), strontium (+23.7 %, $p = < 0.0001$), and vanadium (+ 17.7 %, $p < 0.05$). No significant changes were noted for arsenic, copper, lead, zinc, and rubidium in the soil (not shown).

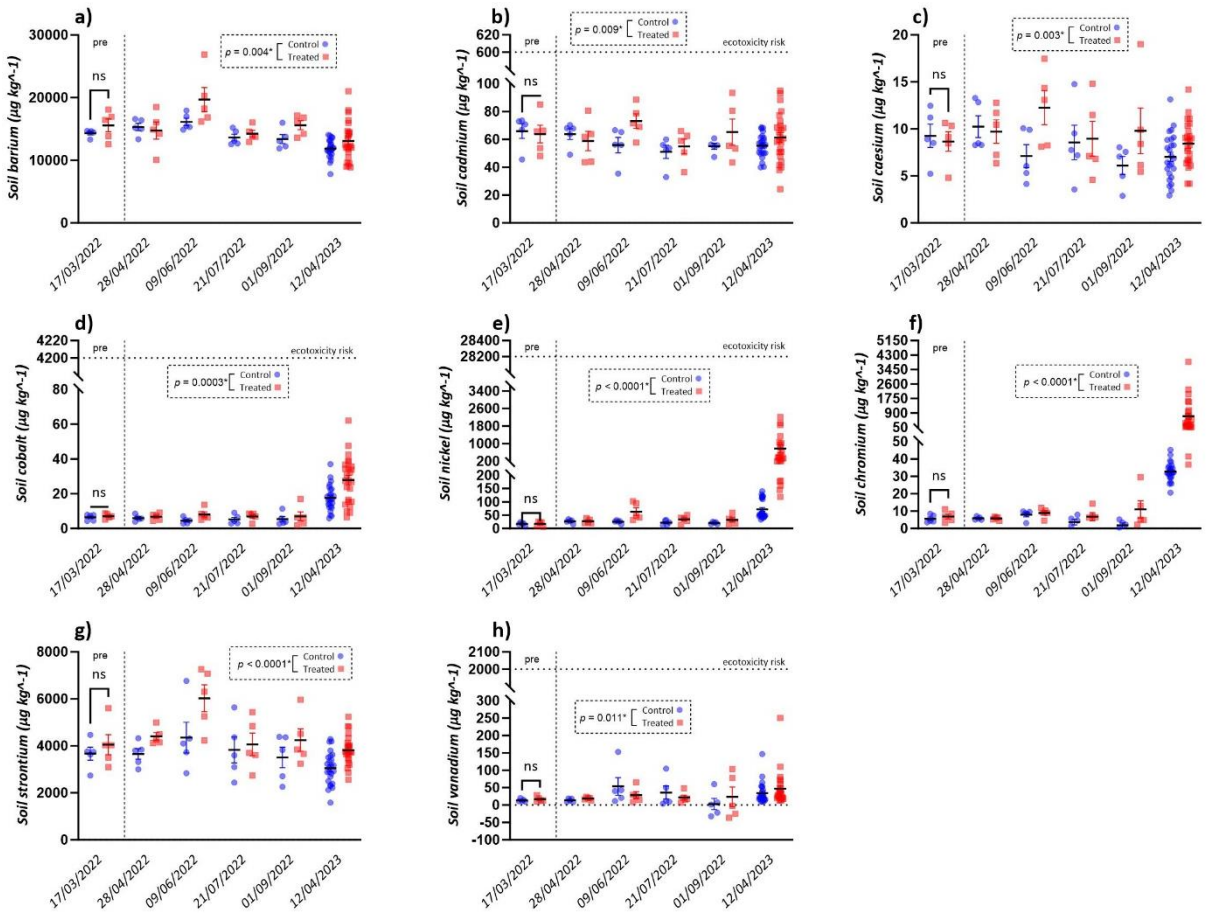

Figure 3. Ammonium acetate soil extractable heavy metals Ba (a), Cd (b), Cs (c), Co (d), Ni (e), Cr (f), Sr (g), and V (h) over time from both control (●) and basalt amended (■) plots. Individual data and standard error depicted in treatment colours while mean in black. Data to the left of the vertical dashed line represent pre-basalt values with T test differences between treatments displayed above. Data to the right of the vertical dashed line represent post-basalt application values, used for subsequent mixed linear effects modelling with treatment *p* value shown. The dotted line denotes the ecotoxicity risk threshold determined by the UK environment agency (Martin et al., 2022); derived from consolidated literature estimates with varying extraction methods, limits were also only given for cadmium, cobalt, nickel, and vanadium as they are considered the primary concern in the UK.

Soil percent organic carbon and total nitrogen, measured prior to application and one year after basalt amendment, showed no significant differences between basalt and control plots (Figure 4).

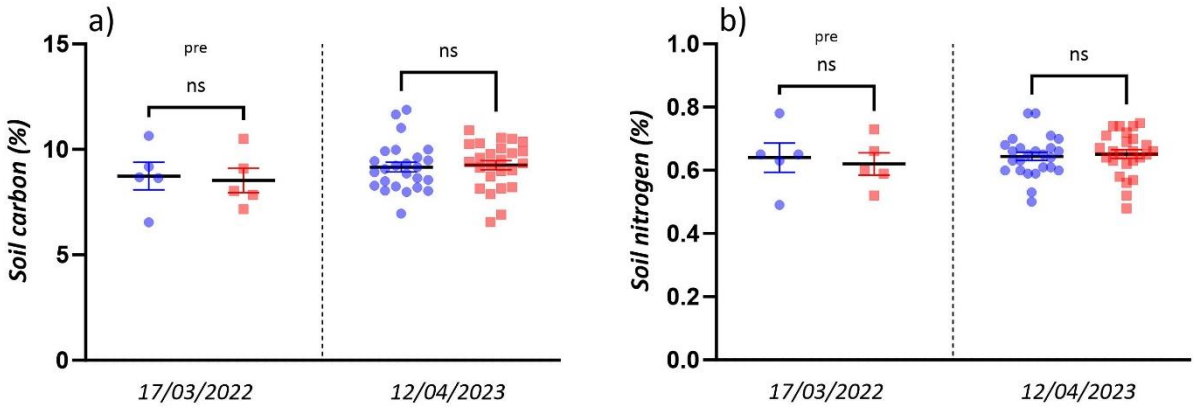

Figure 4. Soil percent carbon (a) and total nitrogen (b) pre and one year post amendment from both control (●) and basalt amended (■) plots. Individual measurements and standard error depicted in treatment colours while mean depicted in black. Data to the right of the vertical dashed line represent post basalt application, used for subsequent statistical analysis. T test significant differences presented alongside the graph with ns = $p > 0.05$.

5.3.3. Plant yield and nutrient analysis

Sub-sampling of above and below ground biomass conducted during the 2022 growing season (Figure 5a, b) showed no significant effect of basalt amendment on plant biomass. This was further corroborated by no significant increases in yearly hay yields in basalt-amended plots (Figure 5c).

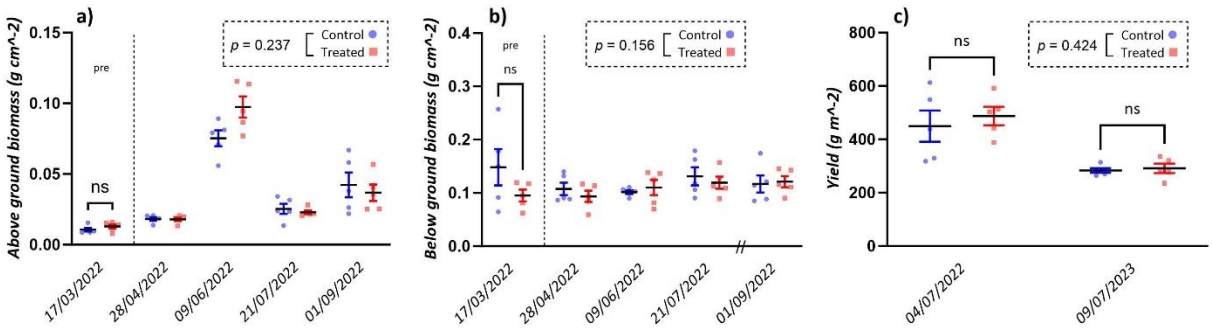

Figure 5. Seasonal variation in plant above (a) and below (b) ground biomass from both control (●) and basalt amended (■) plots in 2022 before and after hay cut, and (c) annual hay cut yield for 2022 and 2023. Individual measurements and standard error depicted in treatment colours while mean depicted in black. For panels a) and b) data to the left of the vertical dashed line represent pre-basalt application values with T test significant differences between treatments displayed above. Data to the right of the vertical dashed line represent post-basalt application values, used for subsequent mixed linear effects modelling. The mixed linear effects models treatment p value displayed in the dashed box with a significant p value denoted by *. In panel c) t test significant differences between treatments are displayed.

The ICP-MS results from the digested hay powders found significant average increases in the shoot concentrations of the micronutrients sodium (780.9 mg kg^{-1}, $p < 0.001$) and magnesium (189.8 mg kg^{-1}, $p < 0.05$) in forage from basalt-amended soil (Figure 6a, 6b). There was also a significant increase in strontium (2.01 mg kg^{-1}, $p < 0.0001$) and a near significant increase in rubidium (2.83 mg kg^{-1}, $p > 0.05$) (Figure 6d,6e). Despite their significant increases in the soil, plant concentrations of calcium (Figure 6f), barium, cadmium, caesium, cobalt, nickel, chromium, strontium, and

vanadium were not significantly affected with basalt application. Additionally, there were no significant effects of basalt amendment on the plant concentrations of phosphorus (Figure 6c), potassium, boron, sulfur, titanium, aluminium, manganese, iron, copper, zinc, selenium, molybdenum, thallium, and lead.

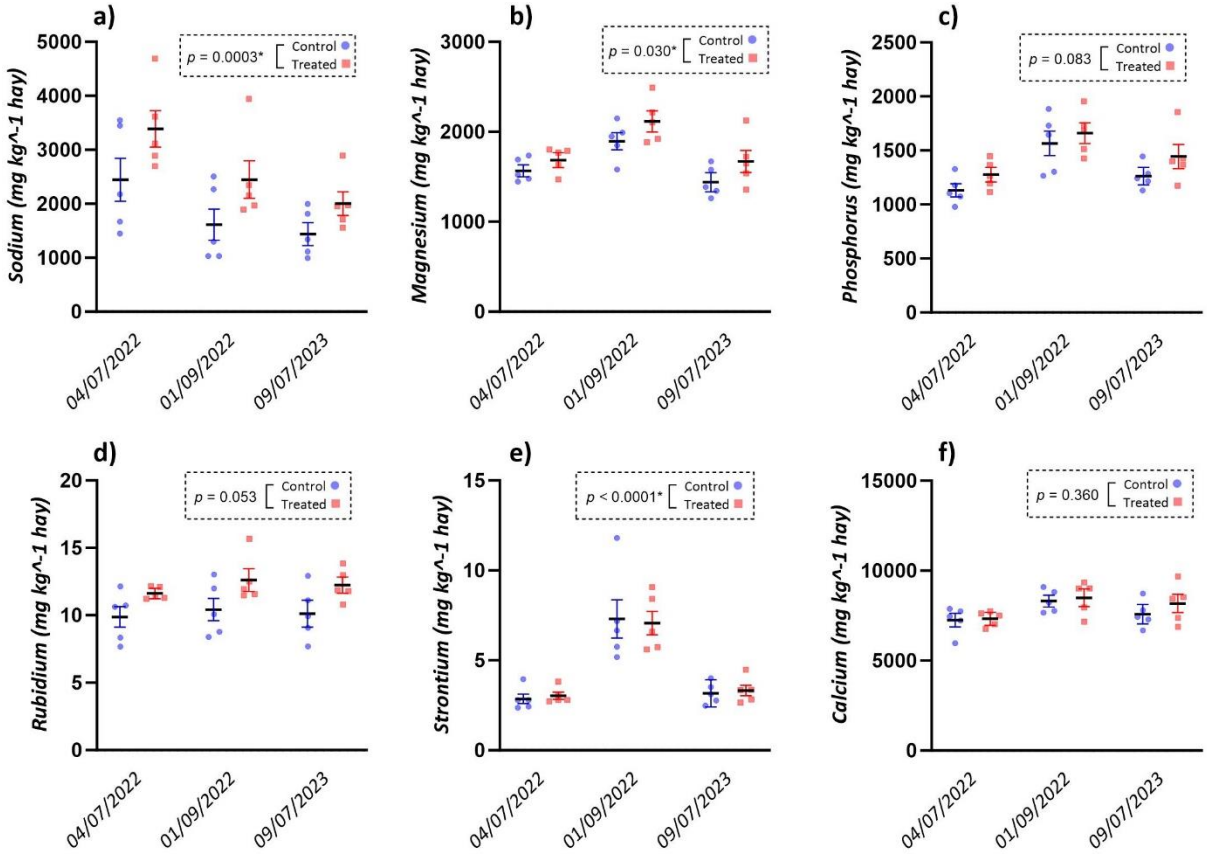

Figure 6. Shoot biomass concentrations of sodium (a), magnesium (b), phosphorus (c), rubidium (d), strontium (e), and calcium (f) from annual hay cuts and the 2022 after growth cut from both control (●) and basalt amended (■) plots. Individual measurements and standard error depicted in treatment colours while mean depicted in black. The mixed linear effects models treatment p value displayed in the dashed box with a significant p value denoted by *.

pXRF analysis of hay and aftergrass show a significant increase of 0.013% ($p < 0.05$) dry mass of the macronutrient phosphorus in basalt amended grassland. However, it also showed that basalt did not significantly affect the concentrations of chlorine, sulphur, potassium, calcium, and silicon (Figure 7).

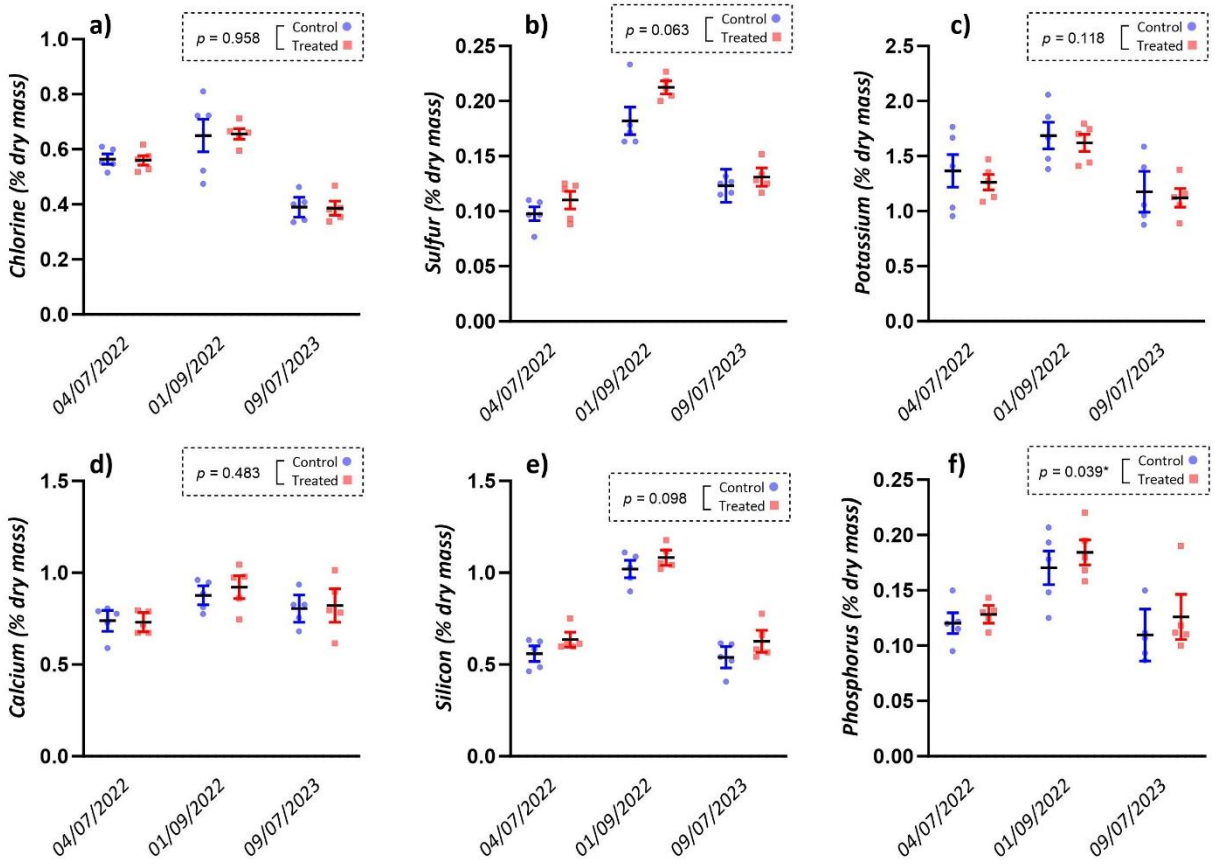

Figure 7. Hay and aftergrass dry matter concentrations of chlorine (a), sulphur (b), potassium (c), calcium (d), and silicon (e) from both control (●) and basalt amended (■) plots. Individual measurements and standard error depicted in treatment colours while mean depicted in black. The mixed linear effects models treatment p value displayed in the dashed box with a significant p value denoted by *.

Hay nutrient analysis, conducted by a commercial laboratory, Sciantec, revealed no significant changes due to basalt treatment in crude protein, oil-B, ash, neutral detergent fibre, acid detergent fibre, sugar, digestive values, metabolised energy, and digestible energy (Figure 8).

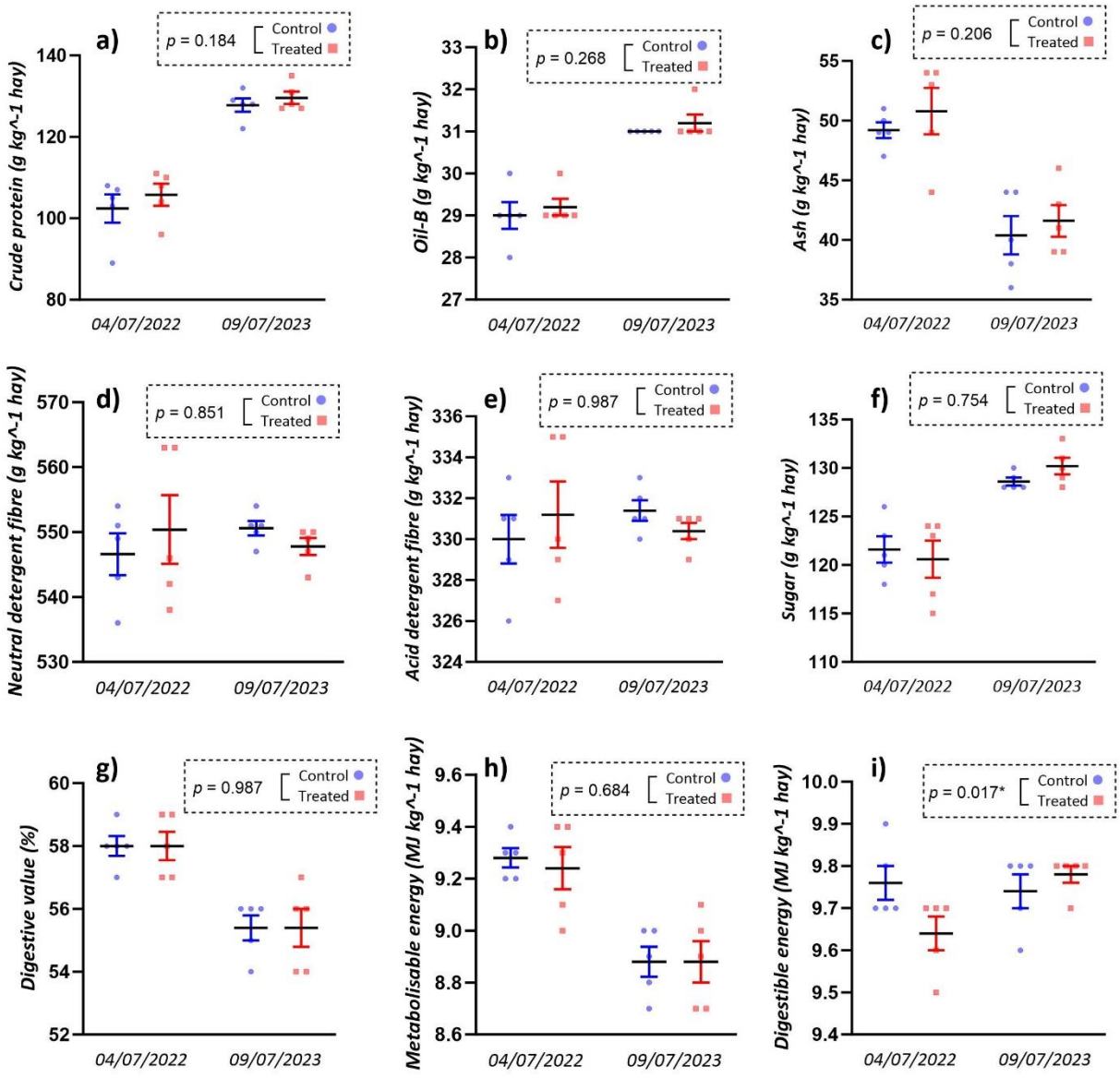

Figure 8. Analysis of annual hay cut crude protein (a), oil-B (b), ash (c), neutral detergent fibre (d), acid detergent fibre (e), sugar (f), d-values (g), metabolised energy (h), and digestible energy (i) from both control (●) and basalt amended (■) plots. Individual measurements and standard error depicted in treatment colours while mean depicted in black. The mixed linear effects models treatment p value displayed in the dashed box with a significant p value denoted by *.

5.3.4. Plant biodiversity

In total 28 vascular plant species were recorded across the total sampled quadrat area (7.5 m^2), including herbs, legumes, grasses, and a wood rush, providing fairly typical hay meadow diversity (Starr-Keddle, 2022). The analysis of the average percent frequencies of different plant species for 2022 (Figure 9) revealed some non-significant initial differences between the control and basalt treated plots. In 2023, similar average percent frequencies were observed for almost all identified plant species. However, one exception was *Hypochaeris radicata*, which exhibited a significant reduction in average percent frequency by 13.9% ($p < 0.01$). It is important to note that for several species identified including *Trifolium repens*, *Lolium perenne*, *Festuca ovina*, *Taraxacum officinale*, *Scorzoneroides autumnalis*, *Cardamine pratensis*, *Prunella vulgaris*, *Bellis perennis*, *Alopecurus pratensis*, *Vicia sativa*, and *Veronica chamaedrys* the impact of basalt amendment could not be statistically assessed. This occurred because of the non-normal distribution of residuals.

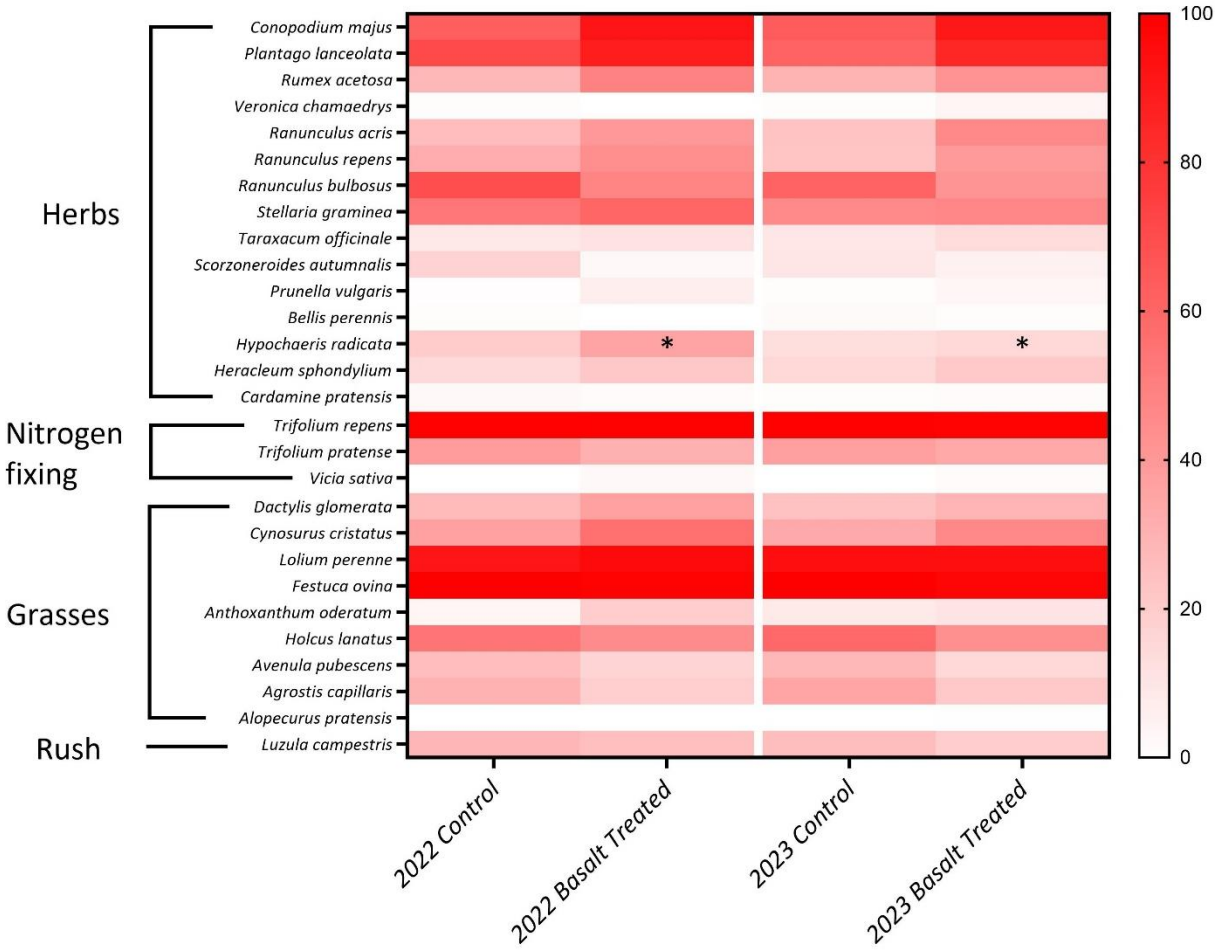

Figure 9. Average percent frequency (0 – 100%) heat map of functionally grouped plant species identified within both control and basalt treated plots in 2022 and 2023. Statistically significant differences ($p < 0.05$) relative to the control plots denoted by *.

The PCA plot (Figure 10) below further supports that basalt did not significantly alter plant community composition as no obvious clustering effect due to either year or basalt amendment was seen. Furthermore, only 39.2% of the cumulative proportion of variance was accounted for with the two largest eigenvalue principal components (PC).

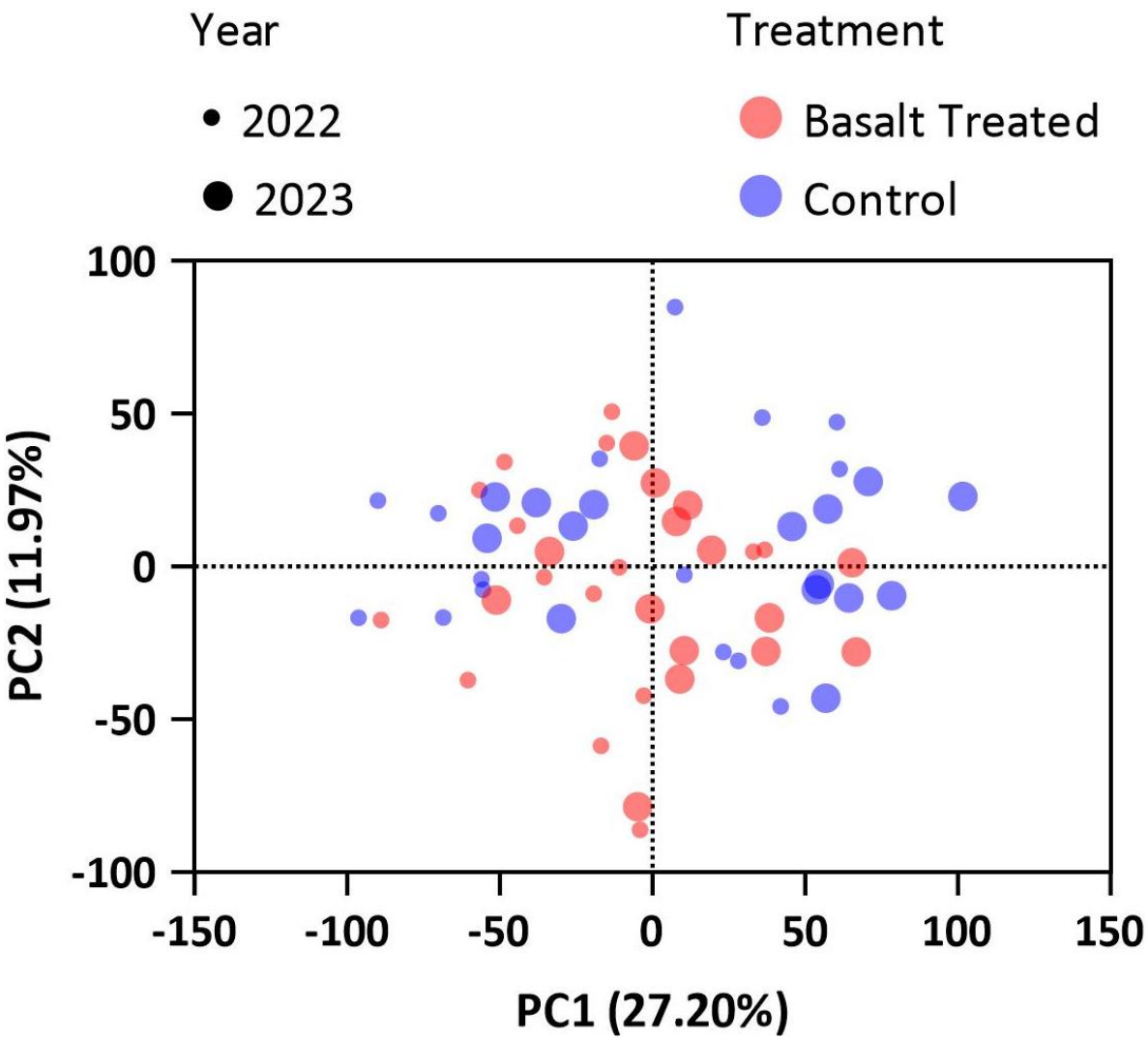

Figure 10. PCA on covariance matrix calculated with average plot percent frequency with the two greatest eigenvalues for axis PC1 (2281) and PC2 (1004). Groupings of points by treatment and year denoted by colour and size, respectively.

The cumulative species-area curves revealed high diversity at small spatial scales, with most species being found within single 0.25 m^2 quadrats, and the curves approaching a plateau value by 0.75 m^2 (Figure 11). Basalt amendment tended to increase rather than decrease species richness even in 0.25 m^2 areas, and the species-area curves remained similar between years and treatments, but tended to show greater basalt enhancement of diversity in the second year (Figure 11). Similarly, species richness and biodiversity indexes, including Simpson's and Shannon-Weiner, were not significantly affected by basalt amendment over time (Figure 12). Further,

subdivision of plant species into functional groups non-legume forbs, legumes, and grasses also showed no significant changes to their Shannon-Weiner diversity index values due to basalt amendment (not shown).

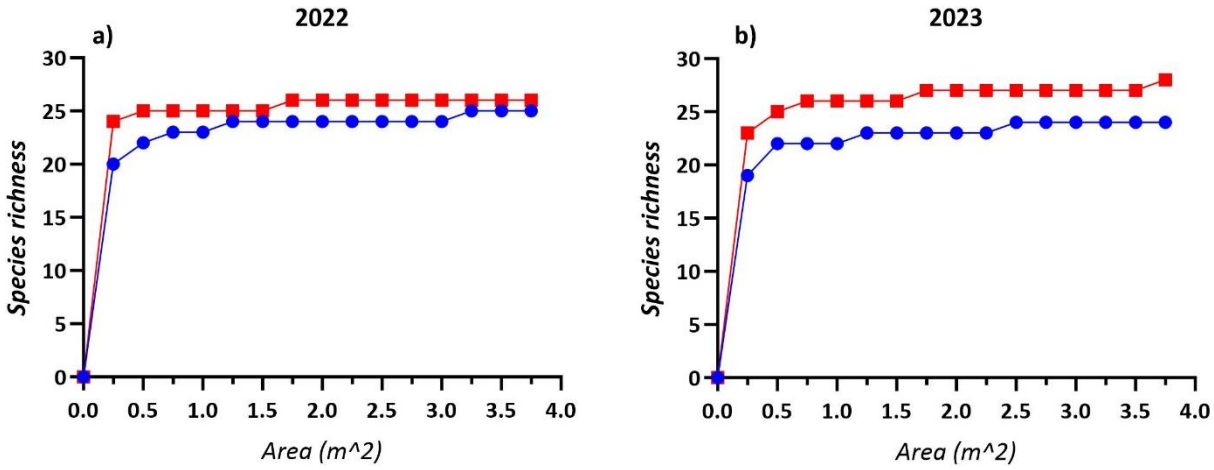

Figure 11. Cumulative higher plant species richness by quadrat (0.25 m²) from control (●) and basalt amended (■) plots from 2022 (a) and 2023 (b) floristic diversity surveys.

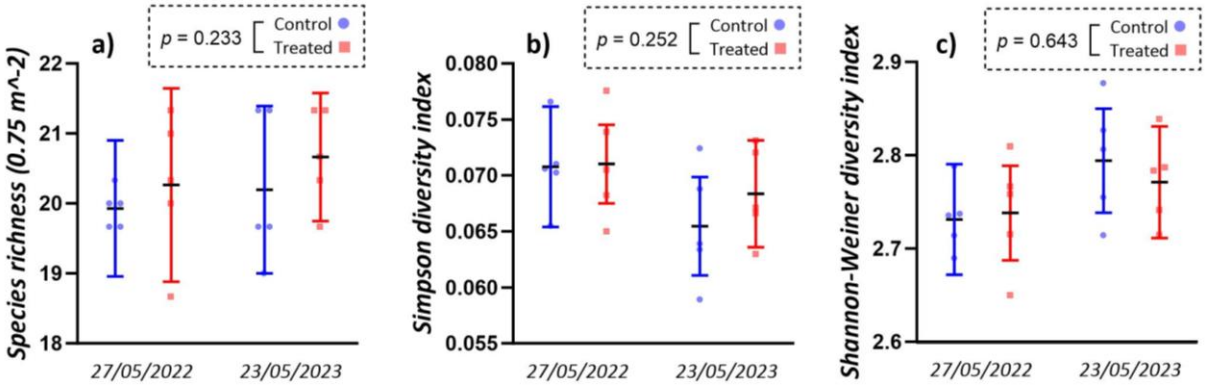

Figure 12. Species richness (a), Simpson (b) and Shannon-Weiner (c) biodiversity index from annual hay cut 2022 and 2023. Individual measurements and standard error depicted in treatment colours while mean depicted in black. The mixed linear effects models treatment p value displayed in the dashed box with a significant p value denoted by *.

5.4. Discussion

Following basalt amendment to the species-rich hay meadow, the exchangeable soil cations magnesium, sodium, calcium, and silicon showed significant concentration increases, indicating basalt weathering, and highlighting grasslands CDR potential for ERW. An initial estimate of potential CDR in the upper 10 cm of soil gives the basalt amendment a maximum carbon drawdown of 542 kg ha^{-1} yr^{-1}. Although this estimate represents a maximum scenario, previous research has reported that weathering is predominantly driven by carbonic acid at pH levels greater than 5 (Hamilton et al., 2007). This suggests that carbonic acid was the primary driver of weathering in the present study, and since the field has no recent history of chemical fertilizer use, the estimate may closely reflect its true CDR potential. Additionally there are approximately 11,000 ha of hay meadows in the UK (National Trust, 2024), so if these weathering rates are typical it would suggest the potential to remove over nearly 6000 tonnes of CO_2 annually by basalt applications, without taking into consideration potential reductions in CO_2 release from reduced lime use. However, more field measurements are needed to confirm this potential.

One year after amendment, pH (dH_2O) within basalt amended plots increased by 0.25 pH units. This change is comparable to the literature, though is lower perhaps than expected considering the 50 tonne ha^{-1} application rate and the year-long duration of the study. For instance, Skov et al. (2024) reported that 256 days after basalt application at a rate of 18.86 tonne ha^{-1} to a crop of *Avena sativa L*, pH rose from 0.2 to 0.29 units, depending on the cultivation type. However, it is important to note that in the present study there was quite large variability between replicates, partially driven by plot variation which contributed on average 0.12 ± 0.35 of the variation seen in the mixed linear effects model over the course of the experiment. Significant increases in soil pH were seen regardless of the extraction method used, dH_2O, 0.01 M $CaCl_2$, or 1 M KCl, but the higher ionic

strength extractants tended to give lower pH differences suggesting that most of the pH change is from the creation of loosely bound salts rather than tightly adhered cations and exchangeable protons on exchange sites. Both the control and the treated plots in this case had pH's above the recommended minimum value of 6.0 for a grassland and forage crops on mineral soil (Agriculture and Horticulture Development Board, 2023). The $CaCl_2$ extraction, intended to address seasonal variation, however, found the pH measured including exchangeable acidity of both the control and basalt amended plots one year after amendment well below the recommended value, at 5.01 ± 0.27 and 5.25 ± 0.41, respectively.

Despite the significant increases in magnesium, sodium, calcium, and silicon, soil potassium concentrations decreased significantly with basalt amendment. Hillhouse basalt, described in more detail by Lewis et al. (2021), is composed of 0.948% potassium approximating to 80.58 g being applied to each plot. This net loss from the soil was not compensated for by increased concentrations within the plants as they were significantly unaffected due to basalt amendment. The loss is potentially supported by the near significant increases seen in plant concentrations of rubidium, commonly used as a tracer for potassium (Drobner and Tyler, 1998). One study noted that the concentration of rubidium within the sedge *Carex pilulifera* significantly decreased with increasing soil concentrations of potassium (Drobner and Tyler, 1998); suggesting that a loss of soil potassium could increase concentrations of plant rubidium. These results pose questions regarding the accessibility of potassium within the Hillhouse basalt and whether it should always be considered as a co-benefit to basalt application. Phosphorus, vital for plant and animal growth (Cherian, 2020), appeared to significantly increase in the plants, when measured with pXRF, despite the soil concentrations of phosphorus not changing with basalt amendment. This could be due to the increased soil pH making both organic and inorganic phosphorus more accessible (Penn and Camberato, 2019),

and/or from a fertilisation effect due to the basalt, which contained 0.108% phosphorus (Lewis et al., 2021).

The single application of 50 t ha^{-1} of basalt led to substantial increases in potentially toxic trace elements within the soil. Of possible concern were concentrations of nickel and chromium, well known for their toxicity to plants (Hassan et al., 2019; Cervantes et al., 2001), soil microorganisms (Li et al., 2015; Dhal et al., 2013), and cattle (O'Dell et al., 1970; Coetzee et al., 2020), which significantly increased by 790% and 187% respectively, relative to the control over the course of a year. Typical nickel concentrations in soils range from 20 to 30 mg kg^{-1} (Echevarria et al., 2006); using data from a nearby permanent grassland, where the bulk soil density is approximately 0.94 g cm^{-3} (UKSO, 2024), a 50 t ha^{-1} application of Hillhouse basalt might be expected to increase the total nickel concentration by 10.37 mg kg^{-1}, although most of this would likely remain bound within the minerals in the rock dust. Previous ERW experiments provide some contradictory results on toxic element responses. For instance, when *Solanum tuberosum* was grown for 99 days in mesocosms containing basalt at rate of 50 t ha^{-1} Vienne et al. (2022) measured a 41% increase in nickel, but no increase in chromium. Beerling et al. (2024) noted no significant increases in the soil for both nickel and chromium following four years of annual 50 t ha^{-1} basalt applications to a soy-maize rotation in the US. The mineralogy of the Hillhouse basalt used in our study may explain these increases as it was composed of 14.7% olivine (Lewis et al., 2021), a mineral known for being enriched in nickel and chromium. This resulted in it having double the nickel and chromium levels compared to the global average for basalts (Dupla et al., 2023). This corroborates the potential risks of using olivine-rich basalts suggested previously (ten Berge et al., 2012; Beerling et al., 2018; Haque et al., 2020; Dupla et al., 2023). The increases in soil trace element concentrations did not lead to any significant increases in the hay; however, these trace elements in soils could still affect

grazing livestock. Cattle grazing in fields ingest 3 to 8% of their dry matter intake as soils (Thornton and Abrahams, 1983), and with sheep this can be up to 30% (Abrahams and Steigmajer, 2003). The impact of this phenomenon remains understudied but has been shown, in the case of fluorine and lead, to be able to expose grazing animals to trace element levels exceeding the recommended dietary guidelines (Abrahams and Blackwell, 2013). The magnitude of direct transfer that ERW could trigger, however, remains unclear.

Contrary to the heavy metal results, the increasing concentrations of sodium and magnesium in soil resulted in significant concentration increases in the forage. These increases in magnesium and sodium could prevent grass tetany, which results in reduced feeding and other symptoms, and reduce the need for supplemental sodium licks (Cherian, 2020). Unexpectedly, although extractable calcium concentrations were increased in the soil by basalt, there were no significant effects on shoot concentrations. It is possible that strontium, which increased significantly in the plant and carries the same charge as calcium, competed for calcium binding sites in the plant cells (Jovanović et al., 2021). The effect of strontium on plant health varies depending on plant type and concentration, with some instances showing potential benefits but more commonly raising concerns of toxicity (Burger and Lichtscheidl, 2019).

The yield and nutrient content from basalt amended plots did not differ significantly from those of the control plots. Figure 4a illustrates that, despite top dressing basalt at a rate of 50 t ha^{-1} there was no apparent smothering effect on the plants four months after application. If anything, there may have been a minor increase in yield as there was a mean increase of 38 g m^{-2} with basalt amendment. Concerns about plant digestibility due to increased silicon concentrations one year after application were unfounded, as there were no significant changes in plant silicon concentrations or digestive values due to basalt amendment. These findings suggest the basalt

amendment would be unlikely to have affected sheep weight gain in the first year after application. However, there may be potential health benefits for animals due to increased concentrations of certain essential minerals in the soil and plants, though it is important to continue to monitor trace metal toxicity in both.

Plant diversity may also provide additional health and product quality benefits through the presence of plant secondary compounds such as phenolics, terpenes, and alkaloids (Villalba et al., 2019). For example, condensed tannins have shown anthelminthic properties (Niezen et al., 1995) and have been linked to reduced emissions of the potent greenhouse gas N_2O (Woodward et al., 2001; Koenig et al., 2018). One year after basalt amendment there was no significant changes to any of the plant biodiversity metrics used including: species richness, Simpson biodiversity index, and Shannon-Weiner biodiversity index. However, species-level effects of basalt could only be determined for 60% of the plant species present, as outlined in section 3.4. Among those that could be assessed only *H. radicata* showed a negative association between its percent frequency and basalt amendment. While the importance of *H. radicata* in agriculture is debated, with some research linking it to the disease stringhalt in horses (McIntosh et al., 2019), it is not reported to have any negative effects on other grazing animals. Notably, *H. radicata* can grow in soils of pH 3.9 to 8.6 (Turkington and Aarssen, 1983) suggesting that the reduction in its percent frequency following basalt amendment is not due to the change in pH, a major factor determinant of floristic diversity (Oldén et al., 2016). One potential reason for the loss of *H. radicata* is the structure of its leaves; also known as "rough cats' ear" the broad and "hairy" leaves may have been adversely affected by the top spreading of basalt, which potentially impaired the plant.

5.5. Conclusion

One year after the addition of basalt to a mildly acidic floristically diverse grassland, rock weathering occurred, resulting in significant increases in soil pH and concentrations of sodium, magnesium, calcium, and silicon within the soil. Additionally, there were increased concentrations of sodium, magnesium, and phosphorus in the plants. Total plant yield, nutrition, and floristic diversity were not significantly affected by basalt amendment, except for the plant *H. radicata* which had a reduced frequency after basalt application. Importantly, basalt amendment significantly reduced soil concentrations of potassium while increasing soil concentrations of potentially toxic trace elements barium, cadmium, caesium, cobalt, nickel, chromium, strontium, and vanadium; however, neither the reduction in soil potassium nor the increase in trace metals was reflected in the plant shoots. These findings underscore the considerable potential benefits of basalt amendment on grasslands, while also indicating that, at least initially, there are no significant changes to plant diversity or farming economics. They also highlight the importance of choosing a suitable rock substrate and the careful monitoring of toxic trace metals to safeguard soil health and prevent plant toxicity.

5.6. CRediT authorship contribution statement

Derek S Bell: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation. Writing – original draft, Writing-review & editing, Visualization, Project administration; **Dimitar Z. Epihov** Conceptualization, Methodology, Validation, Writing – Review & Editing, Supervision, Project administration, Funding acquisition; **Xavier Dupla:** Formal analysis, Writing – review & editing, **David J Beerling**: Validation, Writing – review & editing, **Jonathan R. Leake:** Conceptualization, Methodology, Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition.

5.7. Declaration of Interests

D.J.B. has a minority equity stake in Future Forest/Undo and is a member of the Advisory Board of The Carbon Community, a UK carbon removal charity, and the Scientific Advisory Council of the non-profit Carbon Technology Research Foundation. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

5.8. Funding sources

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5.9. Data statement

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5.10. Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT, an AI language model, to assist with grammar and refining content. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

5.11. Acknowledgements

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Chapter 6: General discussion

6.1. Introductory remarks and brief overview:

In this work, I have attempted to characterise *in vitro* and *in situ* the potential importance of different biota and biological components that could be used in future research to accelerate rates of mineral dissolution and CDR in ERW. Each chapter of experimental work represents a different scale of biological weathering from single strains of bacteria through to natural communities of plants and soil organisms. The first experimental chapter, Chapter 2, explored the role of the enzyme carbonic anhydrase (CA) in bacterial-driven rock weathering *in vitro* using a knockout model of *Burkholderia thailandensis*. The results show that inactivating one of the three CA genes identified within *B. thailandensis* significantly impaired mineral dissolution, suggesting its potential relevance in CDR in soils with ERW.

Chapter 3 builds on these findings by investigating whether there is selective pressure for CA genes in basalt-amended field trial soils and assessing whether literature on CA enzymes accurately reflects their predicted cellular localisations in soil. The results showed that while CA genes are highly abundant among soil organisms, there is no evidence of selective pressure for them in basalt-amended soils. Additionally, intracellular CA genes are far more abundant than extracellular ones, despite the latter being the primary focus of existing research

In Chapter 4 a more holistic approach was used to understand the relative contributions of different biological groups to grassland soil weathering, regardless of the weathering mechanisms they may have employed, with the aim of identifying groups that are most important to weathering. During the growing season, mycorrhizal fungi appeared to play a significant role in grassland weathering, while plant root colonisation

appeared to inhibit weathering rates. However, changes in soil element concentrations were highly element-specific.

Finally, in Chapter 5 a field trial investigation was performed to characterise how ERW could be integrated into grassland agriculture, assessing both its potential benefits and risks. Within one year of amendment, soil pH and some plant and soil nutrients increased, while floristic diversity and yield remained unchanged. These findings suggest that grasslands, which inherently carry a high biological activity, have the potential to support ERW.

The original goals of the research presented in this thesis, across these different scales of biological interactions with minerals, and some of the key findings arising from the work are summarised in Figure 1.

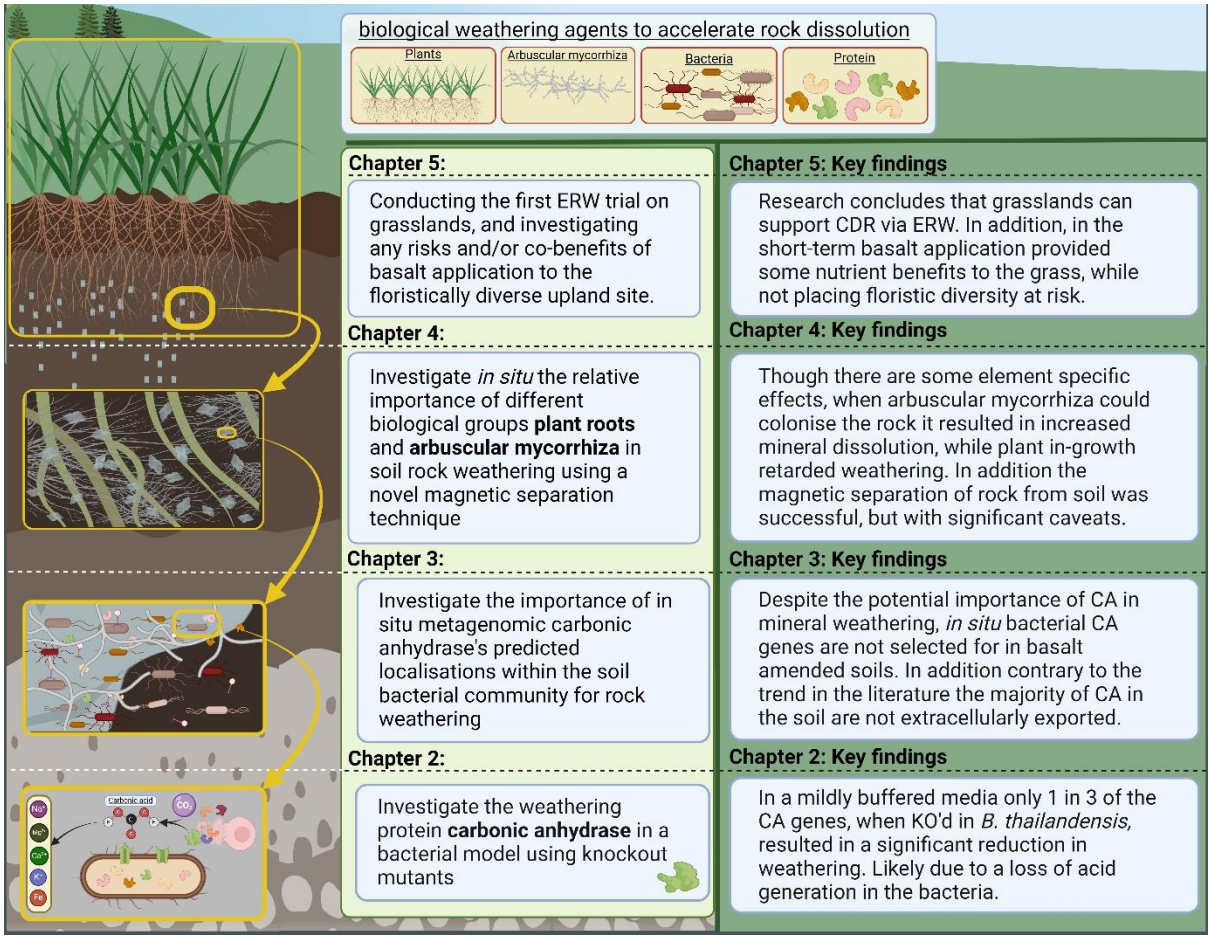

Figure 1. Thesis conceptualisation of research from enzyme to soil biotic community scales with key findings highlighted.

In this general discussion I will provide a summary of some of the important findings from each chapter and how they relate and contribute to the wider literature on biological derived rock weathering. This discussion will also consider the relevance and implications these findings have in relation to government policy and commercial ERW investments, while also discussing some of the uncertainties of the analysis performed and some future research priorities.

6.2. Synthesis of key findings:

6.2.1 Chapter 1:

Chapter 1 presented information about the need for CDR strategies as under most IPCC predicted scenarios an estimated 260 (Smith et al., 2024) to 1000 Gt (Pagano et al., 2020) of CDR is required by the end of the century to limit global average temperature increase to 1.5 °C above the pre-industrial average. Geochemical modelling of ERW suggests that, if rolled out rapidly to croplands globally, it could significantly contribute to these CDR requirements as it has an estimated potential to remove 25 to 100 Gt of CO_2 in the last 50 years of the century alone (Beerling et al., 2020). Furthermore, this assessment does not consider the potential reduction in N_2O emissions that could result from the increase in soil pH due to rock application (Chiaravalloti et al., 2023). A recent model suggests that ERW could reduce N_2O emissions from soils by up to 18% if implemented in the five regions which have the highest potential for CDR with ERW: North America, Brazil, Europe, India, and China (Val Martin et al., 2023). Potential co-benefits of ERW, such as its liming effect, also include the addition of macro- and micro-nutrients to the soil, depending on the mineral applied. Research has shown increases in yields and in some cases plant nutrition; for instance, maize and soy grain concentrations of elements potassium, magnesium, manganese, phosphorus, and zinc all were significantly

increased with basalt application (Beerling et al., 2024). In addition, a recent study has shown improvements in earthworm population numbers in vineyards in Switzerland with a basalt rock dust amendment (20 t ha^{-1}) (Dupla et al., 2024).

Despite the potential benefits of basalt amendments, concerns still exist regarding the bioaccumulation of toxic elements in soils and plants (Dupla et al., 2023), and the possible salinization of soils in relatively dry climates from sodium released from the sodium-bearing silicate minerals in basalt during weathering (Dupla et al., 2024). There are also concerns about the permanence of the CDR, especially in relation to non-carbonic acids (i.e. termed "strong acid weathering") reacting with atmospheric derived HCO_3^{-1} in soils or waters (S. Zhang et al., 2022). In addition, Chapter 1 also described some of the many mechanisms of biological derived weathering used by fauna, plants, fungi, and bacteria and their potential relevance to global weathering reactions both geologically and in the context of accelerating ERW rates in agricultural soils. There was a further specific focus on the limited research regarding grasslands efficacy for ERW along with the characterisation of some of the future steps required to determine if CAs play a role in mineral dissolution in soil bacteria.

6.2.2. Chapter 2:

6.2.2.1. Chapter overview

Chapter 2 introduced and reviewed the existing evidence of the potential role of CAs in microorganism facilitated rock weathering, including evidence that bovine (W. Li et al., 2007; Liu and Dreybrodt, 1997) and bacterially derived CA (Xiao et al., 2015) when incubated with rock can increase rates of mineral dissolution. When *B. mucilaginosus* was grown in an axenic wollastonite amended media for 6 days there was a significant increase in CA gene expression that corresponded to an increase in mineral weathering relative to the control media (Xiao et al., 2015), implicating the

enzyme in weathering. However, direct evidence of the role of CA in bacterial weathering of minerals remains uncertain.

My approach to overcome some of the limitations of previous studies was to use a knockout mutant library of the phosphate solubilising bacterium *B. thailandensis* E264 isolated from soil, in which specific CAs genes were knocked out and thus not expressed. This provided a modern genome-centric view of assessing the role of CAs in bacterial rock dissolution. It was found that some CAs in this organism do not act redundantly, inactivation of the CA gene BTH_I1052, a predicted cytosolic localised protein, significantly reduced the total dissolved base cations, consisting of calcium, magnesium, sodium, and potassium, in a 1% w/v basalt solution by 41% compared to the wildtype *B. thailandensis*. This significant difference in dissolved base cations relative to the wildtype and other KOs was not replicated upon growing the BTH_I1052 mutant in the 10% w/v basalt media. This suggests that in the more highly buffered medium, with a final pH of 7.98, CA was either not expressed to the same extent or the reduction in the CA's ability to generate acid was not significant enough to affect dissolved elemental concentrations, whereas in the mildly buffered medium, with a final pH of 7.11, CA inactivation significantly impaired the bacteria's ability to acidify the solution. However, two of the CA genetic knockouts, BTH_I0345 and BTH_I1199, showed no clear role in mineral dissolution under any of the basalt-supplemented conditions tested. These indicate that as a class of enzyme, bacterial CAs do not universally influence mineral dissolution, but certain CA enzymes may become more important depending on the environmental conditions. As all three of the CA knockout genes identified within *B. thailandensis* were predicted to be cytosolically this work provides some of the first evidence that non-extracellular CA genes may also play a role in mineral dissolution.

6.2.2.2. Relevance for science and business

The differences in the effects of the BTH I1052 CA gene at different basalt concentrations are of particular interest as previous research has shown that adding CA from a bovine source, which would not naturally occur, to soils with different pH levels can significantly impact the carbonic acid linked CO_2 dissolution rate in the soils. Specifically when bovine CA was added to soils with increasing alkalinity of pH 4.1, 6.3 to 7.6, or 8.5 the CO_2 dissolution rate increased by 0.1, ~ 10, and 20 fold relative to the controls, respectively (Sauze et al., 2018). However, my findings indicate that, at least with *B. thailandensis*, despite the enzyme perhaps becoming more efficient at greater pH's it appears to become less relevant in terms of mineral dissolution rate. If this finding is corroborated for CAs across the whole soil bacterial community, then contrary to what the literature may suggest, the optimal conditions for CA activity in the soil may be closer to a circumneutral pH rather than an alkaline pH. The weathering-potent CA gene identified in this work also appeared to be intracellularly localised, which is in contrast with assumptions made in previous work that have only considered extracellular forms of CA as important for mineral dissolution. This discovery further expands the potentially relevant pool of CAs within the soil to those with different predicted localisations. This intracellular CA may play an important role in maintaining cellular pH suitable for core metabolism when the external pH is too alkaline, and this might then indirectly lead to enhanced weathering. More studies are needed to resolve the mechanisms involved and the direct and indirect roles of CA.

Some groups within the ERW industry are currently testing the use of extracellular CA in field trials. Two such companies, Veolia and Fabricnano, are trialling rock immobilised CA and free CA enzyme applications to soils with the hope of seeing increased carbonic acid-driven mineral dissolution and higher CDR rates (Peplow, 2024). Their current estimated costs range from $380 to $510 per t CO_2 removed, which is similar to the estimated cost

of direct air carbon capture and storage that range from $350 to $700 per t CO_2 removed (IEAGHG, 2021; Peplow, 2024). The literature does indicate that these applications of enzymes could work, especially the rock immobilised CA, as the enzyme will be in its optimal alkaline reaction environment immediately adjacent to the rock and perhaps be stabilised in the soils preventing denaturing of the protein (Nannipieri et al., 2018). Importantly however, despite CA also being tested in other carbon capture industries the cost of production is considered quite high, though recent innovations are driving costs down (Mao et al., 2024). Despite this, the cost of producing enzyme may still be a limiting factor to its inclusion in ERW. This work however highlights that CAs found within soil bacteria could perhaps be used to affect weathering rates in soils. Though more research is required, the artificial activation of CA genes or enzymes within the soil could provide a more cost effective method of increasing carbonic acid derived weathering.

6.2.2.3. Uncertainty and future research directions

This work also creates some uncertainty as not all CAs may act the same within an organism, and across different organisms enzyme activities and activation requirements are likely to vary. One recent study on bio-cementation in soils found that adding a zinc co-factor to a bicarbonate-rich cementation solution increased CA activity by approximately 40% in two of the three CA-active bacterial isolates tested: *Bacillus pumilus* and *Bacillus toyonensis*, while the CA activity of *Bacillus licheniformis* appeared unaffected (Mwandira et al., 2024). These differences in activation could make it difficult to leverage the greater soil bacterial communities CAs, but it may be possible to increase the activities of certain members. Another area of uncertainty is that the specific organisms tested in the literature and the model organism I used, *B. thailandensis*, which is a phosphate solubilising bacterium, may not be representative of the weathering potential of the wider soil bacterial community.

Extensive previous literature, as reviewed by Nannipieri et al. (2018), also show that environmental conditions can greatly affect soil enzyme activities and stabilisation within the soil, as is the case with soil pH, mineral concentration, clay, organic matter, and hydrated iron oxide concentrations. Stabilisation in the soil occurs when enzymes are bound to mineral surfaces but retain their active sites, these enzymes, known as abiontic enzymes, could be potentially important for preserving the catalytic functioning of intracellular enzymes released by cell lysis (Nannipieri et al., 2018). As such, it is plausible that intracellular enzymes produced in large amounts could ultimately contribute to extracellular activity after the organism has lysed. Mineral immobilised enzymes could remain active for much longer than free enzymes in soils as the immobilised enzymes cleavage sites may be more occluded from protease activity along with the matrix perhaps inhibiting environmental stressors, like pH, but more research is needed to define enzyme turnover rates in soils (Burns et al., 2013).

The commercial application of CA bound to minerals currently being tested may help to establish whether the enzyme used remains active in the alkaline mineralosphere of basalt, and how effective it is at facilitating weathering compared to the effects of native microbial community, plant roots, and rainfall sources of acidity and carbonic acid. The predicted cytosolic localisation of the weathering CA gene BTH_I1052 also creates uncertainty as to where in the soil CAs are most active, cytosolically, extracellularly, or as abiontic enzyme. Future work is needed to characterise whether in the greater soil community CA does play a role in mineral dissolution and what localisations may appear more relevant. Chapter 3 aimed to address this by testing for *in situ* differences in the genetic abundance of differently localised CA genes within basalt amended soils.

6.2.3. Chapter 3:

6.2.3.1. Chapter overview

As discussed in Section 6.2.2, CA's potential role in bacterially driven weathering appears promising, but not fully resolved. The *in vitro* experiments with model organisms supplied with un-weathered rock dusts in the absence of soil minerals, organic matter, and other soil biota may not accurately reflect how these bacteria or other bacteria would respond *in situ*. In particular, evidence is lacking for the role of bacterial CA in weathering in agricultural soil conditions, where ERW is proposed to be deployed in the UK and more widely (D.J. Beerling et al., 2024; Kantzas et al., 2022).

To address these issues, in Chapter 3 I analysed the soil bacterial community metagenome from samples collected over two successive crops at a maize and soybean rotational ERW field trial site in the U.S. Corn Belt. From the metagenomic communities CA and reference *rpoA* genes were extracted using a specifically designed CA database which also noted each CA genes predicted cellular localisation. This database, which included a total of 126,001 CA genes taken from three databases, KEGG, Pfam, and Tigrfam, was composed of 82% cytosolic predicted CAs, while extracellular CAs, which are predominantly featured in the literature, accounted for only 0.6% of this database. This potentially indicates that extracellular CAs have a less important role than intracellular CAs in bacterial communities. The community based analysis found no significant differences in the total relative abundance of CA's identified from the basalt amended (50 t ha^{-1} yr^{-1}) arable soils, which had received basalt annually for several years, compared to control soils. Similarly, the *rpoA* community composition, which was used as a marker gene, was also not affected by annual basalt amendment, despite there being a pH increase of ~0.9 in basalt treated soils (D.J. Beerling et al., 2024). The relative abundance of the total bacterial CA genes in the soil was approximately 9.6 fold greater than the universal single copy *rpoA* gene. Its ubiquitous and abundant nature in soil bacteria indicate that the

gene may play an important role in cellular functioning, but when basalt and control soils were compared there appeared to be no selective pressure for this gene in bacterial communities exposed to basalt and increased soil pH. Furthermore, there were no significant differences seen between control and basalt treatments regardless of the predicted localisation of the CA. When individual taxonomic groups had their CA relative abundance assessed there were very few taxa that appeared to be significantly affected by basalt treatment, with the majority actually appearing to have decreased in CA relative abundance as a result of basalt treatment.

6.2.3.2. Relevance for science

Almost all the literature regarding CA in mineral dissolution has found that when CAs are incubated with rock they can increase rates of mineral dissolution (Liu and Dreybrodt, 1997; Li et al., 2007; Xiao et al., 2017; Sun and Lian, 2019). Genetic manipulation with KO trials in both the fungus *Aspergillus nidulans* (Sun and Lian, 2019) and the bacteria *B. thailandensis* (Chapter 2) found that CA inactivation could result in a significant reductions in weathering. These findings suggest that CA genes could be relevant for increasing mineral dissolution in soils. However, my research provides little support for bacterial CAs increasing weathering in agricultural soils amended with basalt for ERW and CDR. The soil metagenomic samples used to assess CA's relevance in weathering were taken from soils that have been characterised as having clear signs of weathering and CDR, as analysed by Beerling et al. (2024) and Kantola et al. (2023). Briefly, significant increases in soil calcium and pH, as well as maize and soybean yields by 12% and 16% respectively, along with higher total grain nitrogen content, were observed after four years of annual 50 t ha^{-1} basalt amendment (Beerling et al., 2024). The measured changes in elements in the soils suggested approximately ~3.4 t CO_2 ha^{-1} y^{-1} was sequestered over the 4 year field trial (Beerling et al., 2024). However, this probably did not result in a net CO_2 removal, as the addition of basalt to

these fields was found previously to only reduce total carbon emissions by 23% to 42%, due to large net losses from respiration of soil organic matter stocks, which was only prevented when growing the perennial bioenergy crop *Miscanthus* (Kantola et al., 2023).

Despite the clear signals of mineral weathering in the soils, there appeared to be no selective pressure for CA genes within the bacterial community, regardless of their predicted localisation. Firstly, these results suggest that CA's within the soil bacterial community may not play an important role in mineral dissolution in the field, though there are still some uncertainties. Secondly, they also suggest that extracellular localised CA, though present in the field, represent a small proportion of CAs found within the soil and in terms of future CA research there should be more of a focus to include intracellular localised or perhaps abiontic CAs.

6.2.3.3. Uncertainty and future research

One primary area of uncertainty is in the conclusion, which suggests that bacterial CA may not play an important role in mineral weathering *in situ*. It is important to note that changes to genetic abundance do not always indicate whether a gene is relevant in a cellular process. In this case the CA gene is highly abundant within the soil community in both the control and basalt amended soils so there may not have been a selective pressure for the gene because of this abundance. Furthermore, the presence of the gene does not reflect whether it is being transcribed and translated, and whether the subsequent protein is active. In this case CA's could still be playing a highly important role in rock weathering as it was beyond the scope of the study to characterise its transcription, translation, or activity in basalt amended soils. As such all three of these could be researched in the future to determine CAs relevance more definitively in bacterially derived mineral weathering in soils.

Another uncertainty, which is an area of concern for almost all metagenomic research, is any bias found within the databases themselves. This concern was perhaps mitigated as the CA database was composed of genes acquired from multiple different databases, which have in turn collated their gene sequences from a variety of different environmental and biomedical experiments. However, during our collation of CA genes we identified that these databases had a low number of genes that localised extracellularly or to the outer membrane, accounting for 0.6% and 0.00002% of the final CA database, respectively. Though this may indicate that these localisations of CA are generally a rarity, if the database has some bias towards cytosolic localised CA, which represented 82% of the database, the results may artificially underestimate the relevance of the other predicted localisations. This uncertainty can only be addressed as more genomes are included in these databases. In addition, it is important to remember that at least in *B. thailandensis*, as discussed in Chapter 2, CAs can act differentially within an organism so it may be difficult to delineate the weathering capable CAs to the non-weathering CAs.

Finally, there remains uncertainty whether CA may play a more important role in soil organisms other than bacteria, for example in soil fungi, which have been shown to enhance weathering and in which CA appears to play a role in axenic laboratory culture experiments (Sun and Lian, 2019). In addition, mycorrhizal fungi, in symbiosis with plant roots and ubiquitous in croplands have been shown to be actively involved in increasing silicate and basalt weathering in soils (Quirk et al., 2012, 2014). The final two experimental chapters of this thesis evaluate the role of the wider groups of soil organisms including plant roots and their mycorrhizal fungal partners in basalt weathering.

6.2.4. Chapter 4:

6.2.4.1. Chapter overview

Considering that there is a plethora of different weathering mechanisms employed by bacteria, as reviewed by Ribeiro et al (2020), studies focused at the level of individual organisms or genes involved in weathering provide important functional insights, but leave unaddressed the actual weathering rates achieved by natural communities of organisms in soils. In Chapter 4 the aims of the work were to conduct the first field study of ERW using basalt dust additions to grassland, and to seek to gain insight into the relative importance of plant roots and associated soil microorganisms in weathering. One of the challenges in moving from laboratory studies of weathering using rock grains added to solution cultures to rock grains added to soil in the field, is how to recover the weathered rock grains and physically separate them from clays and organic materials that have high cation exchange capacities that can accommodate ions released from weathering.

Specifically, Chapter 4 explored two main questions: first, whether rocks can be reliably analysed after being magnetically reclaimed from soil following *in situ* incubation? And second, what are the relative contributions of different biological groups, such as plant roots and mycorrhizal and root-carbon dependent fungi, to rock weathering in a mildly acidic floristically diverse grassland?

The magnetic rock extraction technique was developed to address some of the persistent problems found in ERW validation. Briefly, no method of measuring rock weathering and ERW's potential CDR has been widely adopted within the literature, industry, or government. Some of the complexity in calculating weathering and CDR is due to the complex environments weathering products, such as HCO_3^-, have to move through before it is considered CDR, with there also being debate regarding when

CDR should be considered stable with ERW. One primary method of estimating CDR potential used in research is charge balancing cations released from the rock matrix with those of the anion HCO_3^{-1} (Manning et al., 2024). This method effectively creates a maximum CDR scenario as it assumes all weathering occurred with carbonic acid, which *in situ* is not accurate. However, research has tried to address this through estimating when strong acid (such as nitric acid from nitrification of ammonium fertilizers) generated weathering does significantly contribute to weathering rates (Dietzen and Rosing, 2023). Many different methods, such as TiCAT and fused bead XRF, have been proposed and developed to try to accurately determine the rates of cation release from the rock, but the majority have issues with scalability. Both TiCAT and fused bead XRF require specialist equipment and in the case of TiCAT it requires to digest the soil and rock with hydrofluoric acid thus necessitating specialist laboratory environments. One method, which could be more cheap and scalable, is through measuring the total cation depletion from the mineral elemental matrix following weathering with portable XRF (Beerling et al., 2024).

As discussed in Chapter 4 rock reclamation from the soil for subsequent analysis is problematic and previous researchers have sought to reduce the extent of contamination of rock grains by soil minerals and organic matter by incubating mineral grains in mesh bags for easy recovery (Quirk et al., 2012; Epihov, 2018). As such these rock grains may be incubating in hydrologically, biologically, and chemically distinct microsites as a result of not having normal soil colloids around them, and it seems likely that bagged grains may not represent the natural state of weathering relative to when loosely spread and mixed with soil. The magnetic rock extraction technique was developed to allow the rock to be more realistically distributed in the soil.

The results in Chapter 4 indicate that despite pre-screening the soil to remove magnetic particles, magnetic soil contamination was a significant problem in any subsequent soil or rock pXRF analysis. Soil analysis was significantly impacted by magnetic screening as results indicate that in the lab incubated controls the magnetic recovery of rock from soil resulted in significant reductions of 19% and 27% of soil calcium and magnesium, respectively. Subsequently, magnetic soil contamination of rock also appeared to affect the pXRF analysis as mean increases 413% and 105% of elemental lead was seen relative to the fresh unmixed rock from both native sand and basalt containing columns, respectively.

Through modifying a method to functionally exclude arbuscular mycorrhizal fungal colonisation in soils, created by Johnson et al. (2001), both plant roots and AM fungal relative contributions to weathering in the floristically diverse mildly acidic grassland was assessed. Extensive research, as discussed in section 1.4, has characterised the many different methods of mineral weathering used by different biological groups found within soil, but no research has previously characterised the relative contributions of these groups to basalt weathering *in situ*. My results indicate that plant colonisation of the soil-filled mesh columns appeared to generally inhibit weathering as significant reductions in the extractable fraction of the elements magnesium, calcium, strontium, titanium, and silicon were noted relative to the columns where the mesh excluded roots but permitted in-growth of mycorrhizal fungal hyphae. Although some of these decreases could be the result of the roots absorbing these elements, the reduction in titanium, a non-mobile element not actively taken up from soil by plants (Reershemius et al., 2023), implies that mineral weathering was probably inhibited by plant roots. This was possibly a result of root water uptake and plant transpiration depleting the soil moisture content faster than when roots were excluded, and dryer soil conditions acted to then slow rates of weathering (Cipolla et al., 2021). When plant roots were

excluded from the soil columns, but AM fungi could colonise them and the basaltic rock grains within, there appeared to be increased mineral weathering. This was shown by significant increases in the soil concentrations of calcium, strontium, and titanium relative to soil columns where both plant roots and AM fungi were excluded. However, there did appear to be an element specific effect as some soil elemental concentrations were unaffected by AM hyphal colonisation or in some cases decreased; as was the case with potassium and molybdenum (Mo), which may have been taken up by the fungi and passed on to plant partners as essential elements. The nitrogen-fixing legumes present in the hay meadow, including the vetch, *Vicia sativa*, and the highly mycorrhizal clovers *Trifolium repens* and *Trifolium pratense,* will have a high demand for Mo in their root nodules due to its essential role in the nitrogenase N_2-fixing enzyme within their rhizobia bacterial symbionts (Bursakov et al., 2023). Furthermore, previous research suggesting that Mo extraction from dunite rock grains was greater under N_2-fixing legume trees than under non-fixing trees (Epihov et al., 2021). Research has shown that plant uptake of Mo can be significantly increased by AM symbiosis in a soilless medium (Vultaggio et al., 2024) and furthermore that certain AM fungi can be highly enriched in some legume root nodules relative to other legumes and non-legumes (Scheublin et al., 2004), suggesting a role in supporting the specific nutrient requirements in nodules.

6.2.4.2. Relevance for science, industry, and policy

Though broadly the magnetic reclamation results did not achieve 100% pure basalt extraction with some in-passing soil contamination, the technique still holds potential for further development and application. However, any future use in research should address any soil magnetism through either only using this technique on soils with little native magnetism or through testing additional soil separation steps such as size or density fractionation, ultrasonic cleaning, and other approaches to clean

and isolate the mineral grains either physically or post-operatively. This work also highlighted some of the problems which arise from analysing a complex mineral like basalt with pXRF, as variation within the mineral greatly contributed to the final error making short-term weathering difficult to measure which advocated for the need of pre-scanning, homogenization, or greater replication. Ideally, in retrospect said basalt grains could have been ball-milled into fine powder to achieve greater homogeneity in the sample prior to the pXRF scans. However, this approach was not feasible with the relatively small samples that were recovered after soil incubation.

The findings in Chapter 4 indicate that AM and root-associated fungi appears to play a significant role in mineral weathering, in at least a mildly acidic grassland, consistent with previous studies of mineral weathering by AM under trees and small herbaceous plants (Quirk et al., 2012, 2014). Their contribution to calcium dissolution if extrapolated to field scale resulted in a potential CDR increase of 0.344 t CO_2 ha^{-1} yr^{-1}. Though this increase is small in relation to some other ERW CDR estimates, such as the 3.4 t CO_2 ha^{-1} yr^{-1} reported for the maize to soy rotation in the US corn belt (Beerling et al., 2024), it does highlight the potential of fungi in increasing ERW CDR rates in soils. Though research on ERW's efficacy in grasslands is lacking, as discussed in Chapter 5, the results indicate that grasslands which facilitate successful mycorrhizal networks in soils could have higher rates of weathering, with these likely including grasslands that are not ploughed or heavily fertilized with available phosphorus, both of which can adversely affect mycorrhiza (Peng et al., 2024). Importantly, sustainable farming practices, such as some of those being subsidised by the UK sustainable farming incentives (DEFRA, 2024a), including reduced fertiliser inputs and organic amendments could increase AM abundance (Bradley et al., 2006) and species richness (Pagano et al., 2020; Maria et al., 2009). However, AM responses to land management strategies and the environment are complex

and situation dependent (Field et al., 2020). Although ERW is not currently included or mentioned in the UK sustainable farming incentives, it could potentially be co-deployed without jeopardising the sustainability goals, but more research is needed in both grasslands and croplands to ensure this. AM's weathering potential in croplands is even more complex as not all plants respond positively to AM colonisation (Khan et al., 2022). Management of farmland with approaches that favour weathering, such as not adding lime but basaltic rock dust instead to ameliorate soil acidity, minimizing soil disturbance by grazing management, reseeding to reintroduce herbs and legumes lost by grazing, and using direct drilling rather than ploughing are all likely to regenerate mycorrhizal communities. In contrast there is no clear scientific case to justify adding commercial mycorrhizal inoculum to established grasslands, since commercial inoculants of AM are not always as advertised, often contain pathogens, and typically have no effect or adverse effects on plant growth compared to no inoculum (Faye et al., 2013; Watts et al., 2023; Koziol et al., 2024). However, in grassland restoration from arable cropping the use of native microbial inoculants derived from original grasslands that were not cultivated has been shown to help support native plant biodiversity and to suppress undesirable weeds (Koziol et al., 2023).

6.2.4.3. Uncertainty and future research

One area of uncertainty in using magnetically extracted minerals from the soils for subsequent weathering analysis is the potential bias in their weathering rates. Magnetite, the primary iron rich mineral in basalt, relative to other minerals, like biotite, has a relatively fast weathering rate (Lewis et al., 2021), so measuring elemental release from the mineral matrix may result in overestimates of weathering. Complex calculations would be needed to characterize the weathering rate of the non-magnetic basalt fraction, beyond simply comparing general mineral dissolution rates. For instance, the surface area-to-volume ratio may change more quickly,

potentially increasing weathering; conversely, the iron-rich minerals could oxidize, potentially forming weathering inhibiting layers (Schott and Berner, 1983).

An important consideration with the biological simplification columns is that they are additive in nature. For instance, in the mycorrhiza and plant restricting columns multiple sources, including small invertebrates, abiotic processes, bacteria, saprophytic fungi, and archaea, could be contributing to weathering. So, any increases identified with mycorrhizal colonisation should be viewed in the context of other soil processes. As such AM and root-associated fungi in this case may not have directly, but indirectly increased weathering rates through stimulating other biological groups or abiotic processes. For instance, when AM fungi could access the column the soil appeared more aggregated relative to when they were restricted. This aggregation perhaps occurred due to increases in water stable soil aggregates through AM producing glomalin (Wilkes et al., 2021). This change in soil properties could have indirectly increased weathering rates during the growing season through increasing water retention allowing for more weathering reactions to occur. One remaining uncertainty with the biological simplification columns is their physical impact on weathering rates as the columns may have influenced water flow through the system as well as plant root and mycorrhizal hyphae colonisation through the restrictive pore sizes, or the regularly disturbed columns to prevent AM fungal proliferation may have poorer hydrological continuity with the surrounding soil matrix.

One area of future research is expanding the biological simplification experiment to try to distinguish more unique biological groups contribution to weathering *in situ*, such as earthworms. The importance of seasonality could also be addressed through performing the experiment over the autumn and winter, where biological contributions could be different. Finally, the experiment could be trialled in different grasslands and

croplands under varying management practices to try and identify groups that ubiquitously increase weathering *in situ*, thereby, potentially identifying a universal target for ERW acceleration in many different agricultural settings.

6.2.5. Chapter 5:

6.2.5.1. Chapter overview

Chapter 5 attempted to provide the first published insight into ERW's potential in grasslands. Aforementioned modelling studies, such as the UK modelling study by Kantzas et al. (2022) which estimated a cumulative CDR potential of 0.3 – 1.5 Gt CO_2 in the last 50 years of the century, only considered ERW rates derived from croplands. In the UK both un-managed and managed grasslands account for 90 M ha of land, greater than double the UKs total cropland area (DEFRA, 2023). The lack of published research on ERW in grasslands is especially surprising in the UK considering that grasslands theoretically should have a greater weathering potential to croplands, since they are generally most abundant in the wetter western and northern areas of the country where soils are mostly acidic and have benefitted from regular liming to maintain productivity (Holland et al., 2018). As discussed in section 1.3, a perennial plant system like grasslands could increase CDR through having a greater root biomass compared to other crops (Kantola et al., 2022; DuPont et al., 2014) and perhaps, as discussed in Chapter 4, by having more stable mycorrhizal symbionts (Ma et al., 2023).

Chapter 5 aimed to provide a preliminary estimate of CDR along with characterising any potential co-benefits and risks of basalt application to grasslands. To do this one basalt application of 50 t ha^{-1} was applied to a mildly acidic, floristically diverse upland grassland, and subsequent soil chemistry, floristic diversity, plant nutrition, and hay yields were assessed for changes resulting from the amendment. Results provide a preliminary

extrapolated CDR estimate of 0.542 t CO_2 ha^{-1} yr^{-1}, with only one 50 t ha^{-1} basalt amendment. Given that there are about 12,000 ha of traditional hay meadows in the UK, if these rates of weathering occurred across all these hay meadows it would sequester 6504 t CO_2 y^{-1}. Compared to the total greenhouse gas emissions from UK agriculture of 48 M t CO_2e in 2022, of which 1.5 M t CO_2e are estimated to be released form liming and use of urea fertilizer (DEFRA, 2024b) this sequestration would make a modest contribution to net zero targets. However, if basalt was applied more widely to lowland acid grassland (55,000 ha) or semi-natural acid grasslands (2.1 million hectares) in the UK, the potential to sequester millions of tonnes of CO_2 annually might be theoretically possible, although this would not be acceptable in sites with important conservation value and relatively rare acid-loving species, as in the Breckland grasslands.

Though the CDR potential of grasslands at least initially appear modest, basalt amendment did provide some benefits including significantly increasing soil pH by approximately 0.25 pH units and soil exchangeable concentrations of calcium, magnesium, sodium, and silicon by 13%, 14%, 22%, and 35% relative to the control soils, respectively. Along with these soil increases, there were also significant increases in plant nutrients such as phosphorus, sodium, and magnesium, but no increases in yields. The increases in soil pH may bring additional benefits in acid grasslands as a recent study in Wales has suggested that the cessation of liming of acid upland grasslands is associated with declines in earthworm abundance and potential reduction in soil infiltration rates, increasing the flooding risks from runoff (Freeman et al., 2023). In terms of ERW risks, basalt amendment did not significantly alter floristic diversity, species richness or Simpson and Shannon-Weiner diversity indices. Surprisingly, despite the basalt containing potassium, there was a significant reduction in soil potassium, perhaps as a result of other cations releasing it from soil exchange sites and/or soluble potassium being adsorbed onto secondary

mineral phases developing on the surface of the weathered rock grains. Furthermore, an additional concern arose from the significant increases in the soil exchangeable concentrations of trace metals barium, cadmium, caesium, cobalt, nickel, chromium, strontium, and vanadium, but of the elements that have been reviewed by the UK environment agency (Martin et al., 2022) basalt amendment did not result in any ecotoxicity thresholds being exceeded. The absence of increases high enough as to represent an ecotoxicity concern is particularly interesting given the use in this study of the Hillhouse quarry basalt feedstock high in olivine which is a known risk-factor due to the high content of nickel and chromium in olivine ores. In fact, high-olivine basalts are avoided in commercial operations for ERW-CDR on the carbon credit volunteer market performed by companies such as Undo (UK) and Lithos (US) exactly because the gain of using such basalts cannot outweigh their risk.

6.2.5.2. Relevance for science, industry, and policy

As mentioned above, this work opens up a new and surprisingly overlooked field of ERW research as grasslands efficacy in weathering has been critically understudied. The only exception being a study of the giant perennial bioenergy grass miscanthus, which was much more effective at weathering and sequestering organic C in soil than was maize or soybean in trials in the US (Kantola et al., 2023). Despite the absence of published research on ERW's potential in grazed grasslands or hay and silage meadows, the ERW industry, including UNDO, are currently trialling it, which highlights the need for more independent assessment of its potential benefits and risks. As mentioned in section 6.2.4.2 the UK government is offering sustainable farming incentive subsidies for farms to promote sustainable agricultural and land management practices (DEFRA, 2024a). Currently there are at least 32 different subsidies that relate to promoting the protection and/or the establishment of grasslands or grass segments in croplands (DEFRA, 2024a). One of these subsidy options is the GRH6

subsidy “to manage priority habitat species rich grasslands” which amounts to £646 ha^{-1} yr^{-1} for the farmer (DEFRA, 2024a). Though no specific long-term analysis on the effects of ERW on species diversity has been performed, long-term liming experiments on grasslands could act as a proxy. The long-term Park Grass experiment run by Rothamsted found that lime individually did not affect species richness, but when in conjunction with other individual or combined additions such as nitrogen, phosphorus, and/or potassium it could be significantly increased or reduced depending on the combination (Crawley et al., 2005). These results create uncertainty as to how basalt may affect species richness as one of its benefits is it that, depending on the mineral, it can contain plant nutrients including phosphorus and potassium, which based on Crawley et al.’s (2005) results could when in combination with alkalinity result in a decline in species richness. Liming however with calcium carbonate can result in CO_2 emissions if the carbonate is weathered with a non-carbonic acid, although these emissions in some cases are considered minimal in relation to the potential reductions seen in N_2O and CH_4 emissions resulting from increasing soil pH, as reviewed by Abdalla et al. (2022). Basalt on the other hand appears to pose no risk of increasing emissions once on the field (Beerling et al., 2018), so should result in the same potential N_2O and CH_4 emission reductions as lime, and if weathered with carbonic acid can result in 2 moles of CO_2 absorption relative to 1 mole through calcium carbonate (Hartmann et al., 2013). Importantly, due to basalts lower reactivity it may create additional emissions as it could take more rock to reach the same pH increases seen with calcium carbonate. There also remain some uncertainties as to the effect of basalt rock dust on soil organic matter, as liming is associated with substantial increases in soil respiration (Zhang et al., 2022). However, earlier studies have claimed no significant depletion of soil organic C stocks by lime, despite a fairly consistent trend for modest decreases in croplands and grasslands (Paradelo et al., 2015), so the respiration increase could be from soil respiration.

ERW in grasslands may also act to replenish soil stocks of silica. The silica cycle has been largely overlooked in terms of geological relevance, but it appears that cropping with silica accumulating plants like cereals has resulted in a depletion of soil biogenic silica globally (Carey and Fulweiler, 2016). Although increasing silica in agricultural soils with ERW could have some important benefits in terms of increasing shoot Si concentrations in crops and conferring increased pest resistance (Liang et al., 2015; Keeping, 2014), there could be unintended downstream consequences to silica in waterways (Street-Perrott and Barker, 2008) and grass palatability (Massey et al., 2009). Grasslands, which are considered silica accumulators, can carry a large pool of biogenic silica ranging from 30 to 100 t Si ha^{-1} (Haynes, 2017). If biomass is removed, as is the case with hay meadows, and manure not re-applied there is a risk of soil silica reduction (Haynes, 2017), which basalt amendments could ameliorate.

6.2.5.3. Uncertainty and future research

The primary point of uncertainty with this work is the short-term nature, the small scale of the field trial, along with the summer of 2022 being one of the driest ones on record in the past 46 years (MET-Office, 2022). This is especially relevant due to the significant increases in soil trace metals, the noticeable decrease in soil potassium, and the associated longer term risks to floristic diversity. In the case of trace metals the basalt rock chosen had the highest concentrations of nickel (195 mg kg^{-1}) and chromium (350 mg kg^{-1}) seen for all the basalts analysed by Lewis et al. (2021), indicating that a different basalt could be used to lessen the risk of bioaccumulation. A calculated value with extensive uncertainty is the CDR estimate which despite it appearing low relative to other cropland estimates, may actually represent a maximum CDR potential as it assumes that the majority of weathering was driven by carbonic acid, which at the pH's of the soils analysed was not necessarily unreasonable (Dietzen and Rosing, 2023). However, confounding this is that the soil analysis was only

of the upper 10 cm, which if cations managed to travel further down could have resulted in an underestimate. Whilst substantial leaching is unlikely due to the sandy clay nature of the soil rich in organic matter providing a high cation exchange capacity, there was extensive bioturbation of the soil by earthworms and other organisms, which definitely resulted in mineral grains moving down the soil profile.

A future research goal should be to perform a larger scale field trial, potentially on differently managed grasslands, to remove some of the potential uncertainty that arises in small plot designs. Furthermore, any field trial should consider doing a more in-depth analysis of the soil profile, leachate, and gas exchanges to aid in creating a more accurate emission avoidance and CDR estimate. One final field of interest is characterising how the below ground diversity is affected with basalt amendment including the native bacteria, fungi, and fauna.

6.3. Conclusion

This thesis has aimed to characterise the relevance of carbonic acid generating enzymes and different groups of biota in enhancing rock weathering. Specifically, this work has shown that though some CAs do appear to significantly affect weathering rates *in vitro* there is no selective pressure for CA genes within soils amended annually with basalt in terms of total CA abundance in soil microbial communities. Though not definitive these results suggest that the total abundance of CA genes within the soil may not play a direct role in the survival of microorganisms in response to mineral dissolution. Subsequent research on the CA gene expression, translation, and protein activity within the soils is required to expand on these findings. This work inspired the later aim of attempting to identify functional groups of biology within soils that could, with simple management practices, be used to increase rates of weathering in soils. The biological simplification experiment, with the associated magnetic separation technique, was developed to try to establish which biotic

components were responsible for weathering *in situ*. The experiment performed in grassland suggested that at least during the growing season AM appeared to play a significant role in mineral weathering, whereas plant root colonisation appeared to inhibit rates of weathering. Though interestingly there were element specific patterns identified, which could carry additional meaning when considering mineral amendments on grasslands. In the same grassland one basalt amendment to plots led to significant increases, at least in the short term, to soil pH and cation concentrations, indicating potential CDR along with confirming basalts potential as a liming agent. Given the potential area of semi-improved grasslands in the UK that could benefit from basalt substitution of liming, which extend to millions of hectares, further work is urgently required across these other grassland communities to establish their suitability and potential to help meet net zero targets for UK agriculture. Potential co-benefits for soil hydrology, reductions in N_2O emissions, forage quality and quantity, livestock nutrition and productivity, and livestock greenhouse gas emissions would need to be further investigated to build a full life-cycle net greenhouse gas budget for such actions.

In the present study the significant increases in the plant concentrations of the micronutrients magnesium and sodium and the macronutrient phosphorus, could all potentially benefit livestock health. While the basalt amendment did not lead to noticeable changes in yield or other nutrient levels in the first year after basalt treatment, suggesting limited potential for cost savings, there was also a concerning reduction in soil potassium and increases in trace metals that may have resulted from the choice of basalt feedstock high in olivine, highlighting the importance of the right choice of rock feedstock for field trials aimed at ERW-CDR. Positively the application of 50 t ha^{-1} of basalt did not appear to immediately impact floristic diversity, but longer-term trials are essential to fully understand how annual basalt amendments might influence both

biodiversity and soil chemistry over time. Though each results chapter provides a unique insight on a different aspect of biological weathering, together they express the widespread and potentially vital role biology-derived rock weathering could play in ERW and furthermore highlight its potential to be leveraged for improved CDR.

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