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Turnover of S-containing amino acids (cysteine and methionine) in grassland soils and their availability to plants

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Turnover of S-containing amino acids (cysteine and methionine) in grassland soils and their availability to plants

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Thesis Summary

Sulphur (S) is one of the most important elements in nature with large quantities cycling through the biosphere, atmosphere and geosphere annually. In recent decades an increased frequency of S deficiency has been observed in soils from many regions of the world. Consequently, in agricultural systems with low S inputs from fertilizers and atmospheric deposition, plants must rely heavily on the release of S from soil organic matter. Typically, less than 10% of the soil S pool occurs as inorganic sulphate with most soil S held in an organic form. Sulphate, together with simple organic S compounds (low molecular weight organic S compounds such as cysteine (Cys) and methionine (Met)), are regarded as potentially being plant-available. However, little information is available on the mineralization and availability of soil organic S, and this has severely limited our capacity to understand the factors that regulate its persistence, bioavailability, and movement in soil. Therefore, the overall aims of this thesis were to explore the bioavailability and biodegradation of Cys and Met in grassland soils. This was achieved by first investigating the intrinsic dissolved organic sulphur (DOS) and inorganic sulphate concentrations in grassland soils (Chapter 3). The results revealed that DOS is the dominant fraction of dissolved S in grassland soils. These results enabled us to optimise the design of the following experimental chapters. The following experiment (Chapter 4) quantified microbial uptake and mineralization of Cys and Met using ^{14}C labelling. My results revealed that ^{14}C-labelled Cys and Met were directly and rapidly assimilated by soil microbes, with assimilation rates being an order of magnitude (or more) faster than microbial mineralisation rates measured via $^{14}CO_2$ evolution. The considerable delay between uptake and mineralization indicates that the turnover of Cys and Met in soil solution was largely biotically mediated. In a subsequent experiment (Chapter 7), I investigated the concurrent mineralization of S, C and N from Cys and Met, as well as the influence of available C, N and S on this process. My results revealed that while a large proportion of added Cys-C and Met-C were used for microbial respiration and microbial biomass incorporation, N and S were excreted as by-products into soil (NH_4^+ and SO_4^{2-}). We assume this was due to the low C/N ratio of these amino acids (C/N ratio = 3 for Cys, and 5 for Met). This assumption was supported by the results that glucose addition promoted a more complete utilization of both amino acids, whereas nutrient addition had less effect. In Chapter 6, I quantified the gross S mineralization rates and the size of the total labile S pools in a closed incubation experiment (70 days) using a ^{35}S isotope-pool dilution-based method. Another critical question is whether plants can acquire S containing amino acids from soil solution, and if so, how does this compare with the uptake rate of inorganic sulphate? To answer this question, I measured the root uptake of three individual S compounds, namely Cys, Met, and sulphate labelled with ^{14}C or ^{35}S, over short time periods (24 h) at an ecologically relevant concentration (100 µM; Chapter 8). Our results revealed that sulphate is preferred to S-containing amino acids by maize plants. A large proportion of the exogenously applied Cys (66%) and Met (73%) could have been taken up rapidly intact by the roots under sterile hydroponic conditions, indicating that they may supply a significant proportion of S to plants when microbial populations are low. I then studied the microbial-plant competition for S-containing amino acids in a rhizosphere context (Chapter 5). My results revealed that < 10% of the added amino acid-^{14}C was captured by the plant, while the rhizosphere microbial community assimilated > 75%. The addition of inorganic N and S, not C, reduced the uptake of Cys and Met from soil by the maize plants, indicating that amino acid utilization may be regulated by inorganic N and S availability. In conclusion, this research provides a more detailed understanding of the turnover of Cys and Met in soils and their availability to maize plants. In addition, it demonstrated the importance of DOS, its rapid turnover in soils, and the intense microbial and plant competition which exists for this resource. The results will also help develop more accurate models of S cycling in soils.

Thesis Content

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Abbreviations

% – Percentage

°C – Degree Celsius

ANOVA – Analysis of variance

C – Carbon

CO_2 – Carbon dioxide

cm – Centimetre (s)

Cys – Cysteine

d – Day (s)

DOC – Dissolved organic carbon

DON – Dissolved organic nitrogen

DOS – Dissolved organic sulphur

DW – Dry weight

EC – Electrical conductivity

h – Hour (s)

h^{-1} – Per hour

IC– Ion chromatography

ICP – Inductively coupled plasma

kBq – Kilobecquerel

KCl – Potassium chloride

kg – Kilogram

K_2SO_4 – Potassium sulphate

L – Litre

LMW – Low molecular weight

Met – Methionine

mg – Milligram (s)

min – minute (s)

min^{-1} – Per minute (s)

mL – Millilitre

mM – Millimolar

MW – Molecular weight

NaOH – Sodium hydroxide

Na_2SO_4 – Sodium sulphate

N – Nitrogen

NH_4^+ – Ammonium

NO_3^- – Nitrate

PCA – Principal component analysis

S – Sulphur

SEM – Standard error of mean

UK – United Kingdom

µM – Micromolar

w/v – Weight per volume

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Chapter 1

Introduction

1.1 Introduction

Sulphur (S) is increasingly being recognized as the fourth major plant nutrient after nitrogen (N), phosphorus (P) and potassium (K). It is a key element ranking in importance with N and P in the formation of proteins (Scherer, 2001). To be more specific, plants require S for the synthesis of essential amino acids (cysteine and methionine) (Wirtz and Droux, 2005), peptides (e.g. glutathione (GSH) and phytochelatins (PCs)) (Ravilious and Jez, 2012), iron-sulphur clusters (Seeber, 2002), membrane sulfolipids (Shimojima, 2011), cell wall components, vitamins and cofactors, and other secondary metabolites (e.g. glucosinolates and alliins). S is mainly taken up as the inorganic sulphate ion (SO_4^{2-}) against the root's electrochemical potential gradient through the activity of plasma membrane sulphate-specific transporters (De Kok *et al.*, 2017; Hawkesford, 2017). In addition, atmospheric sulphur dioxide can enter the leaf via stomata and be used as a source of S (Spedding, 1969; Van Der Kooij *et al.*, 1997). Therefore, S availability is of great importance for plant growth. However, many European countries are currently facing the problem of S deficiency in their soils as a result of reduced S deposition (Engardt *et al.*, 2017), a consequence of cleaner coal fired power stations, higher yields and consequent greater S removal, and decreased use of S containing fertilisers (McGrath, Zhao and Blake-Kalff, 2003).

Owing to its vital and indispensable role in various metabolic and enzymatic processes of plants, it is important to understand its typical and complex behaviour in soils to manage and use S sources more efficiently. Since soil is the primary source of nutrient supply for plants, it is, therefore, of great importance to understand the complete S cycle. Soil S occurs in organic matter and in mineral forms, among which more than 90% is organic bonded (Jamal, Moon and Abdin, 2010). Although not readily plant available, the large organic S fraction is an important source of S supply to plants due to its rapid mineralization to inorganic SO_4^{2-} (Ghosh, De and Maiti, 2018), which can thereafter be taken up by plants. Therefore, apart from

anthropogenic inputs of SO_4^{2-}, the mineralization of organic S to SO_4^{2-} may represent an equally important source of S for plants, especially in areas where there is a deficiency of available inorganic sulphate and organic S is the only S available. There is a great diversity of organic S compounds in soil, among which S-containing amino acids may constitute an important proportion (Scott, Bick and Anderson, 1981). Cysteine and methionine are the only two S-containing amino acids from twenty essential amino acids that make up proteins (Colovic *et al.*, 2018). Since the mineralization of cysteine and methionine to sulphate in soil represents a critical process in S availability to plants, they will be the main focus of the research described in this thesis.

More recently, a number of studies have confirmed that various plant species from diverse ecosystems such as boreal forests (Nordin, Högberg and Näsholm, 2001; Persson and Näsholm, 2001b; Lupi *et al.*, 2013), agricultural plants (Jämtgård, Näsholm and Huss-Danell, 2008; Gioseffi, de Neergaard and Schjørring, 2012), arctic plants (Kielland, 1994) as well as bromeliads plants (Endres and Mercier, 2003) have the capacity to take up amino acids and oligopeptides as a source of N, bypassing the common mineralization process and the need to take up ammonium (NH_4^+) and nitrate (NO_3^-). There is also evidence that cysteine and methionine can be actively transported into cultured tobacco cells (Wright, 1962; Harrington and Smith, 1977; Persson and Näsholm, 2001b), indicating plants may have active mechanisms for enhancing their access to amino acid-S in soil. While the root uptake of amino acids demonstrates the potential for plants to acquire organic-S, understanding the quantitative significance of this process in soil S cycling remains unknown. In addition, the concentration of amino acids in soil solution is known to vary both temporarily and spatially, suggesting that plants may acquire both organic and inorganic S depending on the local conditions. Further, whether plants can compete with soil microorganisms for amino acid-N and S sources in natural environments is still unclear.

1.2 Thesis aims and objectives

1.2.1 Thesis aims

Based on the knowledge gaps highlighted above, this PhD thesis mainly focuses on the potential importance of two S containing amino acids (cysteine and methionine; Cys and Met) as a S source in the soil-plant system. Radioisotope tracers (^{14}C and ^{35}S) were applied to quantify the turnover of these labile organic S compounds in a series of experimental chapters. This thesis quantified the mineralization of cysteine and methionine to inorganic sulphate in soil solution and measured the importance of abiotic and biotic factors affecting this process. In addition, this thesis also explored the direct uptake of Cys and Met by maize roots under sterile conditions, as well as the rhizosphere competition between plant roots and soil microorganisms for these amino acids in soil solution. The overall hypotheses were that 1) microbial mineralization of Cys and Met from soil solution is extremely rapid, releasing plant available inorganic N and S, and 2) plant roots have the capacity to compete with soil microorganisms for Cys and Met in soil solution to enhance their access to low molecular weight organic-S.

1.2.2 Thesis objectives

1.2.2.1 Objective 1: Determine the size of the inorganic and organic S pools in grassland soils

Dissolved S in soil solution was fractionated into dissolved organic S (DOS) and inorganic sulphate. In chapter 3, the size of the DOS and sulphate pools in grassland soils were compared, providing evidence of the spatial variation of these S fractions. In chapter 4, DOS (cysteine and methionine) biodegradation rates were investigated using an isotopic labelling approach (^{14}C), highlighting their capacity to supply S-containing amino acids to plants due to their high rates of replenishment in soil solution. In chapter 6, the size of the labile organic S (LOS) pool in grassland soils was estimated using a ^{35}S isotope dilution technique.

1.2.2.2 Objective 2: Investigate the effects of nutrient amendment on the mineralization of cysteine and methionine in soil

Soil microorganisms play an important role in mineralizing organic S to inorganic forms that are potentially more plant available. In chapter 6, the mineralization and immobilization rates of S from grassland soils was estimated in a closed laboratory incubation system using an isotope-based method. In chapter 7, the concurrent mineralization of C, N and S from Cys and Met was investigated, and the effects of C and nutrient addition on this mineralization process was also studied.

1.2.2.3 Objective 3: Compare the uptake of organic and inorganic S by soil microorganisms and plants

Plant S nutrition is thought to mainly depend on the uptake of inorganic sulphate. However, research has demonstrated that S-containing amino acids (Cys and Met) are also plant-available. Chapter 8 addressed the question: to what extent can plants acquire S-containing amino acids, and how does this compare with that of (inorganic) sulphate under sterile conditions? In chapter 5, the competition between maize roots and soil microorganisms for three S compounds (SO_4^{2-}, Cys and Met) was quantified in an incubation experiment.

1.2.3 Experimental chapters

A brief summary of each experimental chapter is provided below:

Chapter 3 investigated dissolved organic S (DOS) and inorganic sulphate in soils from across a grassland altitudinal gradient (0-400 m a.s.l.). Sulphate concentration was measured by ion chromatography (IC), total dissolved S (TDS) and other cations were analysed using ICP-OES. DOS concentration was obtained by the difference between TDS and inorganic sulphate. The hypotheses were that: (1) DOS is the dominant fraction of TDS in grassland soils; (2) there is a strong interrelationship between DOS and dissolved organic carbon (DOC) and

total dissolved N (TDN) in soil solution. The data demonstrated that DOS constituted 24 to 95% of TDS in all soils with the amount present strongly correlated with TDS, Al, B, Ba, Fe, K, Mn, NH_4^+, DON, DOC concentrations and pH in soil extracts.

Chapter 4 quantified the rapid microbial uptake and mineralization of ^{14}C-labelled Cys and Met in a series of five contrasting grassland soils from an elevation gradient (32-402 m a.s.l.) with decreasing primary productivity. Substrate depletion from soil solution was measured using a centrifugal-drainage procedure, and the subsequent production of $^{14}CO_2$ from microbial mineralization was measured by 1 M NaOH traps. The hypotheses were that: (1) turnover rates of Cys and Met are faster in soil from more productive and lower altitude grassland than in soil from less intensive, higher altitude grasslands; (2) DOS turnover rates estimated by the centrifugal-drainage procedure are higher than when determined by measuring mineralization rates. These results indicate that alongside SO_4^{2-}, Cys and Met could also be important sources of S for plants.

Chapter 5 evaluated the uptake and rhizosphere competition for Cys and Met between soil microorganisms and maize roots. ^{14}C and ^{35}S labelling was used to quantify the incorporation of amino acids into plant tissues or soil microorganisms. The hypotheses were that: (1) soil microorganisms and maize plants both possess a high capacity to utilize Cys and Met; (2) when allowed to compete, maize plants possess a lower capacity to capture free Cys and Met from soil solution compared to soil microorganisms. Results from the incubation experiment showed that soil microorganisms captured amino acids at around 400 nmol ^{14}C (kg soil)$^{-1}$ h^{-1}, while plant roots incorporated amino acids at around 200 nmol ^{14}C (g root DW)$^{-1}$ h^{-1}. When allowed to compete together in microcosms, maize plants captured around 10% of the ^{14}C-labelled Cys and Met, while the rhizosphere microbial community captured more than 70%.

Chapter 6 explored the intrinsic rates of S turnover in seven contrasting soils along a natural grassland gradient using ^{35}S isotope labelling and a pool-dilution model. In a closed incubation experiment (70 d), we quantified the temporal changes in extractable $^{35}SO_4^{2-}$ and stable $^{35}SO_4^{2-}$ concentrations. The hypotheses were that: (1) the size of the labile S pool derived from the model are compatible with measured extractable concentrations; (2) the addition of non-labelled sulphate would retard the incorporation of ^{35}S-sulphate into the organic-S pool, while the addition of glucose would enhance this incorporation process.

Chapter 7 describes the importance of C and nutrient availability in regulating microbial decomposition of Cys and Met in a grassland soil using dual-isotope labelling with ^{14}C and ^{35}S. This allowed the concurrent mineralization of S, C and N during the incubation to be investigated. The hypotheses were that: (1) Cys and Met mineralization is a biological mediated process, with amino acid-C used for respiration and retained in the microbial biomass, and inorganic N and S released back into soil solution; (2) C and nutrient amendments regulate microbial decomposition of both Cys and Met due to altered microbial elemental stoichiometry. The results showed that microbial communities have an innate capacity to utilize Cys and Met, with inorganic N (NH_4^+ and NO_3^-) and SO_4^{2-} being generated as a result of microbial C acquisition, through which process both soil microorganisms and plants could potentially benefit.

Chapter 8 determined the short-term uptake and efflux of organic and inorganic S (Cys, Met, SO_4^{2-}) by maize plants in sterile conditions. Plants have the ability to take up intact amino acids as an N source, but this has not been thoroughly studied for S-containing amino acids. The hypotheses were that: (1) Cys and Met can be taken up intact by plant roots under sterile hydroponic conditions, yet inorganic sulphate is preferred over organic S by maize plants; (2) root efflux of Cys and Met occurs through passive diffusion. Our results showed that a considerable proportion of Cys (66%) and Met (73%) could have been rapidly taken up intact

under sterile hydroponic conditions, when inorganic sulphate was available, indicating that S-containing amino acids could be important nutrient sources for plants under certain growing conditions.

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Chapter 2

Literature Review

2. Literature review

2.1. The global sulphur cycle

Sulphur (S) is an essential element for all living systems (Mitchell, 1996) and is closely associated with numerous fundamental biotic and abiotic geochemical reactions and cycling processes within the earth's atmosphere, hydrosphere, biosphere, and geosphere (Granat, Rodhe and Hallberg, 1976). This key role is a consequence of its large variety of different oxidation states (Charlson, Anderson and McDuff, 1992; Lomans *et al.*, 2002), ranging from completely reduced (oxidation state, -2) to completely oxidized (oxidation state, +6). Among these oxidation states, only three are abundant in nature, -2 in the form of sulfhydryl and sulphide, 0 in the form of elemental S, and +6 in the form of sulphate. These compounds are continuously converted to each other by a combination of biological (Rennenberg *et al.*, 1990; Rennenberg and Polle, 1994; Sors, Ellis and Salt, 2005; Davidian and Kopriva, 2010), biochemical (Kabdasli, Tünay and Orhon, 1995), and geochemical processes (Maher and Geoscience, 2013; Cao *et al.*, 2018), forming the global S cycle. Therefore, it is useful to consider both the amounts of S in these individual S states and the fluxes between these S pools.

2.1.1. Sulphur cycling in the atmosphere

2.1.1.1. Sources of atmospheric sulphur

S compounds are released into the atmosphere by both anthropogenic and natural processes (Shinn and Lynn, 1979; Galloway and Whelpdale, 1980; Last, 1982). Natural atmospheric sources of S include the following: ocean-derived sea spray containing sulphate, organic compounds produced from the microbial decomposition of organic matter, reduction of sulphate in oxygen depleted waters and soils, volcanoes, and forest fires (Bates *et al.*, 1992). Anthropogenic-derived sources of atmospheric S include the combustion of fossil fuels and the

smelting of non-ferrous ores (Cullis and Hirschler, 1980). Overall, the global emission of all S-containing gases is 251 million tonnes year^{-1}, with about 41% being of anthropogenic origin (Castro, Williams and Ogram, 2000). Due to the ubiquitous presence of S in fossil fuels and metal sulphide deposits, man-made atmospheric S emissions have increased in step with industrialization (Rodhe, 1999; Smith *et al.*, 2011). Atmospheric S occurs in a range of forms, including SO_2 emitted during volcanic eruptions, microbial oxidation and from coal-fired power plants and metal smelters. In contrast, H_2S is emitted by anaerobic bacteria in soils and sediments, from volcanoes, and deep-sea vents. Therefore the majority of SO_2 emitted to the atmosphere is related to human activities, while most H_2S emissions are natural (Rasmussen and Kabel, 1974). In addition, dimethyl-sulphide (DMS) and other volatile S compounds produced by marine algae (Liss, Malin and Turner, 1993) and livestock (Zicari *et al.*, 2013) may also contribute to atmospheric S loading.

2.1.1.2 Deposition of atmospheric sulphur

After being transported and mixed by winds and turbulence, atmospheric S compounds are re-deposited on the land and water surface as either wet or dry deposition. Briefly, SO_2 becomes oxidized to sulphate where it electrostatically interacts with cations (e.g. NH_4^+, Ca^{2+}, H^+) in small particulates or moisture droplets (Park, 2013; Fowler, 1980; Garland, 1978). Both wet and dry deposition processes are efficient in scavenging gaseous and particulate S from the atmosphere, with about half of the SO_2 emitted to the atmosphere removed by dry deposition, and the remainder oxidised to sulphate and removed by precipitation (Garland, 1978; Wałaszek, Kryza and Dore, 2013).

In the 1980s, the amount of S deposited annually in rainfall in SE England ranged from 10 to 15 kg S ha^{-1} from coal-fired power stations, while the absorption of atmospheric-derived S by crops and soil was estimated to be 20 and 50 kg S ha^{-1} y^{-1} (Martin, 1980). However, due

to the move away from coal-fired power stations and S-containing fertilizers, very little S is now deposited directly onto UK agricultural fields (2-4 kg S ha^{-1} y^{-1}; Battarbee et al., 2014). Consequently, atmospheric S deposition can no longer meet crop S demand, resulting in potential crop quality and yields losses. Whilst uncertainties still exist in components of these S budgets, several studies have attempted to provide a holistic circulation of S within terrestrial, freshwater and marine systems (Möller, 1984; Hamilton, 1985; Liss *et al.*, 1997; Granat, 2013).

2.1.2 Sulphur cycling in freshwater ecosystems

2.1.2.1 Sources of sulphur input into freshwater ecosystems

The weathering of S-containing rocks in catchments and the oxidation of organic S from terrestrial sources represent the main sources by which S enters freshwaters (Zak *et al.*, 2021). In addition, S may reach water bodies from atmospheric deposition, the application of S-based products to soil and their subsequent leaching and runoff (e.g, wastes, fertilisers, pesticides and fungicides. Hinckley et al., 2020; Onwona-Kwakye et al., 2020), and in irrigation water (Schuler *et al.*, 2019).

2.1.2.2 Content of sulphur in freshwater sediments

Previous work has established that the sediment and its interface with overlying water are major sites for S accumulation and transformation (Jørgensen, 1990; Holmer and Storkholm, 2001; Zhang *et al.*, 2018). In freshwater sediments, there are many different S compounds including inorganic and organic forms. Inorganic S in freshwater sediments occurs in a variety of forms such as pyrite, other iron sulphide compounds due to redox gradients and elemental S. The organic S fraction can be subdivided into three components: protein, sulphate ester, and non-protein carbon-bonded S (sulfolipids, vitamins, and a large diversity of S containing amino acids; King and Klug, 1982).

2.1.2.3 Transformations of sulphur in freshwater sediments

Freshwater sediments contain a high density and diversity of microorganisms which are involved in a variety of biogeochemical processes including both reductive and oxidative processes. The co-operative action of these gives rise to the S cycle in freshwater sediments. Although based on a mass balance budget, sediment S is predominantly present in an organic form (Mitchell *et al.*, 1984; Losher and Kelts, 1989; Couture *et al.*, 2016). The net mineralization of this organic S released SO_4^{2-} into the water column. In addition, despite low SO_4^{2-} concentrations (Holmer and Storkholm, 2001), the S cycle in freshwater sediments is dominated by SO_4^{2-} reduction in terms of the quantity of S transformed (King and Klug, 1982).

Sulphate reducing bacteria (SRB) are a complex physiological bacteria group (Castro, Williams and Ogram, 2000), including *Desulfovibrio*, *Desulfomicrobium*, *Desulfobulbus*, *Desulfobacter*, *Desulfococcus*, *Desulfosarcina*, *Desulfobacterium*, *Desulfonema*, *Desulfotomaculum* and *Thermodesulfobacterium* (Warren *et al.*, 2005). The groups are characterized by their use of sulphate as a terminal electron acceptor during anaerobic respiration. The first described and best known genus of S-reducing bacteria in sediments and other natural environments is *Desulfovibrio* (Postgate and Campbell, 1966; Odom and Peck Jr, 1981; Fauque *et al.*, 1988; Blumenberg *et al.*, 2006). The concentration of sulphate is a key factor controlling the rate of sulphate reduction in freshwaters. Further, the addition of sulphate to sediments enhances sulphate reduction and promotes the cycling of C, N, P and Fe (Schindler, 1981; Koschorreck, 2008; Chen *et al.*, 2016). Atmospheric precipitation supplies most of the sulphate to natural waters (Fisher *et al.*, 1968) with the concentration of sulphate in freshwater typically ranging from 10 to 500 µM, which is much lower than in seawater (Holmer and Storkholm, 2001).

Sulphides produced during sulphate reduction may undergo re-oxidation by several processes: chemical oxidation with oxygen, bacterial oxidation under oxidized conditions,

phototrophic oxidation, anoxic chemical oxidation and bacterial oxidation under anoxic conditions (Holmer and Storkholm, 2001). Previous studies have shown that microorganisms from the genera *Sulfuricella* (*Betaproteo bacteria*) (Watanabe, Kojima and Fukui, 2014), *Thiobacillus* (*Betaproteobacteria*) (Haaijer *et al.*, 2006) and *Sulfuricurvum* (*Epsilonproteobacteria*) (Haaijer *et al.*, 2008) can undertake S oxidation. Further, aerobic S oxidizing bacteria (SOB) can grow autotrophically using inorganic S as an electron donor and play an important role in the conversion of inorganic S compounds in freshwater ecosystems. Previous research has shown that up to 90% of reduced S was re-oxidized to sulphate (Holmer and Storkholm, 2001), and this high rate of re-oxidation of reduced S compounds in freshwater sediments may revert the sediments from a sulphate sink to a source to the overlying water in some cases.

2.1.3 Sulphur cycling in terrestrial ecosystems

2.1.3.1 Input and output of sulphur in soil

The terrestrial S cycle involves significant interactions between the pedosphere, the hydrosphere, the biosphere, and the atmosphere. In comparison to other elements (e.g. C, N, P), the study of S in soils has received relatively little attention since adequate amounts were supplied from rock weathering, S fertilizers, irrigation water and from atmospheric deposition (volcanoes, atmospheric pollution, and sea spray). Consequently, until recently, there has been little economic drive to fund research on S cycling. Vegetation can also provide an important source of S in soil ecosystems because living roots release organic and inorganic S into soils (i.e., root exudation), and on plant death, decay of plant tissues releases S back to the soil. In addition, S is added to soil due to the widespread use of S-containing fungicides and insecticides (Griffith, Woodrow and Seiber, 2015). A simplified illustration of the sulphur cycle in agricultural soils is depicted in Fig. 2.1.

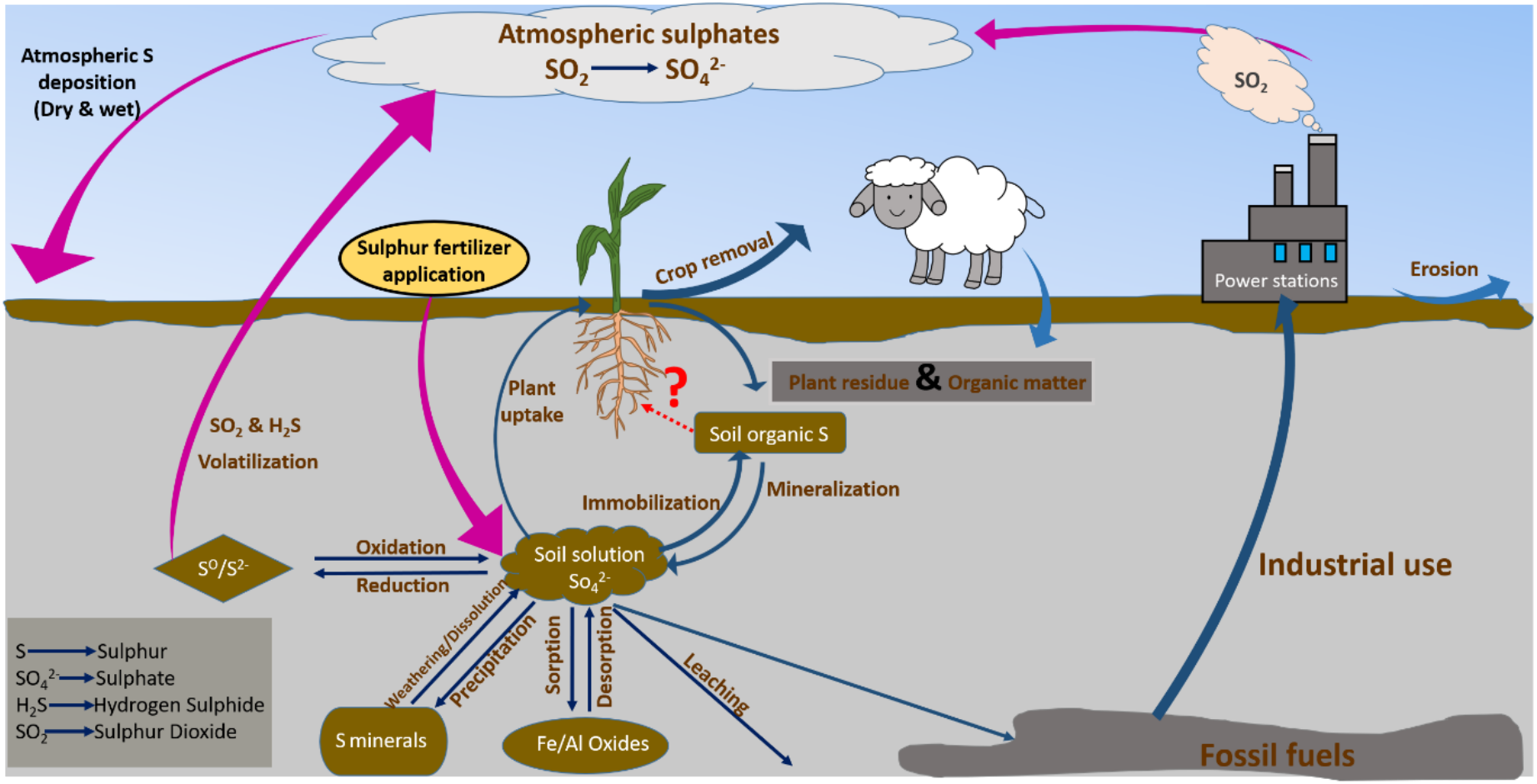

Fig. 2.1 Sulphur cycle in agricultural soils (Chalk, Inácio and Chen, 2017).

S is removed from soil systems by leaching, erosion and surface runoff (Barrows and Kilmer, 1963), S volatilisation (Cullis and Hirschler, 1980; Peñuelas *et al.*, 2014), and uptake by plants (Plante, 2006). Of these, most S is removed from agroecosystems by crop harvesting, although a large amount of S may ultimately be returned to the soil via their residues or in livestock waste. S losses can also occur via leaching of sulphate alongside base cations (Ercoli *et al.*, 2012). Other forms of S (elemental S and organic S) are less mobile in soil (Riley, Zhao and McGrath, 2002), and must be converted to sulphate before significant leaching can occur. The amount of sulphate leaching is dependent on a range of factors including S status of the soil, texture, weather patterns, Fe content, O_2 status and soil structure. In addition, volatile forms of S are emitted from soils (e.g. H_2S from waterlogged soils supplemented with sulphate; Fitzgerald, 1976). Other forms of S are generated and emitted from soils under both aerobic and anaerobic conditions, e.g. CH_3-SH, CH_3-CH_2-CH_2-SH has been shown to be produced following addition of cysteine to soils, and CH_3-SH, CH_3SCH_3 and CH_3-S-S-CH_3 during methionine degradation in soils (W. L. Banwart and Bremner, 1975).

2.1.3.2 Sulphur forms in soil

S is present in soils as both organically-bonded S and inorganic sulphate. Organically bonded S provides the major S reservoir and accounts for about 90% of total S in most soils (Eriksen et al., 1998; Scherer, 2009; Freney et al., 1969; Houle and Carignan, 1992; Kertesz and Mirleau, 2004; Yang et al., 2007), from which plant-available sulphate is released upon microbial oxidation of organic-S. In general, inorganic S species can be operationally fractionated into water-soluble sulphate, adsorbed sulphate, dilute-HCl soluble S, volatile S, and pyritic S (Johnson, Henderson and Todd, 1981; Scherer, 2009). In contrast to inorganic S, the organic S pool contains a diverse mixture of compounds contained within organic matter (SOM) and the microbial biomass (Jamal, Moon and Abdin, 2010). Organic S can be further

divided into two distinct fractions: ester S as well as carbon-bonded S such as S-containing amino acids (Freney, Melville and Williams, 1970; Landerst, David and Mitchell, 1983; Scherer, 2009).

(1). Ester sulphates: Organic forms of S are a heterogeneous mixture of humus and plant residues, and can be subdivided into two main fractions according to the susceptibility to reduction by hydroioidic acid (HI), namely, (i) carbon-bonded S (directly C-S bonded), and (ii) ester organic S (S is bonded to oxygen or N). Ester organic S is easily hydrolysed to inorganic sulphate by mild physical or chemical treatments as well as extracellular enzymes termed arylsulphatases, therefore, they can be considered to be more biologically active (labile) in soil (Scherer, 2001). As the measurement of this form involves reduction with hydroioidic acid (HI), it is often referred to as HI-reducible-S. The organic sulphate fraction includes compounds containing C-O-S linkage (ester sulphate), C-N-S linkage (sulfamates) and N-O-S linkage (sulphated thioglycosides) (Fitzgerald, 1976), and constitutes 30-75% of the total organic S in soil. Ester sulphates include compounds such as choline sulphate, sulphated polysaccharides and phenolic sulphates (Edwards, 1998).

(2). Carbon-bonded S: C-bonded S constitutes up to 30% of the organic S in soil. C-bonded S includes S-amino acids (e.g. methionine, taurine, cystathionine, cysteine and cystine), polypeptides and proteins (Fe-S proteins called ferrodoxodins), and other S-containing compounds such as biotin, thiamine, coenzyme A as well as lipoic acid (Brosnan and Brosnan, 2006; Cronan, 2014; Colovic *et al.*, 2018). S-containing amino acids constitute an important proportion of the soil organic S (Norman M. Scott and Anderson, 1976), however, estimates of cysteine and methionine in soil hydrolysates are generally low because (i) S-containing amino acids are rapidly degraded and metabolized to sulphate in soil; and (ii) amino acids are mostly present as protein or peptides rather than as monomers, making their estimation difficult.

In addition to anthropogenic inputs of sulphate, the mineralization of organic S also represents an important source of sulphate in soils (McGill and Cole, 1981). Both ester sulphates and C-bonded S can be mineralized to plant-available sulphate, and it is generally accepted that soil microorganisms (including bacteria, archaea, and fungi) play a major role in this mineralization process (Kertesz and Mirleau, 2004a; McLaren and Swift, 1977). Previous studies have shown that S present in cysteine can be converted to sulphate by a mixed population of soil microorganisms, with cysteinesulphinic acid, cysteic acid, sulphite and β-hydroxypyruvic as intermediate forms (Freney, 1960). In addition, various fungi and bacteria can convert methionine to sulphate, although in terms of a source of soil sulphate, methionine is thought to be less readily mineralized than cysteine (Fitzgerald and Watwood, 1988). Therefore, for soils lacking significant S or S^{2-} oxidative capacity, mineralization of S-containing amino acids may represent an important mechanism for sulphate generation.

(3). Microbial biomass-S: The soil microbial biomass (i.e. bacteria, fungi and protozoa) generally accounts for ca. 1.5-3% of the total soil organic S (Saggar, Bettany and Stewart, 1981; Chapman, 1987; Smith and Paul, 1990). It plays a vital role in the S cycle as both a source and sink of plant-available S and is frequently considered to be relatively labile and a highly active pool of S turnover in soil (Banerjee and Chapman, 1996; Stevenson and Cole, 1999). The rate at which microbial biomass-S turns over in soil is an important parameter for soil S modelling. One might expect microbial biomass-S to turnover on an annual timeframe since microbial cells assimilate C, N and S in a relatively fixed ratio (Spohn, 2016; Spohn *et al.*, 2016). However, there is evidence that the microbial biomass C:S ratio is not fixed and can vary somewhat (Khan and Joergensen, 2019), therefore microbial biomass-S could turnover at a different rate from that of microbial biomass C.

The major S forms in microbial cells are proteins, amino acids, and other minor metabolites and essential vitamins (Moat, Foster and Spector, 2003). In addition, the microbial

biomass is a major driving force regulating the nutrient transformations in soil (Wu and Syers, 1997). By lysing microbial cells with chloroform, microbial biomass-S can be measured in a similar way to the measurement of biomass C and N (Voroney, Brookes and Beyaert, 2007). Plant residues are a major source of organic inputs to soil, exerting a primary role in maintaining SOM, microbial biomass and activity, and the size of the soil nutrient pool. Following addition of plant residues to soil, the microbial community grows rapidly in response to the addition of labile C, often inducing a rapid immobilization of available soil nutrients, particularly N, P and S by the microbial biomass (Wu, O'Donnell and Syers, 1993). In addition, mineralization of N, P and S may occur. Ultimately, this depends on the stoichiometric C:N:P:S ratio of the residues.

(4). Inorganic sulphate: Inorganic forms of S are usually much less abundant than organic S and can be divided into four major categories: (i) highly mobile sulphate in soil water; (ii) adsorbed sulphate, which can be desorbed; (iii) sulphate minerals, which may precipitate and re-dissolve (Barber, 1995); and (iv) sulphate, which is irreversibly retained (Wang *et al.*, 2011). The content and availability of sulphate in soil is continually undergoing changes due to: 1) rapid mineralization and immobilization by soil microbes; 2) leaching; 3) additions from the atmosphere in gaseous form or from wet deposition, and 4) additions from application of fertilisers and livestock waste (e.g. urine, faeces, manure, slurry).

2.1.3.3 Sulphur transformations in soil

Generally, nutrients are first released into soil due to mineral weathering (dissolution and desorption; Comerford, 2005), as well as from SOM mineralization (Nannipieri, Kandeler and Ruggiero, 2002). Soluble S can than move through the soil solution to the root surface by means of mass flow and diffusion where it can be taken up. Plants mainly acquire S primarily through the assimilation of sulphate, although small amounts of SO_2 can be acquired from the

atmosphere via stomatal uptake (Garsed and Read, 1977). When plant residues and livestock remains are returned to the soil, they are decomposed by microorganisms with the liberation of inorganic S. These inorganic S compounds may be oxidised to sulphates or reduced to hydrogen sulphide.

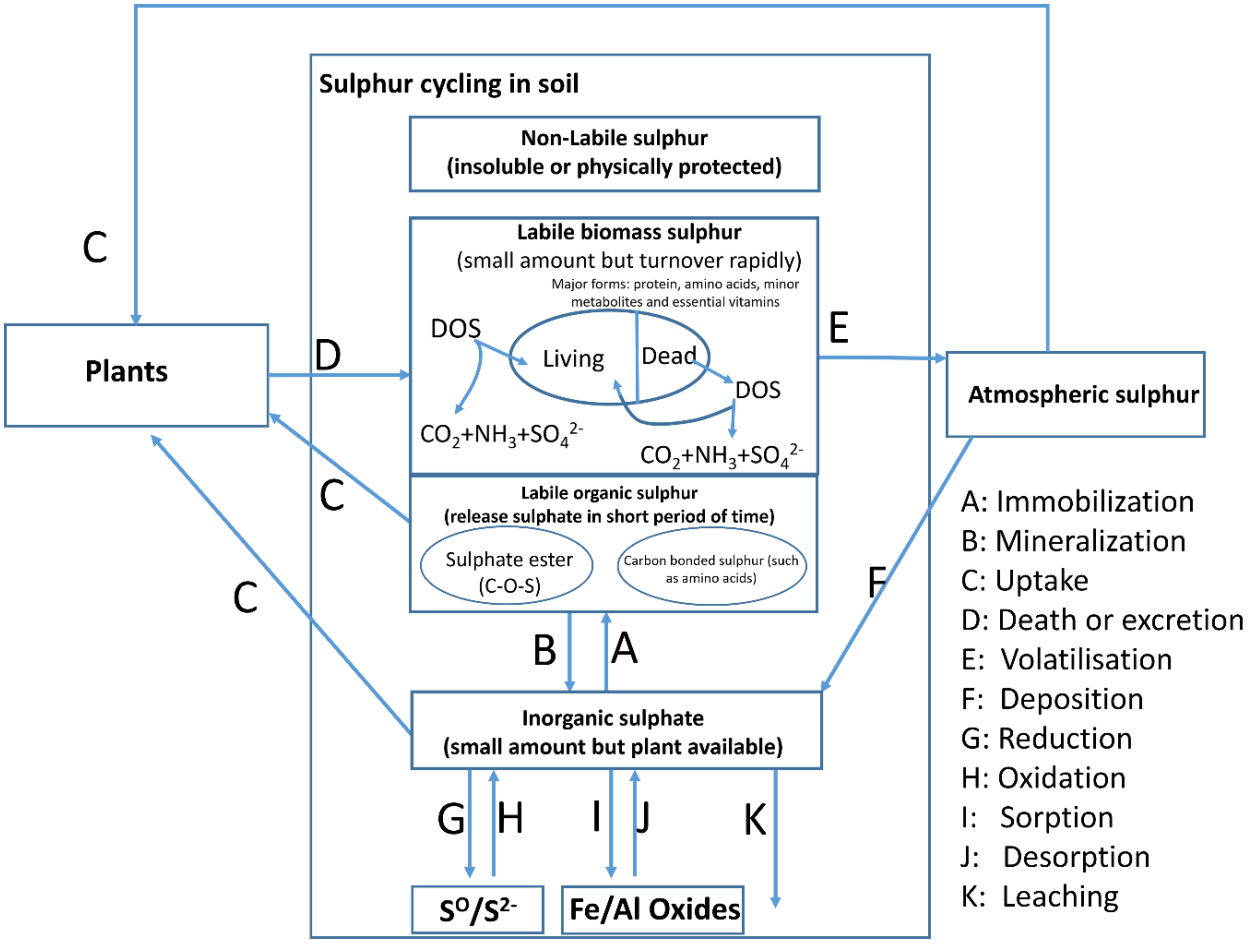

Fig. 2.2 Simplified schematic representation of main processes involved in sulphur cycle within plant-soil-atmosphere systems. Modified from (Brown, 1982).

A simplified schematic representation of the main processes involved in sulphur cycle within plant-soil-atmosphere systems is shown in Fig. 2.2. S compounds undergo many transformations in soils as a result of biological activities including plants, animals and microorganisms, among which the microbial community plays a crucial role in the

transformation of various elements such as S, N, C and Fe due to the combined action of mineralization and immobilization. The key reactions include: 1) mineralization, the decomposition of organic S into inorganic sulphate; 2) immobilization, the assimilation of these inorganic and organic S compounds into microbial tissues; 3) oxidation, the conversion of reduced, inorganic forms (sulphides, thiosulphates, polythionates and elemental S) to sulphate; 4) reduction, the conversion of sulphate and other oxidised forms of S into sulphides; 5) sulphate adsorption-precipitation phenomena; 6) leaching. The importance of these processes is dependent on the form of S present, the physico-chemical environment, and the size, diversity and physiological state of the soil microbial community. Microbial activity is affected by many soil physical and chemical properties, especially pH, temperature, moisture availability, plant type, agricultural management regime etc. (White, 1959; Freney and Spencer, 1960).

(1). Mineralization of S: In well-drained soil surface layers, the amount of inorganic sulphate is often too small to provide adequate S for sustained rapid plant growth (Scherer, 2001), so plants may be largely dependent upon the conversion of soil organic S to sulphate for satisfactory S nutrition. Soil organic S must be broken down into smaller molecules by extracellular enzymes (e.g. proteases) before further mineralization can occur. S mineralization is the transformation of organic S into SO_4^{2-} and CO_2 (McGill and Cole, 1981). The biological process is considered to be driven by the microbial consumption of organic C in response to microbial energy demand, thus S which is directly bonded to C in soil organic compounds (carbon-bonded S, e.g. S containing amino acids) is released as SO_4^{2-} (Blum et al., 2013). In contrast, sulphate can be released due to the cleavage of ester sulphates by extracellular or periplasmic microbial sulphatases (T. Sherene, 2017). This biochemical process is controlled by the supply of S rather than the need for energy, thus when soil sulphate is too low to meet microbial demand, sulphate esters are hydrolysed by sulphatases (McGill and Cole, 1981).

Mineralization of soil organic S has been shown to contribute substantially to plant S uptake and leaching (Pirela and Tabatabai, 1988; Sakadevan, Mackay and Hedley, 1993; Gahan and Schmalenberger, 2014; Coyne, Mikkelsen and Mineralization, 2015). An open system of incubation showed that soil microbes could mineralise C-bonded S to satisfy their S or possibly N requirements, and C-bonded S was identified as a major contributor to mineralised SO_4^{2-} (Ghani, 1989). Therefore, under circumstances where the storage of sulphate is too low to provide plants with adequate S for plant growth, the conversion of organic S to sulphate by the action of soil microbial becomes critical.

(2). Immobilization of S: S applied to soil is subject to physical/electrostatic (sorbed onto soil particles) or biological immobilization. Biological S immobilization is the process by which mineral S is incorporated into the microbial biomass (Freney, 1967; Shahsavani and Gholami, 2009). Since C availability greatly controls microbial activity, the extent to which sulphate-S could be immobilized is determined by both the amount of available-S and the availability of labile C. Addition of labile low-molecular-weight (MW) organic compounds such as glucose can result in a rapid increase in microbial biomass and thus S immobilization (Vong et al., 2008, 2010; Kertesz and Mirleau, 2004a; Scherer, 2009). Incubation studies (Freney et al., 1971; Freney et al., 1975) have also shown that ^{35}S-labelled sulphate is incorporated into two major organic S fractions in soil, namely hydriodic acid reducible S, in which S is not directly bonded to carbon and is reduced to hydrogen sulphide by hydriodic acid, and soluble C-bonded S (e.g. Cys and Met). This is in line with other studies (Williams and Donald, 1957) showing that applied sulphate can be readily recovered in SOM over longer timescales.

(3). Oxidation of inorganic sulphur: Microbial oxidation of S is an important process where reduced forms of S are present, and involves the microbial oxidation of reduced inorganic S forms (e.g. sulphides, elemental S, thiosulfates) to higher oxidation states (e.g. sulphate). Microbial S oxidation is generally beneficial to soil in different ways: (1) solubilized inorganic

salts contain plant nutrients and thereby increase the level of soluble phosphate, potassium, calcium and magnesium for plant nutrition; (2) the acidity produced by oxidation of S can also be used to improve alkaline soils especially under warm and wet conditions (Germida and Janzen, 1993).

Although chemical oxidation of S occurs in soils, this process is mainly microbially mediated and largely involves three groups of microorganisms: (1) chemoautotrophic S bacteria (*Thiobacteriaceae*, *Beggiatoaceae* and *Achromatiaceae*); (2) photosynthetic S bacteria (*Thiorhodaceae* and *Chlorobacteriaceas*); and (3) certain heterotrophic microorganisms including some actinomycetes, bacteria and fungi (e.g. *Pseudomonas* spp., *Arthrobacter* spp., *Flavobacterium spp*. and *Bacillus* spp; Kuenen and Beudeker, 1982; Vidyalakshmi et al., 2009). Of these, *Thiobacilli* are chemoautotrophic bacteria who gain energy from oxidising reduced forms of S (Vidyalakshmi, Paranthaman and Bhakyaraj, 2009). Five species of the genus *Thiobacillus* are regarded as important in S oxidation in soils, *viz*. *T. denitrificans, T. ferrooxidans, T. thioparus* and *T. thiooxidans*.

(4). Reduction of sulphate: Sulphate can be directly reduced to sulphide by sulphate reducing bacteria (SRB) via either assimilatory or dissimilatory pathways (Kushkevych *et al.*, 2020). Both assimilatory and dissimilatory reduction of sulphate begin with the activation of sulphate by adenosine triphosphate (ATP). Adenosine phosphosulphate (APS), formed by ATP and sulphate, is then catalysed by the enzyme ATP sulphurylase (Abdulina *et al.*, 2020). In dissimilatory reduction, the sulphate moiety of APS is directly reduced to sulphite (SO_3^{2-}) by the enzyme APS reductase, while in assimilatory reduction, another P atom is added to APS to form phosphoadenosine phosphosulphate (PAPS), PAPS is then reduced to sulphite (Romero *et al.*, 2014). Once sulphite is formed, it is oxidized to sulphide by the enzyme sulphite reductase. The assimilatory pathway generates reduced S compounds for biosynthesis of amino acids and proteins, and therefore does not lead to direct excretion of sulphide. In contrast, in

dissimilatory reduction, sulphate is reduced to inorganic sulphide by obligatory anaerobic sulphate reducing bacteria (Koschorreck, 2008).

Sulphate can be reduced to hydrogen sulphide (H_2S) by SRB, reducing the availability of S for plant nutrition. The conversion of sulphate to H_2S may seem undesirable from a soil fertility perspective, but it could be of great significance in soils under alkaline and anaerobic conditions, as the acidity produced through this process can be used to lower soil pH. The predominant sulphate reducing bacteria genera in soils are *Desulfovibrio, Desulfotomaculum*, and *Desulfomonas* (Luptakova, 2007), all of which excrete the enzymes desulphurases or bisulphate reductase, which are responsible for reduction of S (Meena and Improvement, 2018).

(5). Sulphate adsorption-precipitation phenomena: The mobility and concentration of sulphate in soil solution can be affected by adsorption and desorption reactions with the solid phase (De Vries and Breeuwsma, 1987). Sulphate can be sorbed to mineral surfaces (e.g. Fe/Al hydroxides) and organic matter in acidic soils (Schoenau and Malhi, 2008), leading to greater retention and reduced leaching losses (Curtin and Syers, 1990; Eriksson, 1988). Sulphate sorption varies widely between soils and is strongly affected by soil pH (Muktamar, 1993), and sulphate concentration (Haque and Walmsley, 1973). Sulphate adsorption and desorption is also important in buffering soil systems against extreme climatic events (Moldan *et al.*, 2012). Sulphate sorption-desorption curves are typically used to predict the concentration-dependent availability of sulphate in soil and thus plant availability (Ghosh and Dash, 2012).

(6). Leaching: Unlike phosphate, sulphate SO_4^{2-} is weakly retained on soil surfaces and is therefore more susceptible to leaching than P in most soils (Barrow, 1978). The amount of S leaching depends on management practices and various soil characteristics, such as the surface charge characteristics of the soil, which can be manipulated by the addition of soil amendments (e.g., P and lime) (Bolan *et al.*, 1988). Addition of lime (with associated pH increase) and P

fertiliser decreases sulphate sorption by increasing the net negative charge of the soil as well as blocking sorption sites. They can also promote S mineralization, thus increasing sulphate movement in soil (Chao, Harward and Fang, 1962). Sulphate is susceptible to leaching and may be lost to the subsoil (Churka Blum et al., 2013). In comparison to topsoils, our knowledge of S cycling in subsoils is very poor. Sulphate leaching down the soil profile mainly occurs in the autumn and winter in response to an increase in seasonal rainfall, however, cover crops can be used to reduce these losses in arable systems (Lehmann and Schroth, 2003).

2.1.4 Enzymes involved in sulphur cycle

A wide range of enzymes are excreted by plant roots and soil microorganisms into soil where they are involved in the cycling of S. The following section summarises the role of these enzymes.

2.1.4.1 Enzymes involved in the soil sulphur cycle

(1). Carbon-S lyases: The release of sulphate from organic S may proceed by two mechanisms: oxidation of the C skeleton to yield energy with sulphate being released as a by-product; or directly after hydrolysis of ester sulphate linkages comprising the organic S. There are two enzymes that belong to the family of lyases, specifically the class of carbon-S lyases. Methionine gamma-lyase (MGL) catalyses the formation of methanethiol, α-ketobutyrate and ammonia from L-methionine (Esaki *et al.*, 1979; Johnston *et al.*, 1981). Cysteine desulfhydrase is an enzyme which catalyses the degradation of L-cysteine to pyruvate, ammonia and hydrogen sulphide (Kumagai *et al.*, 1974). These enzymes have been found in a range of bacteria belonging to the genera *Escherichia*, *Aerobacter*, *Serratia*, *Proteus*, *Alcaligenes*, *Agrobaaterium*, *Miarococcus* and *Sarcina.*

(2). Sulphatases: Sulphatases release sulphate from sulphate esters and have been the focus for much soil S research (Gahan and Schmalenberger, 2014). In soils, different types of

sulfatases occur: arylsulfatases (ARS), alkylsulfatases, steroidsulfatases, glucosesulfatases, chondrosulfatases, and myrosulfatases (Schinner *et al.*, 2012). Of these, ARS is the key enzyme involved in soil organic S mineralization by hydrolysing ester S to sulphate (Tabatabai and Bremner, 1970; Cooper, 1972). It has been detected in plants, animals and soil microorganisms (Stressler *et al.*, 2016). In soils, enzymes are located either in living cells (intracellular enzymes) or outside cells (extracellular enzymes). By use of the chloroform fumigation extraction methods, studies have shown that ca. 57% of the total arylsulphatase was intracellular with the remainder being extracellular (Klose and Tabatabai, 1999). However, increased microbial arylsulfatase activity may not lead to increased amounts of sulphate accumulation, indicating that sulfatase enzyme activity is not the rate-limiting step in the biochemical release of sulphate-S from sulphate esters (Ganeshamurthy and Nielsen, 1990).

The activity of microbial and soil-extracted arylsulfatase increases with increasing sulphate concentration, while barley root arylsulfatase activity can be repressed by sulphate, indicating that arylsulfatase produced by soil microorganisms and plants are controlled differently (Ganeshamurthy and Nielsen, 1990). Current evidence suggests that the activities of ARS are influenced by a range of soil chemical and biological properties. For example, a study in boreal forest soils revealed that maximum sulfatase activity occurred below the humus layer, possibly due to the pH optimum of sulfatase being more typical of subsoils than highly acidic topsoils (Wittmann *et al.*, 2004). Arylsulphatase activity was also found to be positively correlated with the total soil C, total organic S and reducible organic S in soil (Cooper, 1972).

(3). Rhodanese: Rhodanese (Thiosulphate cyanide sulphurtransferase) is another enzyme involved in the S cycle which has been detected and characterized in soil and which catalyses the formation of thiocyanate from $S_2O_3^{2-}$ and cyanide according to the following reaction: $S_2O_3^{2-} + CN^- = SCN^- + SO_3^{2-}$ (Dick and Deng, 1991).

2.1.4.2 Enzymes involved in plant sulphur cycling

In higher plants the enzymes of assimilatory sulphate reduction are localized mainly in the chloroplasts of green leaves, where the reduction is strongly dependent on light intensity (Chen *et al.*, 2018). S assimilation starts with the formation of adenosine phosphosulphate (APS), a reaction catalysed by the enzyme ATP suphurylase, which has been found in many higher plant species (Osslund, Chandler and Segel, 1982; Lunn *et al.*, 1990; Hatzfeld *et al.*, 2000; Herrmann *et al.*, 2014). APS is then phosphorylated by another ATP molecule to form adenosine 3'-phospho-5'-phosphosulphate (PAPS), which is catalysed by the enzyme APS kinase (Coughlan, 1977). Another inorganic S compound produced by the assimilatory reduction of sulphate by photosynthetic organisms is sulfide, which can be incorporated into an amino acid skeleton to form cysteine (Ngo and Shargool, 1974). Cysteine biosynthesis is driven by the sequential reaction of two enzymes: 1) serine acetyl transferase (SAT), which synthesizes the intermediary product O-acetylserine (OAS) from acetyl-CoA and serine; and 2) OAS (thiol) lyase (OASTL), which incorporates sulfide into OAS, producing cysteine, requiring pyridoxal-5'-phosphate as cofactor (Wirtz and Droux, 2005). Depending on their location in the cytosol, plastid, or mitochondrion, a variety of isoforms of SAT and OASTL exist in plants (Romero *et al.*, 2014).

Atmospheric S can enter plants via their stomata where it readily reacts with water to form sulphite, including HSO_3^- and SO_3^{2-} (Rennenberg, 1984). At low concentrations (e.g. < 0.25 ppm), plants are able to utilize SO_2, however, above a certain threshold (e.g. > 0.25 ppm), which differs between plant species, SO_2 toxicity often leads to visible negative effects such as chlorosis, necrosis and long-term yield reduction (Swain and Padhi, 2015). Therefore, plants have developed mechanisms to control sulphite levels by the action of various enzymes, including sulphite oxidase (SO), a key enzyme for protecting plants against SO_2, catalysing the reaction $SO_3^{2-} + H_2O \rightarrow 2H^+ + SO_4^{2-} + 2e^-$ (Brychkova *et al.*, 2007). Sulphite oxidase

belongs to the class of Mo cofactor (Moco) containing enzymes that catalyse electron redox reactions (Hille, 1996), and is essential for detoxifying excessive amounts of sulphite in the cell (Lang *et al.*, 2007).

2.2 Nutrient cycling in the rhizosphere

The rhizosphere is a realm where complex biological and ecological processes occur, encompassing the few millimetres of soil which surrounds the root (Curl and Truelove, 2012). An abundant population of archaea, bacteria, protists, fungi and animals live here alongside plant roots, the activities of each influencing those of the others across spatial and temporal scales (Cardon and Whitbeck, 2011). Rhizosphere soil effectively forms an interface layer between roots and the surrounding soil. As roots and soil act as both sources and sinks for a diverse range of compounds, this interface layer of soil mediates large fluxes of soluble and volatile compounds (Belnap, Hawkes and Firestone, 2003).

Rhizodeposition was first defined as all material lost from plant roots, including water soluble exudates, secretions of insoluble materials, lysates, dead fine roots, and gases such as CO_2 and ethylene (Lynch and Whipps, 1990). In a broad sense, the processes by which C enters the soil e.g. root cap and border cell loss, death and lysis of root cells (cortex, root hairs etc.), flow of C to root-associated symbionts living in the soil, gaseous losses, leakage of solutes from living cells (root exudates), and insoluble polymer secretion from living cells are collectively known as rhizodeposition (D. L. Jones, Nguyen and Finlay, 2009). Rhizodeposition is central to a diverse range of functions in plant nutrition and soil ecology (Cardon and Whitbeck, 2011). Some of these compounds are able to improve nutrient availability, e.g. phytosiderophores for Fe, organic acids for P and phenolics for micronutrients (Carvalhais *et al.*, 2011), while organic acids can relieve Al^{3+} rhizotoxicity (Heim *et al.*, 2001). Furthermore, apart from serving as an important C and energy source for rhizosphere soil

microorganisms, root exudates also act as allelochemicals, or as signalling substances for the establishment of symbiotic relationships between plant roots and microorganisms (Paterson, 2003). Yet despite the involvement in many soil ecological functions, due to the complex pathways and chain of reactions, our knowledge of the amount, composition and turnover of these root-derived compounds, especially those that influence S cycling is still very limited.

Although the quantities of organic compounds exuded from roots is not large (Kuzyakov and Domanski, 2000), they exert a very strong influence on soil microorganisms and may be significant in affecting plant nutrient availability (Rovira, 1969a; Bertin, Yang and Weston, 2003). As soon as a plant germinates, the root starts releasing root exudates (e.g. sugars, amino acids) into the rhizosphere, with the exact composition and quantity excreted dependent upon plant species, age, temperature, light intensity, plant nutritional status, microorganisms, soil bulk density, soil moisture, and root damage (Curl and Truelove, 2012). Root exudates are often divided into two classes: (1) low MW compounds such as amino acids, organic acids, sugars, phenolics, and other secondary metabolites, and (2) high MW compounds, such as mucilage (polysaccharides), peptides and proteins (Bais *et al.*, 2006).

2.2.1 Plant-microbe interactions in the rhizosphere

The rhizosphere microbial community contributes strongly to nutrient cycling, plant growth and root health as they (1) decompose SOM and release inorganic nutrients to the plant; (2) store nutrients in, and release nutrients from the microbial biomass; (3) affect nutrient availability by undertaking solubilisation, chelation, oxidation and reduction reactions; (4) affect plant growth by the release of stimulating or inhibiting substances. Interactions involving plants roots in the rhizosphere include root-root, root-insect and root-microbe interactions, which may be classified as positive, negative associations and neutral associations. Positive interactions include symbiotic associations with mycorrhizal fungi, and root colonization by

bacterial biocontrol agents and plant growth–promoting bacteria (PGPB). Negative interactions include competition or parasitism among plants, pathogenesis by bacteria or fungi, and invertebrate herbivory (Bais *et al.*, 2006). The factors that determine whether the chemical signature of a plant's root exudates will be perceived as a "negative" or a "positive" signal is only beginning to be understood. Due to the release of organic C and N from the root, microorganisms are generally more numerous than in the bulk soil, such that there is fierce competition for nutrients. Soil microorganisms in the rhizosphere can be roughly divided into two categories, those that compete with the plant root for N, P, S, and other nutrients; and others that provide plant inorganic nutrients by the decomposition of SOM.

2.2.2 Carbon input by plants into the rhizosphere

Attempts have been made to achieve total C balances for the soil-plant system (Kuzyakov and Domanski, 2000; Kuzyakov, 2006). Typically, labelling of photo-assimilates with $^{13}CO_2$ or $^{14}CO_2$ is used to trace C flow through the plant into the soil and further transformations in the plant-microbial-soil system monitored. From this, it has been estimated that the amount of C lost in root exudation ranges from 1 to 3% of a plant's net fixed C (Pinton, Varanini and Nannipieri, 2007). Previous estimates suggest that ca. 50% of rhizosphere respiration is due to the turnover of rhizodeposits and 50% to direct root (and mycorrhizal) respiration (Kuzyakov, 2002). Root and microbial respiration can alter the pH of the soil solution and can lead to the creation of anoxic and hypoxic hotpots in soil. This C flow can also flow beyond the rhizosphere by 2 main mechanisms: (1) investment of C in the production of intra- and extra-radical mycorrhizal structures which can extend many cm from the root (mycorrhizosphere; Leake, 2004); (2) release of volatile C that can travel very long distances in soil.

Root exudates can be classified into four groups depending on their mode of delivery: 1) water soluble exudates (e.g. sugars, amino acids, hormones, vitamins) which are lost by passive diffusion and whose loss rate strongly depends upon the concentration gradient between the root cytoplasm and soil solution and the permeability of the plasma membrane (Neumann and Römheld, 2007; Vranová *et al.*, 2010); 2) secretions, such as polymeric carbohydrates and enzymes which are actively excreted (Maseko and Dakora, 2013); 3) lysates, released when cells autolyse (Oburger and Jones, 2018); 4) gases such as ethylene and CO_2 (Jones, Hodge and Kuzyakov, 2004). These groups cover all stages of plant growth and development with the balance of these various processes changing with age of the plant, although water soluble exudates are thought to dominate rhizosphere C flow (Wen *et al.*, 2006).

2.2.3 Secondary productivity by soil microorganisms in the rhizosphere

Microbial growth in the rhizosphere is stimulated by the continual input of readily assimilable organic substrates from roots. In ecological terms, the substrate flowing from the roots is the product of photosynthesis and is thus primary productivity, and the utilization of these organic substrates by rhizosphere microorganisms results in secondary productivity. As the biggest reservoir of terrestrial C, SOM contains more organic C than global vegetation and the atmosphere combined (Lehmann and Kleber, 2015). Plant residues, including roots and rhizodeposits, quantitatively represent the largest inputs of C to soil (Schmidt *et al.*, 2011). In return, SOM retains nutrients, which improves plant growth and protects water quality. To date, SOM research has mainly focussed on the topsoil (0-30 cm) (Rumpel, Eusterhues and Kögel-Knabner, 2004; Song *et al.*, 2005; Hati *et al.*, 2007), yet an emphasis should also be given to deeper soil C to gain a better understanding of the key factors controlling soil C storage. Key processes regulating C dynamics are controlled by microorganisms at the microscale, but the related processes are often studied at larger scales.

2.2.4 DOC flows in rhizosphere

Dissolved organic matter (DOM) is a continuum of organic molecules of different sizes, charges and structures that pass through a 0.45 µm-pore size filter (Bolan *et al.*, 2011). DOM plays an important role in soil formation, mineral weathering and transport of pollutants, and the transport of nutrients from soil to surface waters. Dissolved organic C (DOC) is commonly used as a proxy for DOM (Wang *et al.*, 2012a). DOC is an important fraction of the C pool in soils, and an understanding of DOC dynamics requires a knowledge of its origin, function, and fate. Generally, DOC contains a wide range of molecules, ranging from simple amino acids through to complex high MW materials (Neff and Asner, 2001), which are released from vegetation and SOM. DOC concentrations can vary considerably both spatially and temporally with rates of production dependent on inputs from vegetation and SOM, consumption by soil organisms, sorption by mineral particles and water movement through the soil. As it is relatively mobile, DOC plays an important role in the short- and long-distance transport of N, P and S.

2.2.5 DON flows in the rhizosphere

Dissolved organic N (DON) refers to a diverse array of compounds which can be operationally grouped into high (>1 kDa) and low (<1 kDa) MW components. It is now clear that when soil N mineralization is slow and concentrations of inorganic N are low, plants can take up DON directly from soil (e.g. amino acids, peptides) and that this may contribute significantly to the plant's N demand (Kielland, 1994; Persson and Näsholm, 2001b, 2001a). Therefore, an understanding of DON fluxes between rhizosphere microorganisms and plant roots is critical to quantifying the significance of DON in terrestrial ecosystems (Christou *et al.*, 2005).

DON flow at the soil-root interface is bidirectional with N being lost from roots by root exudation at the same time that DON is also taken up from the soil (Ford *et al.*, 2007; D. L. Jones, Nguyen and Finlay, 2009). Clearly, it is the net balance between these two processes that determines the amount of organic N captured by the root from the soil. The magnitude of N flow into the rhizosphere is dependent upon a wide range of biotic and abiotic factors including plant species, plant ecotype/cultivar, age and environmental conditions (D. L. Jones, Nguyen and Finlay, 2009). As free amino acids and proteins represent only a minor component of root exudates (typically 1–2%), it has been assumed that they contribute little to plant N rhizodeposition (D. L. Jones, Nguyen and Finlay, 2009). Although the size of the DON pool is important, it is the flux rate (pool replenishment rate), however, that is more critical in understanding the importance of DON to plant nutrition (Christou, Avramides and Jones, 2006; Rousk and Jones, 2010). Typically, low MW DON turns over rapidly in soil, persisting from minutes to hours. This has led to the hypothesis that the bottleneck in soil organic N cycling lies in the breakdown of higher MM DON to smaller units that can be directly assimilated by the microbial biomass (Lucy M. Greenfield *et al.*, 2020). Once released into the soil DON undergoes a number of fates including movement away from the root due to diffusion and mass flow, capture by soil microorganisms, and sorption to the soil solid (Sacchi *et al.*, 2000).

2.2.6 DOS flows in the rhizosphere

Dissolved organic S (DOS) makes up a significant part of the S cycle in both terrestrial and aquatic environments (Wang *et al.*, 2012a). Whilst numerous studies have focused on DOC, DON and DOP in soil and freshwater, our knowledge of DOS remains poor, particularly in a rhizosphere context (Marschner, 1992; Wang et al., 2012). Mineralization of DOS in the rhizosphere can be divided into biological and biochemical processes (McGill and Cole, 1981). C-bonded S is thought to be mineralized by biological processes with sulphate released as a

by-product during the oxidation of the C skeleton to CO_2 (Kertesz and Mirleau, 2004b). Although the evidence base is weak, more ester bonded S was found in non-rhizosphere (bulk) soil than in the rhizosphere (Hu *et al.*, 2002), while no significant differences existed between C-bonded S in the rhizosphere and in the bulk soil, indicating that C-bonded S may be more important for plant S nutrition (Hu *et al.*, 2003).

2.3 The role of sulphur for plant nutrition

2.3.1 Sulphur nutrition and sulphur fertilizers

Although required in smaller quantities than other macro elements, S is hugely important in plant nutrition, yet it has received comparatively less attention. This is due to the cheap availability of S fertilizers and atmospheric S inputs which have replenished soil S reserves. During the 25 years, however, S deficiency has become more widespread in the UK and other European countries (Murphy and Boggan, 1988; McGrath, Zhao and Blake-Kalff, 2003; Kühn-institut, 2019b) as a result of strict controls on industrial emissions (Campbell and Smith, 1996). In addition, changes away from S fertilisers (e.g. ammonium sulphate) to low-S or even S-free fertilisers has also led to decreased inputs of S to soils (Ceccotti, Morris and Messick, 1998).

S is a constituent of three essential amino acids namely cysteine, cystine and methionine (Schnug, Haneklaus and Murphy, 1993) which are fundamental to protein/enzyme synthesis. Insufficient S supply therefore affects both the quality and the quantity of plants (Schnug, 1990; Filipek-Mazur *et al.*, 2019) as it prevents protein synthesis and the production of many secondary metabolites (Ahmad *et al.*, 2007). For example, S deficiency reduces nutritional quality due to a re-direction of protein synthesis to low S vicilin proteins at the expense of legumin, which is rich in S-containing amino acids, cysteine and methionine (Byers and Bolton, 1979; Millerd, Thomson and Randall, 1979). The baking quality of wheat is thus

impaired by S deficiency because the S-containing amino acids in the gluten fraction of flour are responsible for the elasticity of the dough and the bread volume (Wrigley *et al.*, 1984). S deficiency can cause phenotypic symptoms that become first visible at chlorosis in leaves, because S is required in the photosynthetic processes. The increasing recognition of the importance of S for plant growth and crop yield, as well as nutritional importance of S for human and animal diets, has led to an increased emphasis on the understanding of S metabolism in plants.

Studies have shown that S fertilization increases N incorporation into protein synthesis, thereby enhancing photosynthetic rates (Ahmad and Abdin, 2000), indicating that S can directly affect the use efficiency of other nutrients. This could be explained by the law of minimum, that balanced supply of all nutrients is the prerequisite of correct crop nutrition (Jungk, 2009). In addition, S can improve the availability of Ca, Mg, P, Cu, Mn and Zn, as acidity produced on oxidation of reduced inorganic S in soil can promote their solubility (Karimizarchi *et al.*, 2014). Leaves can also take up volatile S from the atmosphere (e.g. SO_2 and H_2S) via the stomata and incorporate this S into S organic compounds (Rennenberg and Polle, 1994). In addition, leaves can also emit S in the form of H_2S when leaves or roots are exposed to relatively high concentrations of sulphate (Rennenberg *et al.*, 1990).

S enters soils in both inorganic and organic fertiliser. Inorganic S fertilizers mainly comprise sulphates and elemental S. Sulphate fertilizers (e.g. gypsum, ammonium sulphate) are highly soluble and provide an immediate source of S to the plants, whereas elemental S contains very high concentrations of S (70-100%) and is poorly soluble. Elemental S fertilisers have the potential advantage of offering a continual release of S during the growing season with minimal leaching losses (Boswell and Friesen, 1993; Riley, Zhao and Mcgrath, 2002).

In ecosystems with low mineral fertilizer inputs, organic wastes (e.g. manures, compost, slurry) can be used to supply S to the growing crops. These often contain a more

balanced supply of essential nutrients. However, these S-containing fertilizers often have a low efficiency as S can be released slowly and immobilised in the microbial biomass (Eriksen, 2001). Lloyd (1994) showed that the average annual grass dry matter yield from a cattle slurry application was equivalent to 55% of that when the equivalent quantity of S was applied as gypsum. In less economically developed countries, S is largely delivered to crops in manure collected from cattle and pigs rather than mineral fertilisers (Erikseil and Mortensen, 1999).

Previous studies have shown large differences in S availability between crop residues. For instance, cruciferous crops, such as rape, can provide a large and rapid release of available S, while cereal residues release only minimal amounts of available S (Scherer, 2001). In addition, the release and the subsequent immobilization of S largely depends on the C-to-S ratio of the added organic material (S. J. Chapman, 1997). Previous research has shown that the incorporation of cattle manure compost with a low C-to-S ratio can provide a large amount of plant-available S, while sawdust compost with a high C-to-S ratio results in severe S deficiency, indicating the release and subsequent immobilization of S (Chowdhury *et al.*, 2000).

2.3.2 Uptake and allocation of sulphate by plants

Growing plants have a high demand for S thus inadequate acquisition of S can limit plant growth (Kopriva *et al.*, 2016). The increasing recognition of the importance of S for plant growth (Zhao *et al.*, 1997; Zhao, Hawkesford and McGrath, 1999; Scherer, 2001; Aulakh, 2003; Walker and Booth, 2003; Kühn-institut, 2019a), as well as the nutritional importance of S for human and animal diets (Virtanen, 1962; Donoso-sin, 1963; Kandylis, 1984; Grimble, 2006; Nimni, Han and Cordoba, 2007), has led to an increased emphasis on research on sulphate uptake, transport and assimilation in plants (Coughlan, 1977; Smith, Rae and Hawkesford, 2000; Takahashi *et al.*, 2000; Hawkesford *et al.*, 2003; Buchner, Takahashi and

Hawkesford, 2004). The S requirement of a crop for optimal growth varies between 0.1 and 0.5% of the dry weight of plants. In general, to produce 1 tonne of grain under S-sufficient conditions, winter wheat needs 2-3 kg S (Harwood and Nicholls, 1979), but it should be noted that this is plant cultivar dependent (Vong, Nguyen and Guckert, 2007).

Sulphate uptake across the root plasma membrane is the first step involved in the synthesis of organic-S compounds in plants. This uptake by the root epidermis and cortical cells, as well as sulphate movements to other tissues and organs, requires high affinity transporters. Studies have shown that sulphate uptake is mediated by a combination of saturable high-affinity transporters and non-saturable low-affinity transporters (Epstein, 1966; Yoshimoto et al., 2002). High affinity sulphate transporters are highly regulated by changes in S status of plants, encoded by a group 1 sulphate-transporter clade, while low affinity transporters play an important role in internal translocation of sulphate in plants, and all belong to a group 2 clade (Takahashi *et al.*, 1996, 2000; Gent *et al.*, 1997; Smith *et al.*, 1997). After root uptake, subcellular and long-distance transport can occur (i.e., root to shoot). Sulphate is subsequently delivered to plastids, where assimilatory reductive pathway of sulphate takes place, as well as into storage vacuoles, where the internal nutritional reservoir plays a crucial role in maintaining cytosolic and plastidic sulphate homeostasis (Leustek, 2002).

Sulphate is activated, reduced to sulphide and incorporated into carbohydrate skeletons by assimilatory sulphate reduction before it can be used in protein synthesis in plants (Brunold, 1990). The final product of assimilatory sulphate reduction in plants is cysteine. From this amino acid, all other reduced S compounds including methionine (Rennenberg *et al.*, 1990), glutathione (Strohm *et al.*, 1995), and phytochelatins (Rauser, 1995) are synthesized in a connected set of metabolic pathways. In some cases, these metabolic transformations occur in leaves with newly synthesized organic-S compounds retuned to the root via the phloem. This is of vital importance in plant nutrition, as some plant species have a very low capacity for

reducing N and S in their roots, and thus depend on the translocation of nutrients for synthesis of protein.

The traditional view is that only mineral S (i.e. SO_4^{2-}) can be utilized by plants (Haynes, 1986; Rennenberg *et al.*, 1990). However, observations that plants can take up organic N in the form of amino acids and peptides suggests that thus could also be a potential route of organic-S uptake (Kielland, 1994; Smith and Steenkamp, 1992; Falkengren-Grerup et al., 2000; Näsholm et al., 1998; Nordin et al., 2001; Owen and Jones, 2001; Raab et al., 1996; Schimel and Chapin, 1996). By analogy to N, this uptake of organic S by roots may be particularly important when inorganic S becomes limiting in soil.

2.3.3 Sulphur metabolites in plant metabolism

Cysteine and methionine are the major end-products of sulphate assimilation in plants, comprising up to 90% of the total S in most plants, predominantly bound in protein moieties (99% or more) (Giovanelli, Mudd and Datko, 1980; Rennenberg *et al.*, 1990). S, however, can be found in many co-enzymes and prosthetic groups and various other secondary metabolites. These secondary metabolites are important for plant fitness and their ability to cope with stress. One key example is glutathione which is a tripeptide of glycine, glutamate, cysteine and a major thiol in plants. It functions as a transient storage pool of reduced S, and is a powerful antioxidant being involved in defence against oxidative stress, detoxification of heavy metals or xenobiotics and also in biotic interactions (Mullineaux and Rausch, 2005; Rausch and Wachter, 2005). Another important secondary metabolite group are phytochelatins which consist of repetitive glutamyl-cysteine units (between 2 and more than 10) with a terminal glycine (Inouhe, 2005). They bind heavy metal cations (e.g. Zn^{2+}) via thiol coordination making them non-toxic (Salt and Rauser, 1995; Yadav, 2010). Thioredoxins are low-MW proteins of about 12 kDa with two well-conserved cysteine residues which form a redox-active,

intermolecular disulphide bridge, constituting another important family of thiols in higher plants (Attiwill *et al.*, 1993; Vieira Dos Santos and Rey, 2006). S also plays a vital role in chlorophyll formation (Rahul *et al.*, 2018). When S is deficient, plants are often characterized by yellowing of leaves and generally show symptoms that resemble those of N starvation. In addition, alliins and glucosinolates are also two important secondary metabolites. Alliins (S-allyl-l-cysteine sulfoxide) are abundant in the *Allium* family of plants and are related with defence response reactions. Glucosinolates, the best known example of preformed defence compounds, are stored in vacuoles and their hydrolysis is catalysed by the cytosolic enzyme myrosinase, and are important for plant interactions with herbivores and pathogens (Wittstock and Halkier, 2002; Martínez-Ballesta, Moreno and Carvajal, 2013; Variyar *et al.*, 2014).

2.4 Sulphur analysis methods, including isotopic methods

2.4.1 Extraction of sulphate and organic sulphur in soils

Historically, one of the main reasons why so little work has been done on organic-S, relative to other plant nutrients, is the difficulty of measuring it in plants and soils. In contrast to organic-S, the development of ion chromatography (IC) (Brown and Morra, 1991) and inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Munter and Grande, 1981) has spurred much research on inorganic S. IC with a conductivity detector is suitable technique for rapid determination of ions including sulphate (Raue, Brauch and Frimmel, 1991; Zhao and McGrath, 1994; Turrión, Gallardo and González, 1999; Meneses, Maniasso and Zagatto, 2005). ICP-AES has proved to be an accurate and rapid method for measurement of total dissolved S (TDS) for soil and plant materials (Novozamsky *et al.*, 1986; Perrott *et al.*, 1991), allowing a simultaneous measurement of SO_4^{2-}-S and dissolved organic S by difference with TDS. However, ICP-AES methods fail to identify the exact species of S, therefore near edge X-ray absorption fine structure spectroscopy (NEXAFS), also known as X-ray absorption

near-edge spectroscopy (XANES), the practice of determining oxidation states involves using a set of conventional rules, has been used to identify and quantify multiple organic S composition in coal (Spiro *et al.*, 1984; Kasrai *et al.*, 1990, 1996), minerals (Marusak and Tongson, 1979; Sugiura, 1981; Kubono *et al.*, 2017), biological samples (Frank *et al.*, 1987, 1994; Shadle *et al.*, 1993; Pickering *et al.*, 1998), and humic substances isolated from marine sediments (Vairavamurthy *et al.*, 1994, 1997; Xia *et al.*, 1998), and soils (Jokic *et al.*, 2003; Solomon, Lehmann and Martínez, 2003; Solomon *et al.*, 2005; Zhao *et al.*, 2006; Prietzel *et al.*, 2013).

2.4.2 Determination of volatile sulphur compounds

Research on S cycling in soil was hindered by the lack of sensitive methods for quantifying the flux of volatile organosulfur compounds (VOS) produced by soil microorganisms. However, the advancement of GC-MS and VOS capture technologies now allows us to identify and trace VOS compounds. VOS compounds play important roles in the S cycle, owing to their transfer from solution to the gas phase and *vice versa*. In particular, low MW VOS compounds, such as dimethylsulfide (DMS), dimethyldisulfide (DMDS), methanethiol (MT), carbon disulphide (CS_2) and carbonyl sulfide (COS) are recognised as important source of S to the atmosphere (Kiene, 1996). The release of VOS compounds primarily occurs from soils treated with plant materials (Bending and Lincoln, 1999) or from the breakdown of S-containing amino acids such as methionine and cysteine (W. L. Banwart and Bremner, 1976). Waterlogging appears to be one of the most important factors affecting VOS emissions from soils, with increased VOS emissions following a decrease in oxygen availability (Chapman *et al.*, 1996). Temperature (Staubes et al., 1989) as well as the soil organic S content (Bending and Lincoln, 1999) has also been shown to be important in regulating VOS emissions from soils.

In a water saturated soil, aerobic microorganisms quickly consume all the available oxygen, and anaerobic microorganisms begin to use Fe^{3+}, Mn^{4+} and SO_4^{2-} etc. as terminal electron acceptors to obtain energy for growth (Lovley, Holmes and Nevin, 1991). In wetland soils and freshwater environments with appreciable SO_4^{2-}, microbial reduction of sulphate to S- or H_2S occurs (Pester *et al.*, 2012). Several species of bacteria, such as sulphate reducers of the genera *Desulphovibrio*, *Desulfobacter*, *Desulfococcus*, are predominantly responsible for the volatilization of reduced S gases in waterlogged soils (Brychkova *et al.*, 2007). These bacteria use SO_4^{2-} as an oxidizing agent and produce large quantities of H_2S as one of their main metabolic products. Sulphate can also be reduced to H_2S under well-aerated conditions by the aerobic *Bacillus megaterium* in partially sterilized soil (Brychkova *et al.*, 2007).

2.4.3 Determination of microbial biomass sulphur

The soil microbial biomass acts as the driving force behind mineralization–immobilization, and oxidation–reduction transformations and is therefore central to S cycling (Banerjee and Chapman, 1996). Methods have been developed for estimating microbial biomass-S (Strick and Nakas, 1984; Wu *et al.*, 1994; Banerjee and Chapman, 1996), which accounts for < 3% of organic S in soil (Stevenson and Cole, 1999). The most commonly used method to measure microbial biomass-S is the $CHCl_3$ fumigation-extraction method, in a way analogous to the measurement of microbial biomass-C.

This method involves lysing the microbial cells with $CHCl_3$, evacuating the $CHCl_3$, and then directly extracting the S from both fumigated and non-fumigated soils with 0.01 M $CaCl_2$. Microbial biomass-S is calculated as F/K_s, where F is the total amount of S released from fumigated soil minus that released from non-fumigated soil, and K_s=0.35 represents the efficiency of extraction of microbial biomass-S (Voroney, R.P.; Brookes, P.C.; Beyaert, 2008).

2.4.4 Determination of transformation rates of sulphur in soils

The rates at which nutrients are released to, and removed from, the mineral nutrient pool is an important determinant of nutrients supply to plants. These transformation rates therefore need to be considered when developing nutrient management strategies for sustainable production. Typically, net S mineralisation has traditionally been studied using a closed incubation system which measure net changes in sulphate concentration with time. However, such determinations have contributed very little to the understanding of processes involved in S transformations within the soil system. More recently, by measuring sulphatase activity (A. N. Ganeshamurthy and Nielsen, 1990), gross S mineralization rates can be estimated, yet this only accounts for some of the enzymes responsible for S mineralization. Alternatively, periodic leaching of soils allows the determination of S mineralization rates (D. G. Maynard, Stewart and Bettany, 1983; Ghani, McLaren and Swift, 1991, 1992; Valeur and Nilsson, 1993; Zhou *et al.*, 1999; J., K. and R., 2003), but this method alters soil conditions leading to an overestimation of gross mineralization.

The isotopic dilution technique is also used for determining gross nutrient transformation rates in soil. Isotopic dilution was first described as a method for analysing the lead content of rocks (Lockwood, 1954). Yet it was not until eight years later that this technique was shown to have a great analytical utility and applied for the analysis of complex mixtures of organic compounds (Rittenberg and Foster, 1940). These authors recognized the potential of this method for determining the relative D or L-isomeric content of isolated amino acids. This technique involves labelling a soil mineral nutrient pool, e.g. NH_4^+, NO_3^-, (NISHIO, 1991; Delaune *et al.*, 1998), PO_4^{3-} (Walbridge and Vitousek, 1987; Wanek *et al.*, 2019), or SO_4^{2-} (Di, Cameron and McLaren, 2000; Nziguheba, Smolders and Merckx, 2005), and monitoring the changes of the size of the labelled nutrient pool and the abundance of the rarer isotopes (atom%, if stable isotope is used) or specific activity (if radioisotope is added).

The element S has four naturally occurring stable isotopes. ^{32}S, ^{33}S, ^{34}S and ^{35}S, with approximate abundances of 95.02%, 0.75%, 4.21% and 0.02%, respectively (Day and Moynier, 2014). The only radioactive isotope of S which is suitable for biochemical investigations is ^{35}S. Therefore, ^{35}S has been used to trace S processes in soil, including microbial mineralization, immobilisation, reduction, sorption, leaching and plant uptake (Di, Cameron and McLaren, 2000). By tracing the flow of added ^{35}S from one S fraction to the other, the dynamics of S mineralization and immobilization within soil can be examined. When fertilizer ^{35}S-Na_2SO_4 was added to soil, studies showed that it is readily converted to organic forms of both hydriodic acid reducible and carbon-bonded S (Freney, Melville and Williams, 1971; Schindler *et al.*, 1986). Double labelled fertilizer such as $(^{15}NH_4)_2{}^{35}SO_4$ as well as triple-labelled (^{13}C, ^{15}N, ^{34}S) manure (Andriuzzi and Schmidt, 2014) have been synthesized, yet their experimental application have not been reported.

2.4.5 Quantification of S uptake by plant roots

Current techniques to quantify the S uptake by the roots, include depletion methods (in which uptake rates are calculated from changes in solution concentrations over time), and tracer methods (in which the uptake of tracer into root or shoot tissue is measured) (Lucash *et al.*, 2007). These techniques provide valuable information about the uptake rates, but they are also subject to several methodological problems. First, uptake rates can be determined by analysing tracer accumulation in the roots using the tracer method (Larsson *et al.*, 1991). The tracer method is cost-effective and sensitive and it also offers the advantage of tracking the radioisotope in the shoots by autoradiography, thereby enabling determination of translocation rates of tracer through the plant (Rubio, Sorgona and Lynch, 2004). For the tracer method, both excised and intact roots are used. Of these, the use of excised roots has been used extensively since the 1960s to measure inorganic (Sheat, Fletcher and Street, 1959; Huang *et al.*, 1992) and

organic (McFarlane and Wickliff, 1985; Su and Zhu, 2007) nutrient uptake by roots. However, some scientists have argued that damage to root tissues caused by excision prior to experiments, may often artificially increase the nutrient loss from roots and thereby reduce net uptake, providing less realistic estimates of root uptake than intact root systems (Bloom and Caldwell, 1988).

Second, the depletion method of measuring the concentration of nutrients in solution before and after the experiment, can be used to determine the amount of nutrient taken up by the roots. The depletion method offers a possible improvement over the excised root method, as that the roots are still attached to the plant and can continue nutrient transportation throughout the experiment. Another advantage of this method is that isotopes are not required, so the approach is more affordable, and fewer handling restrictions are involved. The main limitation of the depletion method is that the difference must be detectable and, thus, requires either low starting amino acid concentrations or long uptake periods.

2.5 Summary

The five key findings of this literature review are:

1. Most S held in soils is present in organic matter and the microbial biomass.
2. Many groups of soil microorganisms are involved in the conversion of organic S to SO_4^{2-} .
3. Our understanding of whether plants can take up organic-S from soils is still very limited.
4. The rates of conversion of low MW organic-S to SO_4^{2-} involves many enzymatic pathways but remains poorly understood.
5. While we know what the major pools of S are in the agricultural S cycle, we still know little about rates of flux between these pools and their co-dependence on C and N cycling.

Hence, the main aims of this thesis are to i) study the release and subsequent availability of C, N and S from cysteine and methionine following addition into soil and the factors that control this mineralization process (e.g., nutrient addition, substrate concentrations and soil properties); and ii) compare the uptake and competition of organic and inorganic S nutrients by plant and soil microbes in a rhizosphere context.

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Chapter 3

Dissolved organic and inorganic sulphate in soils along a grassland productivity gradient

Abstract

Nitrogen (N), phosphorus (P) and sulphur (S) predominate in most soils in solid organic forms. However, prior to plant and microbial uptake these must first be converted to dissolved organic or inorganic forms. Although the role of some dissolved organic nutrients in plant nutrition is well established (e.g. N), the role of dissolved organic S (DOS) in plant nutrition remains unknown. However, protein breakdown is predicted to lead to the release of large quantities of DOS compounds into soil solution (e.g. S-containing amino acids and oligopeptides). We hypothesize that, similar to N, the soil solution pool will contain significant quantities of DOS and that the DOS-to-sulphate ratio will be inversely corelated to ecosystem productivity. The aim of this study was therefore to compare the relative concentrations of DOS with inorganic sulphate (SO_4^{2-}) in a contrasting range of grassland soils. The top- (0 – 10 cm) and sub- (10 – 20 cm) soil were collected from 7 grassland pastures along a natural primary productivity gradient. The relative amounts of DOS and SO_4^{2-} present in water extracts from each soil were then evaluated. Our results showed that the concentration of DOS in all soils ranged from 10.6 to 270.3 mg kg^{-1} for topsoils, while it ranged from 41.2 to 309.3 mg kg^{-1} for subsoils. Higher concentrations of DOS, along with higher concentrations of SO_4^{2-}, were found in high altitude soils. The DOS pool constituted 23.8 to 94.8% of total dissolved sulphur (DOS plus SO_4^{2-}, TDS) across the range of soils. Statistical analysis showed that DOS concentration co-correlated with the concentrations of Al, B, Fe, K, Mn, ammonium in the soil extracts as well as DOC. This study shows that DOS is an important contributor of dissolved S in grassland soils, and therefore is important in S cycling and transport in soils. Further investigation is needed to evaluate the relative importance of DOS to plant S acquisition in comparison to inorganic forms of S.

Keywords: Dissolved organic sulphur; Sulphate; Grassland; Productivity gradient; Spatial Variation.

3.1 Introduction

Dissolved organic nutrients are increasing being recognised as a major nutrient loss pathway in some ecosystems as well as being a direct source of nutrients to plants and microorganisms. This pool is often collectively known as dissolved organic matter (DOM) which is operationally defined as the continuum of organic molecules of different sizes, charges and structures that can pass through a 0.45 µm-pore filter (He *et al.*, 2016). DOM also plays a major role in freshwater ecosystems with most of the DOM entering freshwaters originating from soil. DOM is therefore receiving increasing attention due to its key role in nutrient cycling and its ability to transfer from soils to freshwater and marine ecosystems (Stedmon, Markager and Bro, 2003; Zsolnay, 2003; Amon, Benner and Saunders, 2013). Most of the studies to date on soluble organic nutrients have focused on N (Jones *et al.*, 2004; Bronk, Gilbert and Ward, 2007; Neff, III and Vitousek, 2007), with a smaller number on phosphorus (P) (Rowland and Haygarth, 1997; McDowell and Koopmans, 2006). Although sulphur (S) is the second most abundant macronutrient in DOM, information on dissolved organic sulphur (DOS) is lacking. This is surprising considering that, like N and P, organic forms of S represent the dominant form of S in soils. We therefore hypothesize that DOS must play an important role in the soil S cycle.

Sulphur occurs as reduced (e.g. sulfide and thiol) or as oxidized species (e.g. sulfonate and sulfate) in DOM. Incorporation into organic matter is an important pathway to retain S derived from inorganic S fertiliser application or atmospheric deposition. A few studies indicate that DOS constitutes a significant part of the total flux of S from terrestrial to aquatic ecosystems (Wieder and Lang, 1988; Kaiser and Guggenberger, 2005; Goller *et al.*, 2006). However, the DOS content of soil varies considerably with season and space (Peuravuori *et al.*, 2005; Wang *et al.*, 2012a). Therefore, a fundamental mechanistic understanding of the factors governing the spatial and temporal variation of DOS in the landscape will greatly

enhance our ability to develop more sustainable agricultural ecosystems as well as better understanding the different S loss pathways.

In the context of the discussion above, the first aim of this study was to quantify the spatial variations in DOS and SO_4^{2-} concentration in a range of grassland soils. The second aim was to identify parameters governing any patterns of spatial variation in soil S concentration. We approached this by analysing the chemical composition of DOS as well as SO_4^{2-} extracted from both topsoil and subsoil across a grassland productivity gradient.

3.2 Material and Methods

3.2.1 Site description and soil sampling

Soil was collected from seven sites located along a gradient of grassland productivity, altitude, and soil DON availability in Abergwyngregyn, Gwynedd, North Wales (Table 3.1; 53°14'N, 4°01'W; May 2019). The seven selected grassland sites represent a gradient from high productivity, inorganic N dominated grassland to low productivity, high organic matter and organic N dominated grassland. Most of these soils are assumed to be low in absorbed SO_4^{2-} and receive relatively little atmospheric sulphur (S) deposition. Although our study is based on a single elevation gradient (0-400 m a.s.l.), it was selected to include a broad range of vegetation, soil, and climatic conditions. Soil characteristics for all seven sites are presented in Table 3.1.

At each site, three independent samples from randomly positioned replicate plots (5 × 5 m^2; representing 3 replicates) were collected separately from the topsoil (0 - 10 cm) and subsoil (10 - 20 cm). Soil samples were placed in gas-permeable plastic bags and transported immediately to the laboratory, where the soil was gently sieved to < 2 mm for homogenization and to remove roots and big stones. The soil samples were then stored at 4 °C prior to further analysis.

3.2.2 Soil chemical characteristics

Soil bulk density (100 cm^3 cores) and moisture content were determined by oven drying soil at 80 °C. Soil pH and electrical conductivity analysis was performed on a 1:2.5 w/v soil: distilled water suspension using standard electrodes. Soil microbial biomass C and N were determined using the chloroform fumigation-extraction (FE) method (Voroney, Brookes and Beyaert, 2007). For each soil sample, 5 g of field-moist soil was extracted using distilled water (ratio 1:5 w/v). A further 5 g of soil was fumigated in a $CHCl_3$ atmosphere for 48 h and then extracted in the same way. The soil and distilled water mixtures were shaken on an end-over-end shaker for 30 mins at 200 rpm (20 ± 1°C), centrifuged at 3000 rpm for 10 mins and then filtered through a Whatman No. 42 filter paper. The soil solution recovered was analysed for dissolved organic C (DOC) and total dissolved N (TDN) using a TOC/TN analyser (Shimadzu Corp., Kyoto, Japan). Microbial biomass C and N were calculated from the concentration difference from between directly extracted soil and fumigated soil samples, by using K_{ec} and K_{en} factors of 0.35 (Wu *et al.*, 1990) and 0.5, respectively. Field-moist soil (5 g) was shaken for 30 min with 0.5 M K_2SO_4 (1:5 w/v ratio) for analysis of NH_4^+ and NO_3^- with subsequent analysis of the extracts using a SynergyMX microtitre plate reader (Mulvaney, 1996; Miranda, Espey and Wink, 2001). Dissolved organic nitrogen (DON) was calculated as the difference between TDN and dissolved inorganic N (NH_4^+ plus NO_3^-).

Additionally, SO_4^{2-} and other major anions were determined in soil: distilled water extracts (1:5 w/v; filtered through 0.45 µm syringe filters) by ion chromatography (IC; Bradfield and Cooke, 1985). Distilled water was adopted as extract in our study because other strong extracts such as KCL, $NaHCO_3$ etc, may cause large background anion concentration, leading to inaccurate readings of sulphate concentrations by IC. Total dissolved sulphur (DOS plus SO_4^{2-}) and other major cations were determined in 1:5 (w/v) soil: 0.01 M $Ca(H_2PO_4)_2$ extracts using inductively coupled plasma-optical emission spectroscopy (ICP-OES; Anderson

et al., 1992). Since ICP-AES measures total dissolved S, while IC measures only SO_4-S, the difference between the two methods can be taken as extractable organic S (David and Mitchell, 1985; Mitchell *et al.*, 1986). All chemical analyses were made in triplicate and the results expressed on an oven-dry soil weight basis.

3.2.3 Sorption of ^{35}S-Cys, Met and Na_2SO_4 in sterile grassland soil

To determine the sorption of Cys, Met and Na_2SO_4 to grassland soil's solid phase, soil samples were heat sterilised to prevent microbial activity. Briefly, 1 g of soil (top soil from site 1) was placed in pre labelled 15 ml polypropylene tubes and these tubes were capped to prevent soil water loss (six replicates per treatment). Immediately before the sorption experiment, to kill soil micro-organisms, centrifuge tubes containing soils were either heated for 80 °C for 60 minutes, or autoclaved at 121 °C for 60 minutes. Once soil is heat sterilised, 5 ml of either uniformly ^{35}S labelled Cys (specific activity = 0.1 kBq ml^{-1}), Met (specific activity = 0.3 kBq ml^{-1}) or Na_2SO_4 (specific activity = 0.4 kBq ml^{-1}) solution were added to the soil to give five separate concentrations ranging from 0.01 to 100 mM (0.01 mM, 0.1 mM, 1 mM, 10 mM and 100 mM).

The soil samples containing sorption solutions were then shaken at 200 rpm on a flat-shaking bed for 5 minutes at room temperature (21 ± 1°C). Immediately after shaking, 1.5 ml supernatant solution was transferred to a 1.7 ml microfuge tube and centrifuged for 5 minutes at 14000 *g*. 1 ml supernatant was then removed from the microfuge tubes, and the amount of ^{35}S in the supernatant solution was determined by liquid scintillation counting using a Wallac 1404 scintillation counter with automated quench correction (Wallac EG&G, Milton Keynes, UK). The quantity of ^{35}S adsorbed was calculated as the difference between ^{35}S added initially and equilibrium ^{35}S concentration in the solution.

3.2.4 Statistics and data analysis

All data analyses were carried out using SPSS 25.0 (IBM UK Ltd, Portsmouth, UK). Simple correlation analysis and multiple linear regression were used to empirically identify parameters influencing the DOS concentrations from soil solution. These parameters included pH, DOC, DON, NH_4^+, major cations (Mn, Fe, Zn, Na, Al etc.) in soil water. The statistical methods employed were simple Bivariate correlation analysis, stepwise multiple linear regression. All values are presented as mean ± the standard error of the mean (SEM) (n = 3).

3.3 Results and Discussion

3.3.1 DOS and SO_4^{2-} concentration in soils along the grassland productivity gradient

The field site locations and soil property descriptions are reported in table 3.1 and 3.2. All soil samples contained water-extractable sulphate which ranged in amount from 9.7 to 139.5 mg kg^{-1} DW soil (Table 3.1). Higher concentrations of SO_4^{2-} in soil were found at high altitude, low productivity sites (site 4, 5, 6 and 7). We ascribe this to lower rates of S removal by plant uptake at higher altitudes. At all seven sites, the concentrations of SO_4^{2-} in topsoil were higher than that from subsoils (topsoil vs. subsoil; $p < 0.05$ for sites 3, 4, 5 and 6). However, subsoils may contain large amounts of adsorbed sulphate (Schoenau and Malhi, 2008; Anderson, 2020), which may not have been extracted in our study, therefore the contribution of subsoils to plant S uptake can't be ignored.

Sulphate concentrations were consistently smaller than the total extractable S as measured by ICP-AES. The concentrations of total dissolved sulphur (DOS plus sulphate) in most soil samples were found to be less than 400 mg S kg^{-1} soil DW. In general, higher concentrations of TDS were detected from subsoils. DOS concentration accounted for 23.9 to 74.8% of TDS in topsoils, while accounting for between 58.5 to 94.8% in subsoils, this is in line with other studies on soil sulphur fractions (Tabatabai, 2015).

Table 3.1 General site and soil properties (samples collected in late May 2019). Values represent means ± SEM (n = 3) for either topsoil (top) or subsoil (sub). Data is expressed on a dry soil weight basis.

	Site 1 - top	Site 2 - top	Site 3 - top	Site 4 - top	Site 5 - top	Site 6- top	Site 7 - top
Altitude (m)	23	87	134	209	277	333	404
NO_3^--N (mg kg^{-1} soil DW)	8.1 ± 0.2	7.6 ± 1.5	11.5 ± 0.2	16.6 ± 1.3	23.4 ± 0.5	33.9 ± 4.7	21.6 ± 2.3
NH_4^+-N (mg kg^{-1} soil DW)	2.7 ± 0.6	15.3 ± 2.1	5.0 ± 0.3	12.0 ± 2.4	10.6 ± 1.0	20.8 ± 3.5	64.5 ± 0.7
Organic matter (mg kg^{-1} soil DW)	7.3 ± 0.1	12.4 ± 7.4	18.7 ± 2.5	22.4 ± 0.4	20.4 ± 0.2	23.8 ± 0.5	85.9 ± 0.8
pH	6.0 ± 0.04	5.1 ± 0.02	4.6 ± 0.03	4.8 ± 0.04	5.1 ± 0.04	4.5 ± 0.12	4.1 ± 0.05
Water content (%)	19.7 ± 0.2	24.3 ± 12.5	25.3 ± 0.6	34.6 ± 0.2	38.5 ± 0.2	26.3 ± 0.6	65.2 ± 0.3
Sulphate-S (mg S kg^{-1} soil DW)	32.8 ± 2.7	65.8 ± 30.7	60.3 ± 13.2	46.9 ± 16.5	83.4 ± 15.7	84.9 ± 5.7	137.9 ± 16.5
DOS (mg kg^{-1} soil DW)	10.6 ± 3.7	186.0 ± 21.8	232.8 ± 66.7	196.3 ± 17.8	65.5 ±22.7	79.3 ± 15.8	270.3 ± 17.4
DOC (mg kg^{-1} soil DW)	108.0 ± 1.2	192.3 ± 30.6	174.1 ± 8.7	390.1 ± 8.0	226.8 ± 6.2	329.3 ± 1.2	1537.2 ± 18.6
DON (mg kg^{-1} soil DW)	20.7 ± 0.5	38.6 ± 6.9	29.4 ± 1.0	74.4 ± 1.5	50.7 ± 1.1	65.1 ± 1.2	202.3 ± 3.8
MBC (mg kg^{-1} soil DW)	81.8 ± 1.0	158.7 ± 25.5	143.5 ± 8.8	349.6 ± 7.8	190.0 ± 4.6	286.1 ± 2.1	1348.1 ± 20.7
MBN (mg kg^{-1} soil DW)	10.1 ± 0.1	20.8 ± 3.7	16.7 ± 0.9	51.5 ± 0.9	22.8 ± 1.5	40.1 ± 0.4	148.7 ± 4.7

	Site 1 - sub	Site 2 - sub	Site 3 - sub	Site 4 - sub	Site 5 - sub	Site 6- sub	Site 7 - sub
NO_3^--N (mg kg^{-1} soil DW)	6.2 ± 0.1	7.7 ± 0.2	10.2 ± 0.8	10.5 ± 0.2	12.5 ± 0.9	19.0 ± 0.5	44.5 ± 2.9
NH_4^+-N (mg kg^{-1} soil DW)	2.3 ± 0.6	7.6 ± 0.3	4.4 ± 0.4	4.7 ± 0.3	7.9 ± 1.6	6.5 ± 0.3	23.5 ± 2.2
Organic matter (mg kg^{-1} soil DW)	6.9 ± 0.2	10.8 ± 0.8	12.8 ± 0.3	14.7 ± 0.4	14.5 ± 0.5	14.9 ± 0.2	66.2 ± 0.5
pH	6.4 ± 0.03	5.2 ± 0.04	4.6 ± 0.02	4.9 ± 0.02	5.2 ± 0.02	4.6 ± 0.02	3.9 ± 0.02
Water content (%)	17.8 ± 0.1	25.8 ± 1.2	30.4 ± 0.5	32.2 ± 0.3	32.6 ± 0.6	29.9 ± 0.2	65.3 ± 0.1
Sulphate-S (mg S kg^{-1} soil DW)	9.7 ± 2.1	18.2 ± 9.6	17.0 ± 2.9	12.1 ± 3.3	48.5 ± 7.8	47.2 ± 4.4	139.5 ± 25.3
DOS (mg kg^{-1} soil DW)	41.2 ± 8.1	120.4 ± 16.9	317.5 ± 24.3	350.0 ± 241.0	87.1 ± 5.6	97.9 ± 6.9	209.5 ± 85.9
DOC (mg kg^{-1} soil DW)	96.5 ± 4.4	140.1 ± 1.9	127.4 ± 2.2	189.5 ± 3.5	160.1 ± 0.7	177.7 ± 0.4	1241.7 ± 23.5
DON (mg kg^{-1} soil DW)	15.7 ± 0.4	25.6 ± 0.4	20.8 ± 0.1	32.3 ± 0.4	28.9 ± 1.0	34.6 ± 1.1	149.0 ± 3.5
MBC (mg kg^{-1} soil DW)	69.8 ± 4.2	111.8 ± 2.7	98.5 ± 0.6	158.8 ± 3.3	128.0 ± 0.8	147.3 ± 1.5	1073.7 ± 27.3
MBN (mg kg^{-1} soil DW)	9.4 ± 0.2	14.9 ± 0.5	12.4 ± 0.5	20.7 ± 0.3	15.0 ± 1.0	18.3 ± 0.9	99.4 ± 4.5

Table 3.2 Major soil cation concentrations measured by ICP-OES (samples collected in late May 2019). Values represent means ± SEM (n = 3) for either topsoil (top) or subsoil (sub). Data is expressed on a dry soil weight basis. NA indicates not applicable.

	Site 1 - top	Site 2 - top	Site 3 - top	Site 4 - top	Site 5 - top	Site 6- top	Site 7 - top
Al (mg kg^{-1} soil DW)	8.6 ± 1.2	67.6 ± 29.4	120.9 ±28.5	114.7 ± 24.8	56.3 ± 12.9	111.8 ± 2.2	518.4 ± 3.1
B (mg kg^{-1} soil DW)	2.6 ± 0.3	3.6 ± 1.3	2.8 ± 0.8	7.3 ± 0.7	6.6 ± 3.5	3.3 ± 0.5	10.2 ± 9.1
Ba (mg kg^{-1} soil DW)	4.1 ± 0.2	1.5 ± 0.7	0.9 ± 0.3	3.6 ± 0.9	0.9 ± 0.2	3.6 ± 0.02	28.5 ± 0.4
Co (mg kg^{-1} soil DW)	0.1 ± 0.01	0.4 ± 0.06	0.5 ± 0.03	0.2 ± 0.04	0.1 ± 0.04	0.3 ± 0.02	0.7 ± 0.04
Cr (mg kg^{-1} soil DW)	0.6 ± 0.03	0.6 ± 0.11	0.7 ± 0.02	0.8 ± 0.01	1.0 ± 0.02	0.7 ± 0.02	1.6 ± 0.01
Fe (mg kg^{-1} soil DW)	2.3 ± 0.7	20.4 ± 6.7	14.9 ± 1.3	37.7 ± 5.8	21.2 ± 7.0	33.0 ± 0.2	68.2 ± 1.8
K (g kg^{-1} soil DW)	1.0 ± 0.03	1.2 ± 0.19	0.2 ± 0.01	1.4 ± 0.03	0.8 ± 0.02	1.5 ± 0.03	6.9 ± 0.1
Mg (g kg^{-1} soil DW)	0.6 ± 0.01	0.9 ± 0.14	0.3 ± 0.01	1.3 ± 0.01	1.1 ± 0.01	1.1 ± 0.01	2.5 ± 0.02
Mn (mg kg^{-1} soil DW)	47.5 ± 1.0	217.9 ± 34.3	34.1 ± 0.9	253.5 ± 6.0	110.8 ± 3.7	350.6 ± 8.6	691.3 ± 3.5
Na (g kg^{-1} soil DW)	0.2 ± 0.01	0.9 ± 0.14	0.3 ± 0.01	0.4 ± 0.01	0.5 ± 0.01	0.5 ± 0.01	1.5 ± 0.02
Si (mg kg^{-1} soil DW)	43.2 ± 0.3	37.8 ± 4.5	46.6 ± 1.0	58.8 ± 4.5	57.3 ± 6.2	55.3 ± 2.1	111.5 ± 1.2
Sr (mg kg^{-1} soil DW)	38.4 ± 0.2	17.4 ± 2.9	10.3 ± 0.1	17.9 ± 0.4	17.5 ± 0.1	16.0 ± 0.1	48.9 ± 0.3
Zn (mg kg^{-1} soil DW)	1.0 ± 0.1	10.1 ± 2.6	2.7 ± 0.1	17.4 ± 1.5	8.9 ± 0.4	17.8 ± 0.5	46.3 ± 0.3

	Site 1 - sub	Site 2 - sub	Site 3 - sub	Site 4 - sub	Site 5 - sub	Site 6- sub	Site 7 - sub
Al (mg kg^{-1} soil DW)	6.8 ± 1.9	40.7 ± 7.5	134.5 ± 16.2	66.4 ± 43.7	40.2 ± 2.7	134.0 ±0.6	441.7 ± 7.0
B (mg kg^{-1} soil DW)	3.3 ± 1.3	5.3 ± 2.5	2.6 ± 0.1	5.5 ± 1.9	4.0 ± 2.1	3.3 ± 1.6	4.1 ± 1.1
Ba (mg kg^{-1} soil DW)	4.0 ± 0.3	0.7 ± 0.2	1.6 ± 0.9	1.1 ± 0.9	0.3 ± 0.03	2.3 ± 0.01	33.5 ± 0.6
Co (mg kg^{-1} soil DW)	NA	0.2 ± 0.01	0.5 ± 0.03	0.2 ± 0.02	0.1 ± 0.01	0.3 ± 0.02	0.7 ± 0.06
Cr (mg kg^{-1} soil DW)	0.6 ± 0.01	0.6 ± 0.02	0.7 ± 0.04	0.7 ± 0.07	0.9 ± 0.03	0.7 ± 0.03	1.6 ± 0.03
Fe (mg kg^{-1} soil DW)	1.8 ± 0.9	7.5 ± 1.4	23.1 ± 6.4	13.9 ± 9.8	8.6 ± 0.4	20.3 ± 1.8	69.3 ± 2.4
K (g kg^{-1} soil DW)	1.1 ± 0.02	0.5 ± 0.01	0.5 ± 0.40	0.6 ± 0.03	0.4 ± 0.01	0.8 ± 0.01	5.5 ± 0.1
Mg (g kg^{-1} soil DW)	0.5 ± 0.01	0.7 ± 0.01	0.4 ± 0.12	0.7 ± 0.01	0.7 ± 0.01	0.6 ± 0.01	2.3 ± 0.01
Mn (mg kg^{-1} soil DW)	18.8 ± 0.5	76.7 ± 2.3	64.6 ± 35.8	87.1 ± 1.0	53.9 ± 1.2	97.5 ± 0.9	1123.0 ± 3.4
Na (g kg^{-1} soil DW)	0.2 ± 0.01	0.4 ± 0.01	0.4 ± 0.12	0.4 ± 0.01	0.5 ± 0.01	0.3 ± 0.03	1.3 ± 0.02
Si (mg kg^{-1} soil DW)	49.5 ± 0.9	32.6 ± 1.2	51.0 ± 4.0	51.6 ± 2.1	37.2 ± 1.8	39.9 ± 3.1	78.6 ± 2.4
Sr (mg kg^{-1} soil DW)	35.9 ± 0.3	15.4 ± 0.4	12.3 ± 2.0	11.8 ± 0.3	12.7 ± 0.1	11.9 ± 0.1	50.9 ± 0.1
Zn (mg kg^{-1} soil DW)	0.6 ± 0.1	5.4 ± 0.1	4.5 ± 1.9	5.8 ± 0.5	4.3 ± 0.4	8.4 ± 0.2	53.4 ± 0.8

3.3.2 Factors influencing DOS concentration in grassland soils

Concentrations of the major cations in soil solution were summarized in Table 3.2. Stepwise linear regressions were employed to identify parameters which may help explain the spatial variation in DOS concentrations across the seven selected grassland sites. In this method, DOS was selected as the dependent variable, and TDS, Al, B, Ba, Fe, K, Mn, Na, Sr, Zn, NH_4^+ content from soil water, and soil organic matter, pH, DOC, DON, were selected as possible independent variables. According to our linear regression analysis, TDS, DON along with pH were found to be the significant factors in the model, together they are able to explain over 95% of the spatial variation in DOS in soil solution (Table 3.3).

The data were then analysed using Bivariate correlation, this analysis results confirm that TDS, DON concentration and pH in soil plays a significant role in governing the DOS concentration. The analysis results were summarized in table 3.4, results showed that Al, B, Ba, Fe, K, Mn, NH_4^+, DOC and soil organic matter also co-correlate with DOS concentration in soil solution, while Na, Sr and Zn correlated less well (Table 3.4). Al and Fe concentrations are positively correlated to DOS in the multiple regression, this may be due to that they are inherently and positively correlated to SO_4^{2-}. In contrast, soil pH is negatively corelated to DOS content. Soil pH affects the microbial population and their activities, therefore, a pH increase would lead to higher DOS mineralization (Neina, 2019).

Previous studies indicated that the major organic sulphur constituents in soil solution are carbon-bonded sulphur, including some amino acids, and ester sulphates such as choline sulphates and sulphated thioglycides (Zhao *et al.*, 2006). Hence, there is often a close correspondence between organic C and organic S in soils since carbon-bonded sulphur is generally the dominant organic sulphur (Homann *et al.*, 1990; Houle *et al.*, 2011). In our study, a strong relationship between DOS and DOC was also found, indicating that soil organic

sulphur is conceptually an explanatory parameter for DOS as both parameters are inherently related to the amount of DOM.

Table 3.3 Stepwise multiple linear regressions results.

Model	Predictor in model	B	SE	R	Adjusted R^2	R^2 change
1	(Constant)	-13.41	11.55	0.92	0.85	0.85
	TDS	0.78	0.05			
2	(Constant)	-20.46	7.34	0.97	0.94	0.94
	TDS	1.02	0.05			
	DON	-0.71	0.09			
3	(Constant)	-176.31	45.28	0.98	0.96	0.95
	TDS	1.13	0.05			
	DON	-.69	0.08			
	pH	26.64	7.66			

Predictor in model: variables selected into the model; B: partial regression coefficient; SE: standard errors.

3.3.3 Sorption of ^{35}S-Cys, Met and Na_2SO_4 in sterile grassland soil

Previous studies indicated that most UK soils have limited capacity to adsorb SO_4-S (Curtin and Syers, 1990; Zhao and McGrath, 1994), this is also found in our study, according to our results (Fig. 3.1), less than 4% of ^{35}S-Na_2SO_4 was adsorbed by our selected grassland soil, this in line with previous studies. Although the SO_4-S sorption capacity of soils could vary widely depending on factors such as the pH, Fe, Ca and Al content, clay minerals, soil organic matter content, temperature, water content etc. (Tabatabai, 1987; Sokolova and Alekseeva, 2008). In general, the soil sorption capacities of ^{35}S-Cys and Met were slightly higher than that of inorganic sulphate, with 5-10 % of amino acid-^{35}S lost during our experiment. Due to the large solution to soil ratio and rapid experimentation, we assume that microorganisms played little, if any, part in our sorption experiment.

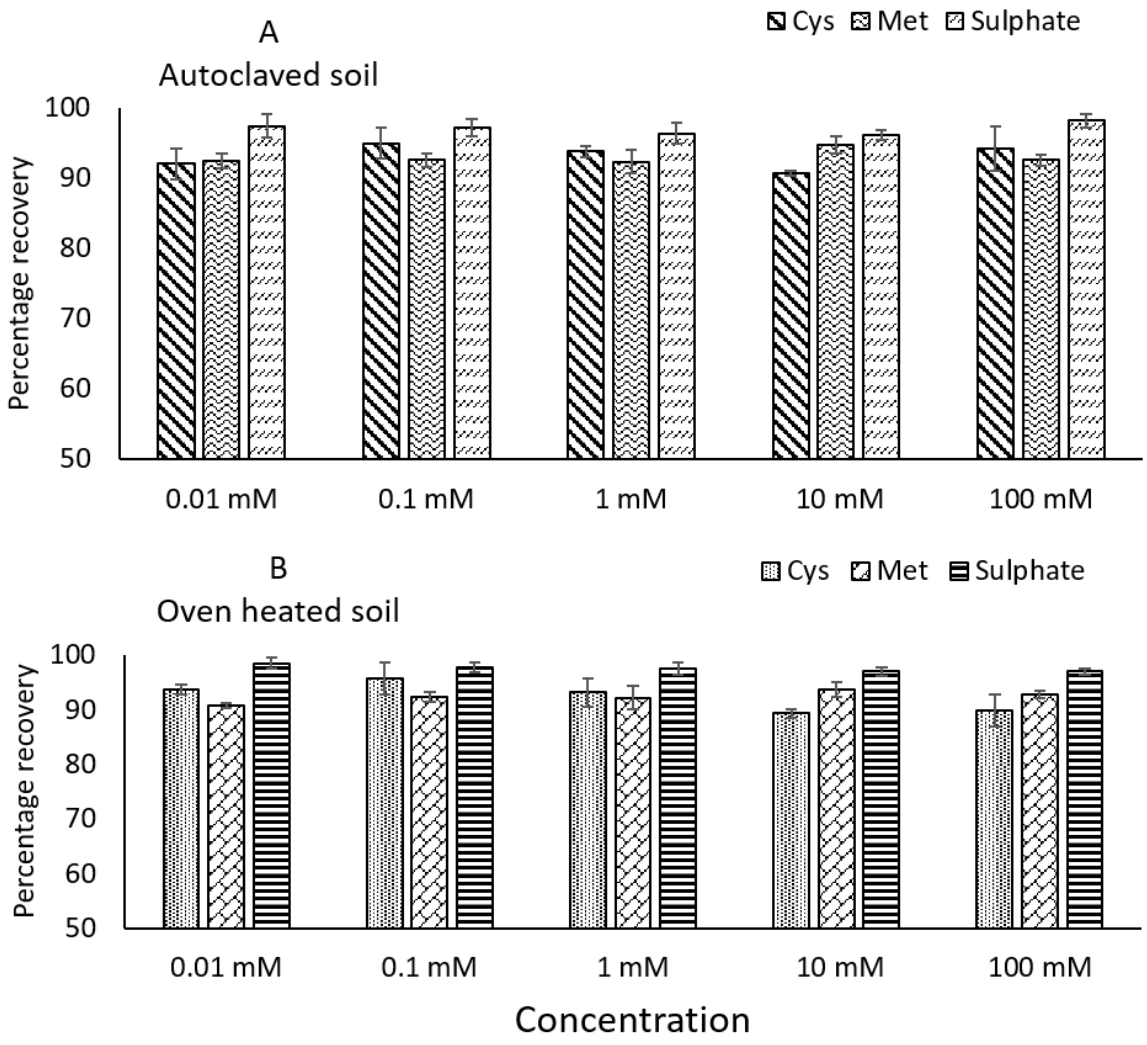

Fig. 3.1 Sorption of Cys, Met and Na_2SO_4 to the soil's solid phase, panel A shows sorption onto autoclaved soils, panel B shows sorption onto oven heated soils. The legend is the same for both panels. Values represent means ± SEM (n = 6).

Table 3.4 Correlation matrixes between DOS concentration and cations, ammonium, and MBC. ** and * represents significance at level $p < 0.01$ and $p < 0.05$, respectively.

	DOS	TDS	Al	B	Ba	Fe	K	Mn	Na	Sr	Zn	NH_4^+	Organic matter	pH	DOC	DON
DOS	1	0.92 **	0.68 **	-0.49 **	0.35 *	0.57 **	-0.56 **	0.41 **	0.31 *	-0.36 *	0.32 *	0.41 **	0.47 **	-0.63 **	0.44 **	0.41 **
TDS		1	0.86 **	-0.66 **	0.59 **	0.74 **	-0.60 **	0.66 **	0.43 **	-0.3	0.59 **	0.65 **	0.73 **	-0.78 **	0.70 **	0.68 **
Al			1	-0.65 **	0.78 **	0.90 **	-0.48 **	0.82 **	0.50 **	-0.2	0.74 **	0.74 **	0.87 **	-0.86 **	0.82 **	0.78 **
B				1	-0.46 **	-0.48 **	0.72 **	-0.52 **	-0.3	0.3	-0.45 **	-0.60 **	-0.62 **	0.58 **	-0.64 **	-0.66 **
Ba					1	0.69 **	-0.02	0.95 **	0.45 **	0.44 **	0.82 **	0.77 **	0.92 **	-0.49 **	0.95 **	0.90 **
Fe						1	-0.36 *	0.77 **	0.69 **	-0.2	0.76 **	0.66 **	0.79 **	-0.84 **	0.72 **	0.68 **
K							1	-0.2	-0.1	0.75 **	-0.2	-0.23	-0.29 **	0.68 **	-0.19	-0.02
Mn								1	0.53 **	0.2	0.89 **	0.68 **	0.91 **	-0.64 **	0.92 **	0.87 **
Na									1	-0.1	0.59 **	0.53 **	0.53 **	-0.46 **	0.55 **	0.54 **
Sr										1	0.05	0.12	0.15	0.53 **	0.27	0.22
Zn											1	0.76 **	0.87 **	-0.72 **	0.85 **	0.85 **
NH_4^+												1	0.90 **	-0.55 **	0.89 **	0.93 **
Organic Matter													1	-0.68 **	0.96 **	0.95 **
pH														1	-0.58 **	-0.58 **
DOC															1	0.98 **
DON																1

3.4 Conclusions

DOS and SO_4^{2-} concentration in seven grassland soils in North Wales were studied. We found that the DOS concentration in all soils ranged from 10.6 to 270.3 mg kg^{-1} DW soil from topsoils, while it ranged from 41.2 to 309.3 mg kg^{-1} DW soil in subsoils; concentrations of SO_4^{2-} in all seven sites ranged from 46.9 to 137.9 mg kg^{-1} DW soil in topsoil, while ranged from 9.7 to 139.5 mg kg^{-1} DW soil in subsoil. The concentrations of total dissolved sulphur (DOS plus SO_4^{2-}) in most of grassland soils were found to be < 350 mg kg^{-1} DW soil (Table 3.1). DOS concentrations were found to be higher in highland grassland soil compared to lowland soils. The DOS constituted 23.8 to 93.8% of total dissolved sulphur (DOS plus SO_4^{2-}) in all soils; this may be because our sites currently receive little atmospheric S deposition and are low in extractable SO_4^{2-}, therefore soil S stored is primarily in the organic form (Table 3.1). Soluble organic S seems to be intimately related with soluble carbon, suggesting the production of dissolved organic matter and mineralisation of SO_4^{2-} may be regulated by the same factors.

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3.6 References

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Chapter 4

Rapid microbial uptake and mineralization of cysteine and methionine along a grassland productivity gradient

Abstract

Cysteine (Cys) and methionine (Met) constitute important sources of directly available carbon (C), nitrogen (N), and sulphur (S) for both plant nutrition and microbial growth and are central to terrestrial S cycling. The factors controlling turnover rates of these S-amino acids in soil, however, remains understudied. Due to their multi-nutrient composition (C, N and S) we hypothesized that intense competition would exist in the microbial community for these substrates. Further, we hypothesized that that this competition would be inversely related to plant productivity and thus C inputs to soil. To address this, we studied the short-term, concentration-dependent microbial uptake and mineralization of ^{14}C-labelled Cys and Met in five grassland soils taken from an altitude-driven primary productivity gradient. Substrate depletion from soil solution was measured over a 60-minute period using a centrifugal drainage procedure and the subsequent evolution of $^{14}CO_2$ measured over a 48 h-period. Our results revealed that ^{14}C-Cys and Met are directly and rapidly assimilated by soil microbes, with half-times ranging from 0.34 - 2.14 mins. This is an order of magnitude (or more) faster than when determined from measurement of $^{14}CO_2$ evolution. This considerable delay between microbial ^{14}C removal from soil solution and subsequent $^{14}CO_2$ evolution indicates that the degradation of Cys and Met in soil solution was mainly through biological processes. The use of different substrate concentrations suggested that when lower concentrations of ^{14}C-Cys and Met were added to soil (0.01 - 0.1 mM), soil microbial uptake occurred via a single carrier system, while a second transport system/mechanism became important when the substrates were added at higher concentrations (1 - 100 mM). Rates of substrate mineralization by soil microbes declined in less productive, nutrient limited grassland soils with lower levels of microbial biomass, suggesting that the turnover of organic N and S, and subsequent availability for plant uptake is likely to be controlled by the size and activity of the soil microbial community. We conclude that despite the relatively low concentration of Cys and Met in soil solution, these DON/DOS compounds could be of great significance in terrestrial nitrogen and sulphur cycling due to their rapid microbial turnover.

Keywords: Cysteine, methionine, biodegradation, mineralization, grassland productivity gradient, free amino acid

4.1 Introduction

Recent decades has seen a major reduction in global anthropogenic SO_2 emissions due to a decrease in fossil fuel burning and greater regulatory controls on sulphur (S) release to the atmosphere (Fowler et al., 2007; Hinckley et al., 2020). In combination with the adoption of low S-containing fertilizers and greater crop yields (Ceccotti and Messick, 1997), this has resulted in a negative S balance in many agricultural systems (McGrath and Zhao, 1995; Hioang et al, 2020; Pariasca-Tanaka et al., 2020). As a consequence, these agroecosystems are becoming more reliant on the supply of S from native soil organic matter (SOM). In comparison to other macronutrients (e.g. N, P), however, relatively few studies have focused on the mineralization of organic S to SO_4^{2-} and its capacity to alleviate S deficiency in plants (Fitzgerald et al., 1988; Fitzgerald and Andrew, 1984; Chalk and Inacio, 2017).

Typically, more than 95% of the S present in soil is held in a solid organic form. Most of this S enters soil from plant residues and therefore includes an abundance of free and combined S-containing amino acids (i.e. cysteine and methionine; Cys and Met) in addition to a range of other S compounds such as thiols (e.g. glutathione), sulfolipids and secondary S compounds (e.g. allicins, glucosinolates, phytochelatins; Falloon and Smith, 2000). Unsurprisingly therefore, studies have shown the presence of large quantities of S-containing amino acids in hydrolysates of SOM (Freney, Stevenson and Beavers, 1972; Scott, Bick and Anderson, 1981).

In contrast to SOM, several studies have reported that the concentration of free S-containing amino acids in soil solution is very low (ca. 0.1 to 10 µM; Broughton et al., 2015), particularly in relation to the levels of SO_4^{2-} (ca. 50 - 1000 µM; Cambier et al., 2014; Kölling and Prietzel, 1995). This may suggest that inorganic S dominates plant and microbial uptake. However, studies in freshwaters suggest that this assumption may not always be valid, especially when both pool size and flux rate are considered together (Brailsford *et al.*, 2020). Their low concentration in soil solution could be explained by (i) their low rate of production,

(ii) their rapid removal by roots and microorganisms, (iii) their external breakdown by free sulfatases, or (iv) sorption to mineral surfaces (Broughton et al., 2015; Ma et al., 2020; Sanchez-Arenillas et al., 2017). The relative importance and complementarity of these mechanisms in regulating free Cys and Met levels in soil, however, remains unknown. Cys and Met can be considered multi-nutritional as they contain C, N and S. It might be expected therefore that C- or N-limited microbial communities would prefer these substrates to SO_4^{2-}. From a thermodynamic perspective, it is also energetically more efficient to take up amino acids which can be directly incorporated into common metabolic pathways that to take up and assimilate SO_4^{2-} (Kertesz, 2000). We hypothesize that S mineralization from C-bonded organic forms (e.g. Cys and Met), occurs mainly through biological processes (McGill and Cole, 1981; Jones and Shannon, 1999), driven by microbial demand for energy and C skeletons for maintenance and growth, with excess SO_4^{2-} and NH_4^+ being released as by-products (Ruiz-Herrera and Starkey, 1969a; Sohn and Ho, 1995; Zhao, Wu and McGrath, 1996). These processes may be regulated by many physical, chemical and biological factors which would ultimately determine the quality and timing of inorganic N and S becoming available for plant growth (Zehr, Axler and Goldman, 1985; Grant, Juma and McGill, 1993).

The purpose of this study was therefore to evaluate the uptake and subsequent mineralization of Cys and Met in a contrasting range of grassland soils. We chose Cys and Met as they are likely to represent the dominant forms of organic S entering grassland soils (Scott, Bick and Anderson, 1981). The specific aims of this study were therefore to: (1) determine the effects of substrate concentration on the mineralization of Cys and Met; (2) compare the turnover rates of Cys and Met under differing pasture productivity conditions; (3) ascertain whether the microbial uptake and mineralization of Cys and Met were temporally uncoupled in soil, and (4) determine if Met and Cys had different microbial C use efficiencies.

4.2 Materials and methods

4.2.1 Soil sampling and characteristics

Five soils were sampled along an altitudinal gradient on a north facing slope at the Henfaes Research Station, Abergwyngregyn, Gwynedd, North Wales, UK (53°14'N; 4°10'W; Table 4.1). They were not chosen to be intrinsically linked, rather just to provide a diverse array of soils with inherent differences in fertility and grassland productivity. This elevation gradient is characterised by a reduction in soil fertility and grassland productivity with increased elevation (Farrell, Hill, Farrar, *et al.*, 2011). Further characteristics of the altitudinal catena sequence are described in Wilkinson et al. (2014a), Greenfield et al. (2020) and Withers et al. (2020). The mean annual temperature ranged from 11 ℃ at low altitude (Site 1) to 8 ℃ at the top of the gradient (Site 5), with annual rainfall ranging from 960 mm at Site 1 to 1800 mm at Site 5. Five sites with contrasting soil characteristics were selected along the gradient. More soil characteristics are provided in Table 4.1. At each site, 2 kg of topsoil (0 - 10 cm) was collected from three independent randomly positioned replicate plots (5 × 5 m^2; Withers et al., 2020). The soil was placed in gas-permeable plastic bags and transported immediately back to the laboratory where it was sieved (2 mm) and stored at 4 ℃ (< 48 h) until required for further experimentation.

Moisture content was determined by oven drying (80 ℃, 24 h). Soil pH and electrical conductivity (EC) were determined in a 1:5 (w/v) soil: distilled water suspensions with standard electrodes. Soil bulk density was determined by taking intact cores (100 cm^3) from the field followed by oven drying and stone correction (Rowell, 2014). Available NH_4^+ and NO_3^- were determined by extracting 5 g of field-moist soil with 25 ml 0.5 M K_2SO_4 (200 rev min^{-1}; 20℃, 30 min), filtering the suspensions (Whatman No. 42 filter paper) before colorimetric analysis using a Synergy-MX microplate reader (BioTek Ltd, Swindon, UK). Nitrate was determined according to the vanadate procedure of Miranda et al. (2001) while ammonium was determined

using the salycylic acid proedure of Mulvaney (1996). Extractable phosphorus (P) was extracted using a 0.5 M acetic acid (1:5 w/v) shaken for 1 hour (200 rev min^{-1}; 20°C), then centrifuged for 10 min at 3220 *g* before passing through a Whatman 42 filter (Quevauviller, 2007). P was then analysed by the colorimetric method of Murphy and Riley (1962). Total free amino acids were determined in the 0.5 M K_2SO_4 extracts according to Jones et al. (2002). Microbial biomass C (MBC) and N (MBN) were calculated from the differences between C and N concentrations in $CHCl_3$-fumigated and unfumigated samples after extracting the soil with 0.5 M K_2SO_4 as described above and analysis of extractable C and N using a TOC-V-TN analyser (Shimadzu Corp., Kyoto, Japan;(Brookes *et al.*, 1985)). Total soil C and N were determined on oven dry soil using a TruSpec CN analyser (Leco Corp., St Joseph, MI). Extraction efficiency conversion factors (Kec and Ken) of 0.35 and 0.50 were used to calculate MBC and MBN, respectively (Voroney, Brookes and Beyaert, 2007). Sulphate and other major anions were analysed in 1:5 w/v soil: distilled water extracts (200 rev min, 20°C, 30 min; (Zhao and McGrath, 1994)) using an ICS-2011 ion chromatograph (Dionex Inc., Sunnyvale, CA). Total dissolved S (dissolved organic and inorganic S) and other major cations were analysed by inductively coupled plasma-optical emission spectroscopy after extraction of the soil (200 rev min, 20°C, 30 min) in 1:5 w/v soil: 0.01 M $Ca(H_2PO_4)_2$ extracts using a Varian 710ES ICP-OES (Agilent Technologies, Santa Clara, CA). Dissolved organic sulphur (DOS) content was calculated from the difference between total dissolved S and inorganic SO_4^{2-} (David and Mitchell, 1985).

Differences in soil properties along the altitudinal gradient were assessed using ANOVA with Tukey's LSD post hoc test using SPSS version 25.0 (IBM UK Ltd., Portsmouth, UK). $P < 0.05$ was used as the cut-off for statistical significance.

Table 4.1 Characteristics of the 5 grassland soils used in the experiment. Values represent means ± SEM, n = 3. Superscript letters represent significant differences between soils at the $P < 0.05$ level. Data are presented on a dry weight basis.

	Site1	Site2	Site3	Site4	Site5
Altitude (m)	32	53	222	342	400
Bulk density[a] (g cm^{-3})	0.96 ± 0.04[a]	0.72 ± 0.03[b]	0.47 ± 0.06[cd]	0.51 ± 0.05[c]	0.55 ± 0.08[c]
Water content[b] (%)	17.1 ± 0.2[e]	53.5 ± 2.1[d]	81.9 ± 1.3[b]	77.1 ± 1.5[c]	84.6 ± 0.5[a]
pH[c]	5.92 ± 0.02[a]	5.74 ± 0.06[b]	5.67 ± 0.01[c]	5.43 ± 0.01[d]	4.20 ± 0.01[e]
Electrical conductivity[c] (µS cm^{-1})	73.5 ± 3.6[a]	56.8 ± 1.0[d]	90.1 ± 0.8[b]	42.1 ± 1.1[e]	153.0 ± 1.4[a]
NH_4^+-N[d] (mg kg^{-1})	6.6 ± 0.6[c]	10.2 ± 6.1[c]	9.7 ± 1.1[c]	19.9 ± 9.2[b]	61.5 ± 4.2[a]
NO_3^--N[d] (mg kg^{-1})	0.89 ± 0.06[e]	12.6 ± 0.1[c]	21.4 ± 2.1[b]	4.0 ± 0.3[d]	49.2 ± 1.2[a]
Available P[d] (mg kg^{-1})	0.47 ± 0.05[ab]	0.42 ± 0.01[b]	0.51 ± 0.02[a]	0.52 ± 0.09[a]	0.58 ± 0.03[a]
Dissolved organic C[e] (mg kg^{-1})	35.1 ± 8.7[a]	40.5 ± 23.0[a]	41.3 ± 3.7[a]	40.6 ± 1.2[a]	45.8 ± 7.0[a]
Microbial biomass-C[e] (g kg^{-1})	0.67 ± 0.02[e]	2.26 ± 0.16[d]	2.94 ± 0.20[c]	3.23 ± 0.38[b]	94 ± 0.32[a]
Total dissolved S[f] (mg S kg^{-1})	31.6 ± 2.1[d]	34.4 ± 3.7[c]	42.8 ± 0.6[b]	42.9 ± 2.4[b]	62.9 ± 6.6[a]
Sulphate-S[g] (mg S kg^{-1})	4.37 ± 0.79[c]	4.65 ± 0.33[c]	5.53 ± 0.54[bc]	6.71 ± 0.48[b]	11.89 ± 1.77[a]

[a]Dry bulk density measured by taking intact 100 cm^3 cores in the field and then oven drying at 105 °C overnight.
[b]Measured by drying at 105 °C overnight and expressed on a wet weight basis.
[c]Measured in a 1:5 (v/v) soil-to-distilled water extract.
[d]Measured in a 1:5 (w/v) 0.5 M K_2SO_4 extract followed by colorimetry analysis via a Synergy-MX microplate reader.
[e]Measured in a 1:5 (w/v) 0.5 M K_2SO_4 extract using a TOC-V-TN analyser.
[f]Measured in 1:5 w/v soil: 0.01 M $Ca(H_2PO_4)_2$ extracts using a Varian 710ES ICP-OES.
[g]Measured in 1:5 w/v soil: distilled water extracts using an ICS-2011 ion chromatograph.

4.2.1 S-amino acid depletion from soil

A centrifugal-drainage (snap elution) approach was used to measure the rate of Met and Cys depletion from soil solution as described by Hill et al. (2008) and Wilkinson et al. (2014). Briefly, 1 g of field-moist soil was placed in a 1.5 cm^3 polypropylene micro-centrifuge tube in which a small hole (0.55 mm dia.) had been drilled in the base. This tube was then placed into another intact 1.5 cm^3 micro-centrifuge tube. A uniformly ^{14}C-labelled Cys or Met solution (200 µl, 0.5 kBq ml^{-1}; PerkinElmer Inc., Waltham, MA) was added to the soil surface. Infiltration of the Cys and Met solution into the soil took less than 5 s. The range of concentrations used (0.01, 0.05, 0.1, 1, 10 or 100 mM) were chosen to reflect those likely to be naturally found in soil solution (0.01 - 0.05 mM), those present in the rhizosphere due to root exudation (0.1 - 1 mM), those present in soil after a root cell bursts (1 - 10 mM), and those in amino acid-based fertilisers (10 - 100 mM) (Jones and Darrah, 1994; Moe, 2013). After incubating ^{14}C-Cys and ^{14}C-Met in the soil for varying times (1, 3, 5, 10, 20, 30, 40, 60 mins), soil solution was recovered in the lower receptacle by centrifuging the soils for 1 min at 14000 *g* at 20°C. 100 µl of the recovered soil solution was mixed with Optiphase Hisafe 3 scintillation fluid (PerkinElmer Inc.) and ^{14}C determined by liquid scintillation counting using a Wallac 1404 scintillation counter with automated quench correction (Wallac EG&G, Milton Keynes, UK). All treatments were carried out in triplicate.

To account for any abiotic effects (e.g. imperfect mixing, dilution with the intrinsic non-labelled soil solution and sorption; Hill et al., 2008), the same experiment was carried out on field-moist soil that had been autoclaved (121°C, 30 min) and allowed to cool down immediately prior to use. This treatment was used to eliminate the microbial activity in the samples.

4.2.2 S-amino acid mineralization

Cys and Met mineralization was measured by placing 5 g of field-moist soil into sterile polypropylene tubes (50 cm^3). Subsequently, 1 ml of uniformly labelled ^{14}C-Cys or ^{14}C-Met (0.5 kBq ml^{-1}; 0.01 to 100 mM; PerkinElmer Inc.) was added to soil surface. A 6 cm^3 polypropylene vial containing 1 ml of 1 M NaOH solution was then placed above the soil to trap any $^{14}CO_2$ emitted. All tubes were then sealed and maintained at 20°C in the dark. The NaOH traps were changed after 0.25, 0.5, 1, 3, 6, 24 and 48 h. The $^{14}CO_2$ in the NaOH traps was determined by liquid scintillation counting as described earlier. All treatments were carried out in triplicate. The same experiment was also carried out on field-moist soil that had been autoclaved as described above.

4.2.3 Statistical and data analysis

After addition of the ^{14}C-labelled amino acid solution to the soil, the ^{14}C-Cys or Met solution gradually equilibrates with the intrinsic soil solution. The theoretical final concentration of ^{14}C-amino acid in the soil solution ($^{14}C_{max}$) after equilibration is therefore:

$$^{14}C_{max} = {}^{14}C_{added} \times V_{added} / (V_{added} + V_{native}) \quad \text{(Eqn. 1)}$$

where $^{14}C_{added}$ is the activity of the ^{14}C solution added (kBq ml^{-1}), V_{added} is the volume of ^{14}C solution added and V_{native} the volume of water already present in the soil. To investigate the time required to achieve perfect isotopic mixing, the dynamics of isotopic pool dilution was described in the autoclaved soil (i.e. no microbial removal) by a single first order exponential decay function:

$$^{14}C_{sol} = A + B \times \exp^{(-k \times t)} \quad \text{(Eqn.2)}$$

Where $^{14}C_{sol}$ is the amount of ^{14}C recovered in autoclaved soil solution over time, A represents the point when perfect mixing is achieved, t is the time after ^{14}C solution addition, and B describes the size of the added amino acid pool and, k is the rate constant describing the time

taken to reach equilibrium. It should be noted that an equilibrium was not reached in some soils. Consequently, the amount of ^{14}C in solution recovered from sterile soils by centrifugation was higher than the theoretical maximum in some cases.

The half-time required to reach equilibrium ($t_{½}$) was calculated from:

$$t_{½} = \ln(2)/k \quad \text{(Eqn.3)}$$

In the non-sterile soils, the depletion of ^{14}C-Cys or ^{14}C-Met from soil solution was described by a range of first order kinetic equations. For the low substrate concentration (0.01 - 0.1 mM) a single first order exponential decay model, was fitted to the experimental data as follows:

$$^{14}C_{sol} = A_1 \times \exp^{(-k_1 \times t)} \quad \text{(Eqn. 4)}$$

Where C_{sol} is the ^{14}C remaining in soil solution, k_1 is the rate constant, t is time and A_1 is the initial amino acid concentration at time, t_0.

At higher ^{14}C-Cys or ^{14}C-Met concentrations (1 - 100 mM) the depletion from soil solution as biphasic. Consequently, a double first order exponential decay model was fitted to the experimental data as follows:

$$^{14}C_{sol} = A_1 \times \exp^{(-k_1 \times t)} + A_2 \times \exp^{(-k_2 \times t)} \quad \text{(Eqn. 5)}$$

Where A_1 and A_2 represent the size of the fast and slow depletion pools, respectively, and k_1 and k_2 represent the rate constants for these two pools, respectively.

It is was well documented that the mineralization of low molecular weight substrates in soils is biphasic (Scow, Simkins and Alexander, 1986; Glanville *et al.*, 2012). Therefore, a double first order exponential decay equation was fitted to the experimental mineralization data as follows:

$$^{14}C_{total} = AA_{catabolic} \times \exp^{(-k_3 \times t)} + AA_{anabolic} \times \exp^{(-k_4 \times t)} \quad \text{(Eqn. 6)}$$

where $^{14}C_{total}$ is the total amount of ^{14}C remaining in soil, $AA_{catabolic}$ is the amount of amino acid-derived ^{14}C partitioned into microbial anabolic processes and $AA_{anabolic}$ is the amount of amino acid-derived ^{14}C partitioned into microbial anabolic processes. The rate constants k_3 and

k_4 describe the turnover rate of these two pools, respectively. For a full description of the model see Glanville et al. (2016). Half-lives of the two C pools was calculated according to Eqn. 3.

Microbial C use efficiency (CUE_{mic}) for each substrate was calculated according to Jones et al. (2018) as follows:

$$CUE_{mic} = AA_{anabolic} / (AA_{catabolic} + AA_{anabolic}) \quad \text{(Eqn. 7)}$$

All experiments were carried out in triplicate. After checking for normality, data were subjected to one-way ANOVA with LSD post hoc test using SPSS version 25.0 (IBM UK Ltd., Portsmouth, UK). $P < 0.05$ was used as the cut-off for statistical significance.

4.3 Results

4.3.1 S-amino acid depletion from soil solution

4.3.1.1 Substrate depletion from soil solution affected by soil conditions

The change in soil solution concentration of ^{14}C-Cys and Met in the autoclaved soils (where biotic removal is prevented) allowed us to calculate the dynamics of isotope pool dilution (i.e. the mixing of the introduced isotope with the native soil solution). After 1 h, 11.6%, 33.0% and 21.9% of added ^{14}C-Cys was removed from the autoclaved soils 1, 2 and 3 respectively due to abiotic factors (such as adsorption, or occlusion within micropores that are not drained by centrifugation), and 32.6% and 27.0% of added ^{14}C-Met was removed in this way from soil 1 and 2 separately (Fig. 4.1). More ^{14}C-Cys was recovered from soil 4 and 5 (107.7%, 120.6% respectively), and more ^{14}C-Met from soil 3, 4 and 5 (145.5%, 108.1% and 140.2% separately) after 1 h than would be predicted following perfect mixing of substrate and soil, indicating that the added substrate had not fully mixed with soil prior to starting the centrifugal drainage procedure. We found that recovery of added ^{14}C from autoclaved soil followed first order exponential decay equation (Eqn. 2; $R^2 > 0.95$). From this, we calculated half-time required for the two solution pools to mix, the majority of ^{14}C Cys and Met mixing

and abiotic depletion occurred rapidly within the first 40 minutess (half times ranged from 3.1 minutes for Cys in soil 3 to 25.4 minutess for Cys in soil 4), this excludes values for soil 1 and 2 which undergo a slower exponential decay.

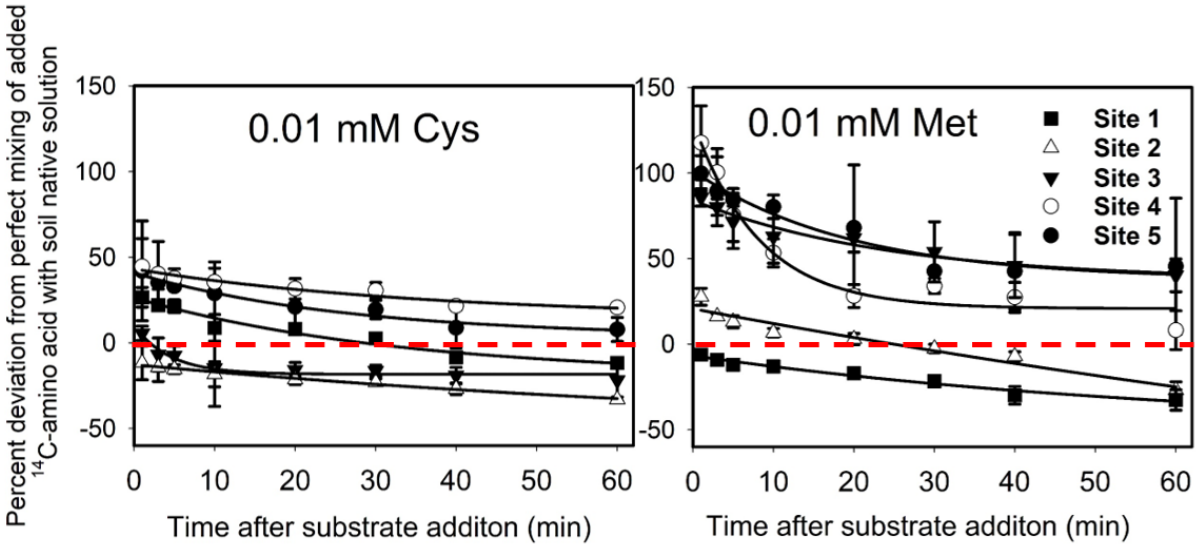

Fig. 4.1 Percent deviation from perfect mixing after addition of ^{14}C-Cys or Met solution (0.01 mM) to autoclaved soils collected from 5 contrasting grassland sites. A 200 µl of ^{14}C-amino acid (Cys or Met) was added to 1g of soil containing non ^{14}C labelled water. Percentages are based on the theoretical amount of ^{14}C available in solution assuming no biotic loss. The dashed line at 0% represents the point at which perfect mixing has occurred throughout the soil. Values above 0% (dotted line) indicates incomplete mixing of added solution and soil native soil solution (values above 0%), values below 0% indicates abiotic sorption. Lines represent first order exponential equations fit to the experimental data. Data points represent means ± SEM (n = 3).

Based on this isotopic pool dilution dynamics in autoclaved soils, depletion of ^{14}C-amino acid from soil solution was corrected for mixing and abiotic processes, the data presented hereafter refer to ^{14}C directly available for microbial uptake. As shown in Fig. 4.2, ^{14}C-Cys removal from soil solution by the microbial community was extremely rapid, with 87.4 ± 1.2% of available ^{14}C-Cys taken up by microbes from soil 1 after only 60 s. The

proportion of ^{14}C-Cys removed from soil solution by microbes fell significantly ($p < 0.05$) in soil from high elevation, low productivity sites to 33.4 ± 7.3% (soil 2), 42.5 ± 12.7% (soil 3), 45.8 ± 8.9 % (soil 4) and 43.3 ± 14.7% (soil 5) within one minute. Similarly, available ^{14}C uptake by soil microbes from soil solution also occurred very quickly after Met addition, especially in soil 1 and 2 where 59.4 ± 2.2% and 57.3 ± 5.7% of the available ^{14}C-Met was taken up by the microbial biomass within 1 min. The amount of ^{14}C-Met removed from soil solution fell significantly ($p < 0.05$) in soils from high elevation, low productivity sites, to 42.0 ± 3.9% (soil 3), 39.5 ± 10.1% (soil 4) and 20.2 ± 8.0% (soil 5) when compared with soil 1 and 2.

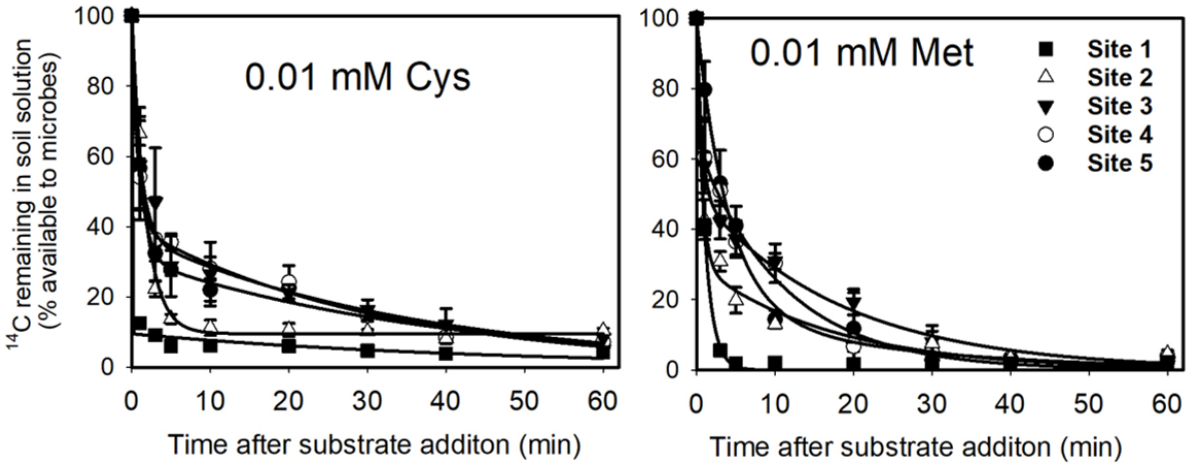

Fig. 4.2 Amount of ^{14}C-label remaining in soil solution after addition of a ^{14}C labelled Cys and Met (0.01 mM) to soil collected from 5 grassland sites of increasing elevation and decreasing productivity within 60 minutes, corrected from autoclaved soil data as described in material and methods section. Percentages are based on theoretical amount of ^{14}C available in solution assuming perfect mixing of the substrate with soil native water (Eqn. 1). Values represent means ± SEM (n = 3), lines represent fits of single first order exponential equations to the experimental data.

The depletion of ^{14}C from soil solution at sites 1 and 2 was best described by a single exponential decay equation (Eqn. 4) for additions of Cys ($r^2 > 0.96$) or Met ($r^2 > 0.89$). The half time ($t_{½}$) for Cys depletion from soil solution was significantly shorter than that for Met ($p <$

0.05) in soil 1, but there were no significant differences between the half times in soil 2. Furthermore, ^{14}C was removed significantly faster ($p < 0.05$) from soil 1 than soil 2 following both Cys and Met addition. By contrast, soils 3, 4 and 5 behaved differently from soil 1 and 2, and ^{14}C depletion from soil solution was best described by a double exponential decay equation (Eq (4); $r^2 > 0.98$). This implies that in grassland soils from higher elevation, lower productivity soils, ^{14}C depletion occurred in two phases. Roughly two thirds of added ^{14}C-Cys was depleted in a rapid initial phase (Table 4.2; $t_{½} < 1$min), while the remaining third was depleted more slowly, with half times up to 27 mins (no significant difference between these three soils). Furthermore, compared to Met, more Cys was taken up by soil microbes after one minute in soil 1 and 2 ($p < 0.05$), there were no significant differences between soil 3, 4 and 5.

4.3.1.2 Substrate depletion from soil solution as affected by substrate concentration

As described earlier, by monitoring the recovery of ^{14}C-amino acid in soil solution extracted from autoclaved soil over time, we obtained deviation from perfect mixing of added ^{14}C labelled solution with native soil solution due to abiotic process including adsorption and occlusion within micro-pores that are not drained by our centrifugal drainage procedure. After one hour, 11.8%, 25.3%, 14.3%, 7.7%, 22.7%, 11.0% of the added ^{14}C-Cys was removed from the autoclaved soil at concentration of 0.01, 0.05, 0.1 mM, and 1, 10, 100 mM respectively due to abiotic factors, and 33.6%, 30.8%, 26.3%, 27.3%, 25.2% and 23.4% of added Met were removed in this way, respectively (Fig. 4.3). ^{14}C-amino acid recovery from sterile soil followed a first order exponential decay (Eqn. 2; $R^2 > 0.86$), with half-lives ranging from 1.8 min at 1 mM (Cys) to 75.3 min at 0.01 mM (Met). Perfect mixing of native soil solution and added ^{14}C-label was achieved within approximately 40 mins after substrate addition.

In the non-sterile soil, the microbial community removed 87.4 ± 1.2% and 59.4 ± 2.2% of the added ^{14}C-Cys and Met from the soil solution within 1 minute when added at 0.01 mM,

respectively. This fell to 30.2 ± 1.9% and 29.1 ± 3.9% when added at the highest concentration (100 mM). In absolute terms, however, the actual amount of Cys and Met removed from soil solution by soil microbes increased with increasing amino acid concentration, at the highest added amino acid concentration (100 mM), uptake rates were equivalent to 7.1 mmol ^{14}C-Cys (kg^{-1} DW soil) min^{-1} and 6.8 mmol ^{14}C-Met (kg^{-1} DW soil) min^{-1}, respectively after only one minute.

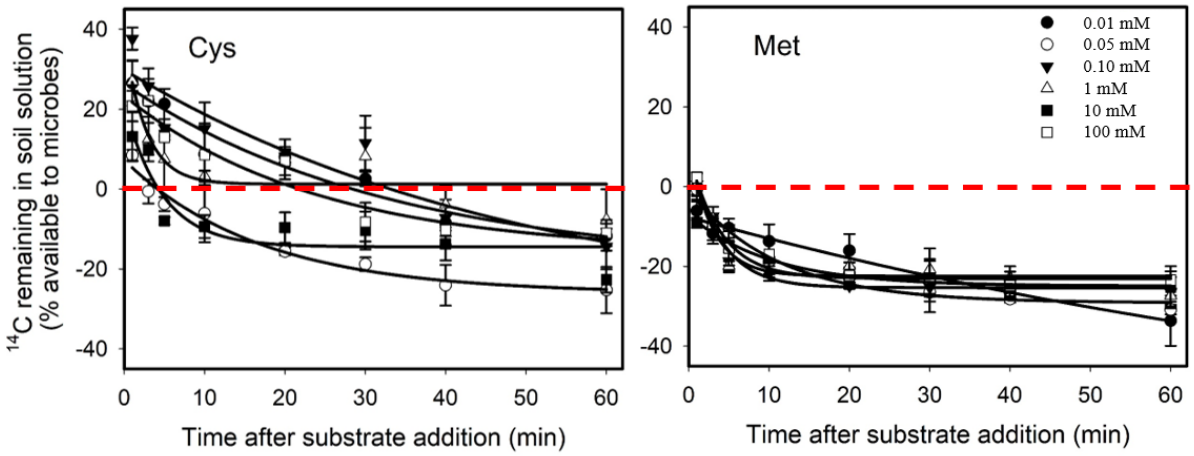

Fig. 4.3 Deviation from perfect mixing when a ^{14}C-Cys and Met solution (0.01 to 100 mM) is added to autoclaved soil containing non ^{14}C labelled water. Percentages are based on the theoretical amount of ^{14}C available in solution assuming perfect mixing of the substrate with the native soil solution. Deviations from 0% (dotted line) indicates incomplete mixing (values above 0) or abiotic sorption (values below 0%). Lines represent fits of first order kinetic equations (Eqn. 2) to the experimental data. Values represent means ± SEM (n = 3).

The dynamics of both Cys and Met depletion from soil solution were dependent upon the amount of substrate added to the soil (Fig. 4.4). The depletion of ^{14}C-Cys and Met from soil solution was best described by a single exponential decay equation ($R^2 > 0.92$ in all cases) when added at 0.01, 0.05 and 0.1 mM, but better described by a double exponential kinetic decay model ($R^2 > 0.98$ in all cases) when added at higher concentrations (1 mM, 10 mM, 100 mM; Table 4.3). The exponential coefficients and half-lives ($t_{½}$) describing the loss of Cys and Met from the soil solution are presented in Table 4.3.

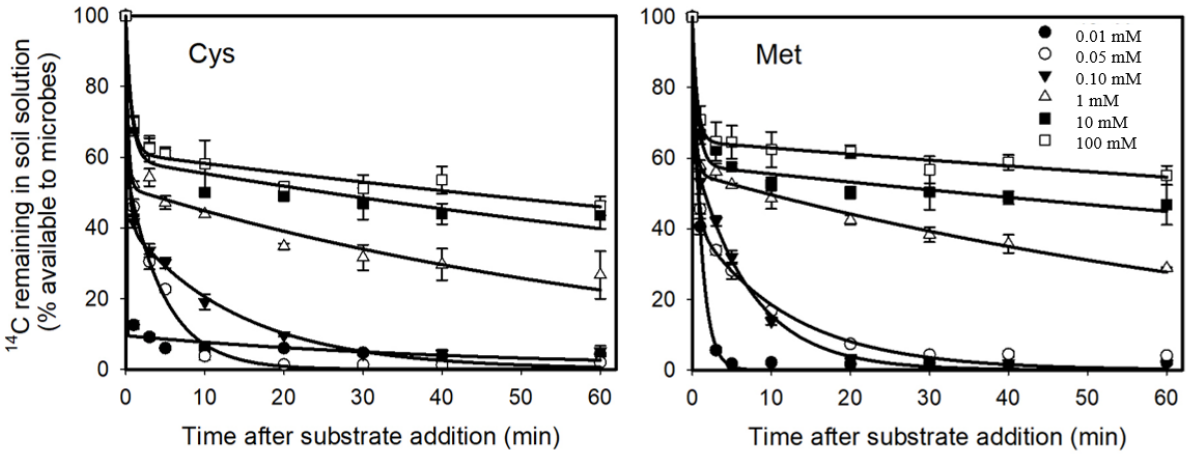

Fig. 4.4 Amount of ^{14}C label in soil solution after the addition of a ^{14}C labelled Cys and Met to non-sterile grassland soil (site 1). Lines represent fits of single (0.01 to 0.1 mM) and double (1 to 100 mM) first order kinetic equations to the experimental data. Values represent means ± SEM (*n* = 3).

4.3.2 Microbial C use efficiency for individual substrates

4.3.2.1 S-amino acid mineralization from autoclaved soils

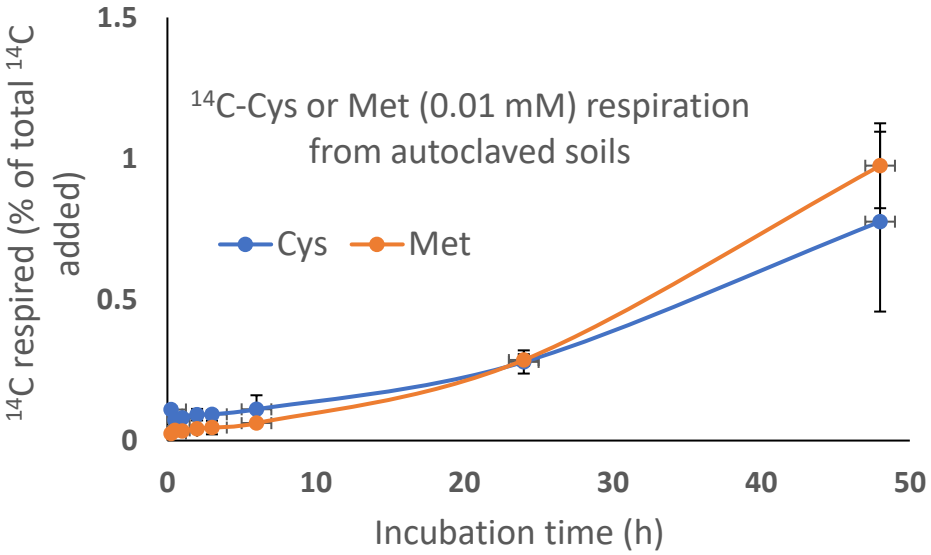

Fig. 4.5 Kinetics of the ^{14}C released from cumulative microbial respiration after addition of ^{14}C labelled Cys or Met (0.01 mM) to autoclaved grassland soil (site 1) within 48h. Values represent means ± SEM (*n* = 3).

The supply of ^{14}C-Cys or Met to autoclaved grassland soil led to little $^{14}CO_2$ respiration within 48 hours, the amount of ^{14}C-Cys or Met released as $^{14}CO_2$ corresponded to less than 1% of total ^{14}C-substrate input (Fig. 4.5). The low recovery of ^{14}C from microbial respiration indicates that autoclaving process was successful at killing the active soil microorganisms in short term, these results concur with the findings of similar studies on autoclaved soils (Carter, Yellowlees and Tibbett, 2007).

4.3.2.2 S-amino acid mineralization affected by soil conditions

The ^{14}C-amino acid mineralization was divided into two main phases: the first phase, rapid respired $^{14}CO_2$ may be attributable to the immediate use of the added substrate for catabolic process, in the second phase, $^{14}CO_2$ is released much more slowly following microbial turnover thought catabolic processes (Eqn. 5; K_3 and K_4; Table 4.4). This is in line with what has previously been reported (Vinolas, Vallejo and Jones, 2001; Wilkinson, Paul W. Hill, *et al.*, 2014). The first order double exponential decay equation gave a good fit to these biphasic experimental data (Fig. 4.6; $R^2 > 0.99$). The exponential coefficients and half-lives of substrate mineralization are presented in Table 4.4. As little $^{14}CO_2$ evolution from soil solution was observed in the autoclaved soil treatments (Fig. 4.5), we assume that ^{14}C-Cys and Met mineralization from soil solution occurred almost entirely due to microbial activity.

In soil 2, 3 and 4, more ^{14}C-Cys was allocated to the initial rapid mineralization pool than in the secondary mineralization pool ($p < 0.01$), yet there were no significant differences ($p < 0.01$) in two Cys pools in soil 1 and 5, where marginally more ^{14}C-Cys was allocated to the fast turnover pool. Half-lives for the ^{14}C-Cys fast mineralization pool c_1 ranged from 0.6 h (site 3) to approximately 1.8 h (site 5), while the slower turnover pool has a much longer half-life, ranging from ca.10.1 d (site 5) to 67.8 d (site 4). On the other hand, between 7.9% (soil 5)

and 38.0% (soil 2) of ^{14}C-Met was allocated to the rapid mineralization pool in soils, with the slow turnover pool accounting for 66.4 - 92.4% of added ^{14}C-Met.

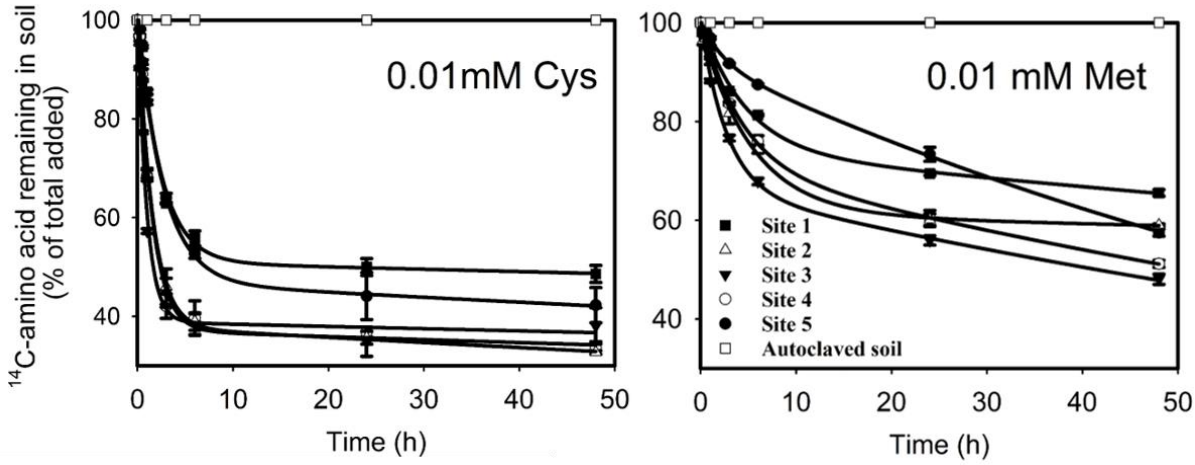

Fig. 4.6 Amount of ^{14}C-label remaining in soil solution and microbial biomass after the injection of ^{14}C labelled Cys and Met (0.01 mM) to soil collected from five contrasting grassland sites. Values represent means ± SEM (n = 3). Lines represent fits a double first order kinetic equations to the experimental data.

More ^{14}C-Met was retained in soil solution and soil microbial biomass one hour after incubation from all five sites compared to Cys (0.01 mM; Fig. 4.6; $p < 0.001$), due to faster mineralization of Cys than that of Met. One hour after substrate addition, the cumulative $^{14}CO_2$ evolved from added ^{14}C-Cys was 16.7 ± 0.5% (site 5) 31.4 ± 1.1% (site 4), 42.7 ± 0.5% (site 3), 31.0 ± 0.9% (site 2), and 14.5 ± 0.5% (site 1), whereas significantly ($p < 0.05$) less ^{14}C-Met was mineralized in all five soils, 3.0 ± 0.3% (site 5), 6.1 ± 0.6% (site 4), 11.8 ± 0.4% (site 3), 7.9 ± 0.6% (site 2), and 4.6 ± 0.1% (site 1). Furthermore, rates of amino acid mineralization in soil 5 were slower, with the amount of ^{14}C remaining in soil and soil microbial biomass being significantly greater than that in other four soils ($p < 0.05$).

4.3.2.3 S-amino acid mineralization affected by substrate concentrations

The double exponential decay equation gave a good fit to the depletion of both ^{14}C-Cys and Met from soil by microbial mineralization over time (R^2 > 0.98 in all cases). The exponential coefficients and half-lives of double exponential decay equations are presented in Table 4.5.

The proportion of Cys released through microbial respiration in the initial rapid phase of $^{14}CO_2$ evolution range from 3.5 ± 0.6% (100 mM) to 60.4 ± 0.1% (0.05 mM), with half-lives ranging from 1.5 ± 0.1 h to 14.5 ± 0.7 h, while the proportion of Cys respired in the secondary slower phase range from 41.4 ± 1.0% (1 mM) to 96.5 ± 0.6 (100 mM), with half-lives ranging from 6.6 ± 0.04 to 43.8 ± 3.7 d. More Cys was allocated in the first fast-turnover pool than in the secondary slow-turnover pool when added at 0.01 mM, 0.05 mM, 0.1 mM and 1 mM, yet more Cys was allocated in the second slow-turnover pool when added at 10 mM and 100 mM.

On the other hand, more Met was retained in soil solution and microbial biomass compared with Cys at all concentrations (p < 0.001) at all our sampling time points (Fig. 4.7). Less than 50% of added Met was allocated to the first initial rapid mineralization pool at concentrations of 0.01 mM, 0.05 mM, 0.1 Mm and 100 mM with half-lives for the fast turnover of c_1 pool ranging from 4.0 ± 0.1 to 781.7 ± 12.8 h, while the slower pool (c_2) had a much slower half-life, ranging from 17.2 ± 0.5 to 32.5 ± 0.5 d following substrate addition. More Met was allocated to the first initial rapid phase when added at 1mM and 10 mM, with half-life of 17.5 ± 0.1, 114.8 ± 19.2 h for the turnover of fast pool, 57.3 ± 7.7, 4.8 ± 0.8 d for the turnover of slow pool, respectively.

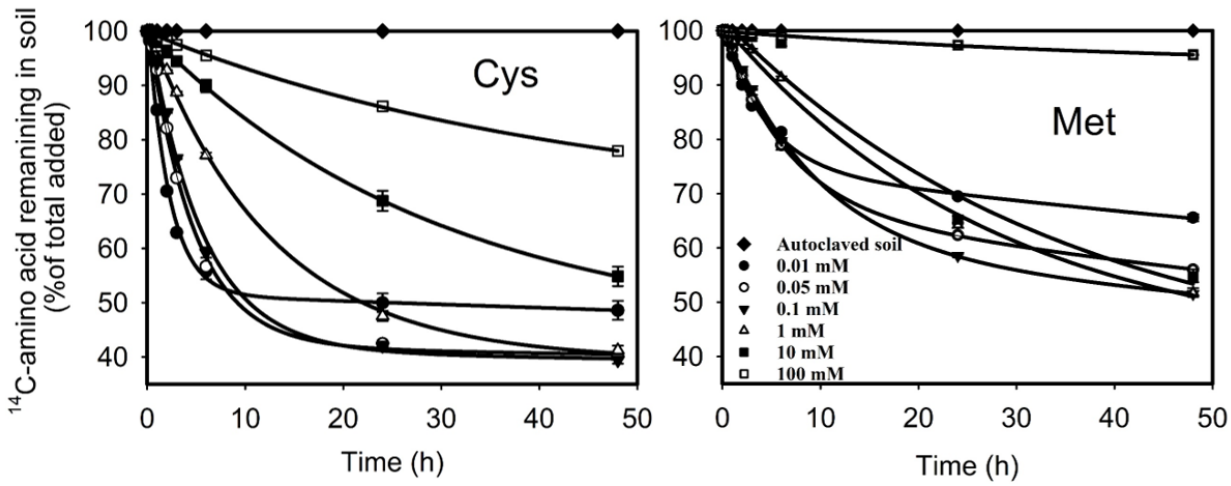

Fig. 4.7 Amount of ^{14}C- Cys and Met remaining in soil solution and soil microbial biomass within 48 hours added at 6 concentrations ranging from 0.01 to 100 mM (soil 1). Data points represent means ± SEM (n = 3). Lines represent fits of single or double first order exponential equations to the experimental data.

In the non-sterile soil, within 30 minutes after substrate application, following addition of the ^{14}C-labelled Cys or Met (0.01 mM) to the soil, ^{14}C released from cumulative microbial respiration accounted for 5.3 ± 0.3% and 1.9 ± 0.01% of the added ^{14}C-Cys and Met, this fell to 0.5 ± 0.1% and 0.2 ± 0.03% when added at the highest concentration (100 mM). In absolute terms, however, the actual amount of Cys and Met removed from soil solution by soil microbial respiration increased with increasing amino acid concentration, at the highest added amino acid concentration (100 mM), mineralization rates were equivalent to 3.7 µmol ^{14}C-Cys (kg^{-1} DW soil) min^{-1} and 1.3 µmol ^{14}C-Met (kg^{-1} DW soil) min^{-1}, respectively after only one minute.

According to our results, 5.63 ± 0.33% and 1.94 ± 0.01% of the added ^{14}C-Cys and Met taken up by the microbial community from the soil solution within 30 minutes were subsequently used for microbial respiration when added at 0.01 mM, respectively (Table 4.6). This fell to 0.98 ± 0.13% and 0.40 ± 0.05% when added at the highest concentration (100 mM).

Table 4.2 Coefficients of single first order curves describing the depletion of Cys and Met from soil solution over time in 5 contrasting grassland soils along an altitudinal productivity gradient. A_1 and A_2 are estimated pool sizes for fast and slow substrate uptake routes, and K_1 and K_2 are the rate constants for the fast and slow uptake routes, respectively. Half time values for pools A_1 and A_2 are represented by $t_{½}$ values and these are determined from K_1 and K_2. Values represent means ± SEM (n = 3).

Substrate	Site	A_1	K_1	A_2	K_2	A_1 $t_{½}$ (min)	A_2 $t_{½}$ (min)
Cys	Site 1	99.9 ± 5.9	2.03 ± 0.46			0.34 ± 0.02	
	Site 2	100.3 ± 8.0	0.44 ± 0.08			1.58 ± 0.15	
	Site 3	61.3 ± 6.6	0.93 ± 0.25	37.3 ± 5.1	0.03 ± 0.01	0.83 ± 0.55	25.6 ± 4.5
	Site 4	59.7 ± 2.8	1.38 ± 0.18	40.4 ± 2.0	0.03 ± 0.00	0.48 ± 0.18	21.8 ± 3.6
	Site 5	68.9 ± 3.2	0.96 ± 0.11	31.1 ± 2.4	0.03 ± 0.00	0.76 ± 0.37	27.9 ± 3.6
Met	Site 1	100.1 ± 1.7	0.91 ± 0.04			0.76 ± 0.03	
	Site 2	91.3 ± 9.8	0.42 ± 0.11			1.64 ± 0.32	
	Site 3	50.0 ± 3.5	1.55 ± 0.28	50.0 ± 2.7	0.05 ± 0.01	0.40 ± 0.24	12.9 ± 3.3
	Site 4	35.8 ± 7.2	2.92 ± 3.11	64.2 ± 6.1	0.09 ± 0.01	0.22 ± 0.38	8.0 ± 1.2
	Site 5	82.4 ± 15.1	0.24 ± 0.05	17.1 ± 15.5	0.04 ± 0.03	2.14 ± 1.65	9.0 ± 7.6

Table 4.3 Influence of amino acid concentration on the coefficients of single (0.01 mM and 0.05 mM) and double (0.1-100 mM) first order curve fits describing the depletion of ^{14}C from soil solution over time. A_1 and A_2 are estimated pool sizes for fast and slow substrate uptake routes, and K_1 and K_2 are the rate constants for the fast and slow uptake routes, respectively. Half time values for pools A_1 and A_2 are represented by $t_{½}$ values and these are determined from K_1 and K_2, respectively. Values represent means ± SEM (n = 3).

Substrate	Concentration	A_1	K_1	A_2	K_2	A_1 $t_{½}$ (min)	A_2 $t_{½}$ (min)	R^2
Cys	0.01 mM	99.9 ± 5.9	2.03 ± 0.46			0.34 ± 0.02		0.98
	0.05 mM	92.1 ± 7.2	0.41 ± 0.08			1.69 ± 0.12		0.97
	0.1 mM	85.0 ± 11.3	0.28 ± 0.09			2.45 ± 0.15		0.92
	1 mM	48.5 ± 4.5	3.42 ± 2.78	51.4 ± 2.6	0.01 ± 0.002	0.17 ± 0.15	53.2 ± 15.9	0.99
	10 mM	40.6 ± 5.2	1.38 ± 0.49	59.1 ± 3.0	0.01 ± 0.001	0.52 ± 0.15	109.7 ± 23.8	0.98
	100 mM	38.8 ± 3.0	1.43 ± 0.32	61.1 ± 1.7	0.004 ± 0.001	0.48 ± 0.14	155.0 ± 41.9	0.99
Met	0.01 mM	100.0 ± 1.7	0.91 ± 0.04			0.76 ± 0.03		0.99
	0.05 mM	87.6 ± 9.8	0.30 ± 0.08			2.29 ± 0.04		0.94
	0.1 mM	88.9 ± 7.0	0.24 ± 0.04			2.85 ± 0.13		0.97
	1 mM	44.2 ± 1.9	2.80 ± 0.69	55.7 ± 1.0	0.01 ± 0.001	0.25 ± 0.04	59.4 ± 3.0	0.99
	10 mM	41.9 ± 3.5	1.45 ± 0.35	57.9 ± 1.9	0.004 ± 0.001	0.48 ± 0.08	185.9 ± 87.6	0.99
	100 mM	35.2 ± 1.7	1.70 ± 0.26	64.7 ± 0.9	0.002 ± 0.001	0.35 ± 0.31	307.5 ± 142.0	0.99

Table 4.4 Kinetic coefficients of single and double exponential models describing the depletion of ^{14}C-Cys and Met from soil and loss in microbial respiration over time in 5 contrasting grassland soils along an altitudinal productivity gradient. $AA_{catabolic}$ and $AA_{anabolic}$ are estimated pool sizes for fast and slow substrate uptake routes, and K_3 and K_4 are the rate constants for the fast and slow uptake routes, respectively. Half-life values for pools $AA_{catabolic}$ and $AA_{anabolic}$ are determined from K_3 and K_4, respectively. Values represent means ± SEM (n = 3).

Substrate	Site	$AA_{catabolic}$	K_3	$AA_{anabolic}$	K_4	Catabolic $\mathbf{t}_{½}$ (h)	Anabolic $\mathbf{t}_{½}$ (d)	R^2
Cys	Site 1	51.4 ± 3.4	0.4 ± 0.1	51.0 ± 3.5	0.001 ± 0.002	1.6 ± 0.1	31.2 ± 6.3	0.99
	Site 2	64.7 ± 3.2	0.7 ± 0.1	37.9 ± 3.0	0.003 ± 0.002	1.1 ± 0.0	11.5 ± 5.0	0.99
	Site 3	62.8 ± 4.0	1.0 ± 0.2	38.9 ± 3.1	0.001 ± 0.002	0.6 ± 0.0	10.4 ± 2.5	0.99
	Site 4	61.2 ± 5.1	0.7 ± 0.1	37.2 ± 4.7	0.002 ± 0.004	0.9 ± 0.2	67.8 ± 50.1	0.99
	Site 5	53.3 ± 0.8	0.4 ± 0.0	46.9 ± 0.9	0.002 ± 0.001	1.8 ± 0.4	10.1 ± 7.8	0.99
Met	Site 1	26.3 ± 2.3	0.2 ± 0.0	74.1 ± 2.3	0.002 ± 0.001	3.2 ± 0.1	11.2 ± 0.6	0.99
	Site 2	38.0 ± 3.0	0.2 ± 0.0	61.2 ± 3.1	0.001 ± 0.001	3.7 ± 0.9	20.7 ± 7.6	0.99
	Site 3	34.0 ± 1.8	0.4 ± 0.0	66.4 ± 1.9	0.007 ± 0.001	1.9 ± 0.1	4.3 ± 0.3	0.99
	Site 4	29.3 ± 1.7	0.2 ± 0.0	71.4 ± 1.8	0.006 ± 0.001	2.9 ± 0.4	4.2 ± 0.5	0.99
	Site 5	7.9 ± 0.6	0.4 ± 0.1	92.4 ± 0.7	0.009 ± 0.000	1.9 ± 0.9	3.0 ± 0.2	0.99

Table 4.5 Influence of amino acid concentration on the kinetic coefficients of double exponential models describing the depletion of ^{14}C-Cys and Met from soil and loss in microbial respiration over time over a 48 h-incubation. Values represent means ± SEM (n = 3).

Substrate	Concentration	$AA_{catabolic}$	K_3	$AA_{anabolic}$	K_4	Catabolic $t_{½}$ (h)	Anabolic $t_{½}$ (d)	R^2
Cys	0.01 mM	52.1 ± 2.0	0.4 ± 0.03	50.5 ± 2.0	0.001 ± 0.0001	1.5 ± 0.1	43.8 ± 3.7	0.99
	0.05 mM	60.4 ± 0.1	0.2 ± 0.001	41.8 ± 0.1	0.001 ± 0.0001	3.2 ± 0.02	33.8 ± 1.9	0.99
	0.1 mM	60.1 ± 0.9	0.2 ± 0.003	41.7 ± 0.8	0.001 ± 0.0001	3.8 ± 0.1	23.3 ± 2.6	0.99
	1 mM	59.2 ± 1.1	0.08 ± 0.001	41.4 ± 1.0	0.001 ± 0.00003	8.4 ± 0.1	28.2 ± 0.9	0.99
	10 mM	41.2 ± 1.9	0.04 ± 0.002	58.9 ± 1.8	0.003 ± 0.0001	14.5 ± 0.7	11.7 ± 0.3	0.99
	100 mM	3.5 ± 0.6	0.2 ± 0.02	96.5 ± 0.6	0.004 ± 0.00003	4.4 ± 0.4	6.6 ± 0.04	0.99
Met	0.01 mM	30.5 ± 0.6	0.2 ± 0.01	69.6 ± 0.6	0.001 ± 0.00003	4.0 ± 0.1	25.9 ± 0.8	0.99
	0.05 mM	40.2 ± 0.2	0.1 ± 0.01	60.2 ± 0.3	0.002 ± 0.00003	5.9 ± 0.5	18.8 ± 0.4	0.99
	0.1 mM	46.1 ± 0.5	0.1 ± 0.002	54.5 ± 0.6	0.002 ± 0.00005	7.6 ± 0.2	17.2 ± 0.5	0.99
	1 mM	57.0 ± 0.9	0.04 ± 0.0002	44.4 ± 0.9	0.001 ± 0.00007	17.5 ± 0.1	57.3 ± 7.7	0.99
	10 mM	50.1 ± 0.3	0.01 ± 0.001	51.1 ± 0.1	0.006 ± 0.001	114.8 ± 19.2	4.8 ± 0.8	0.98
	100 mM	44.1 ± 0.1	0.001 ± 0.00002	55.6 ± 0.1	0.001 ± 0.00002	781.7 ± 12.8	32.5 ± 0.5	0.99

Table 4.6 Influence of amino acid concentration on the uptake and mineralization of either Cys or Met in the first 30 minutes after substrate addition, and the percent of amino acid taken up which was respired as $^{14}CO_2$ during the same time period. Values represent means ± SEM (*n* = 3).

		Rate of amino acid uptake (nmol amino acid kg^{-1} DW soil min^{-1})	Rate of amino acid mineralization (nmol amino acid kg^{-1} DW soil min^{-1})	Percentage of ^{14}C taken up that is subsequently respired (%)
Cys	0.01 mM	63 ± 1	3.5 ± 0.1	5.63 ± 0.33
	0.05 mM	329 ± 1	9.63 ± 0.4	2.93 ± 0.14
	0.1 mM	637 ± 1	15.0 ± 0.5	2.37 ± 0.09
	1 mM	4558 ± 239	92.8 ± 2.5	2.04 ± 0.16
	10 mM	35460 ± 2950	666.3 ± 95.4	1.88 ± 0.24
	100 mM	326084 ± 25950	3196.3 ± 610.4	0.98 ± 0.13
Met	0.01 mM	65 ± 1	1.2 ± 0.0	1.94 ± 0.01
	0.05 mM	319 ± 1	4.7 ± 0.0	1.50 ± 0.02
	0.1 mM	649 ± 2	7.3 ± 0.2	1.12 ± 0.04
	1 mM	4112 ± 139	21.8 ± 2.6	0.53 ± 0.05
	10 mM	33071 ± 3350	148.1 ± 8.1	0.45 ± 0.04
	100 mM	288617 ± 25564	1141.9 ± 201.2	0.40 ± 0.05

4.3.3 Carbon turnover rates in soil

Microbial mineralization of amino acid-^{14}C to $^{14}CO_2$ was extremely fast, but slower than amino acid-^{14}C uptake from the soil solution. A maximum of 59.9% of added amino acid was respired with half times of 1.6 - 4.5 min when taken up at amino acid concentration close to those found naturally in the soil solution (0.01 – 0.05 mM; Table 4.5; Table 4.7). The remainder was cycled through the microbial biomass before mineralization. At steady state, the rates of amino acid removal from the soil solution should be matched with new inputs from microbial/plant root turnover, exudation and hydrolysis of soil organic matter. Therefore assuming 0.01 – 0.05 mM are the closest concentrations to those in natural soil solution, we estimate that the rate of S-containing amino acids inputs to grassland soil was between 6.35 - 329.0 nmol amino acid kg^{-1} DW soil min^{-1}.

Table 4.7 Influence of amino acid concentration on microbial C use efficiency (CUE_{mic}). Values represent means ± SEM (n = 3).

Substrate	Soil solution concentration	CUE_{mic}
Cys	0.01 mM	0.49 ± 0.02
	0.05 mM	0.41 ± 0.001
	0.1 mM	0.41 ± 0.01
	1 mM	0.41 ± 0.01
	10 mM	0.59 ± 0.02
	100 mM	0.96 ± 0.01
Met	0.01 mM	0.69 ± 0.01
	0.05 mM	0.59 ± 0.002
	0.1 mM	0.54 ± 0.005
	1 mM	0.43 ± 0.008
	10 mM	0.51 ± 0.001
	100 mM	0.56 ± 0.001

4.4 Discussion

4.4.1 Kinetics of Cys and Met uptake by soil microbial community

Across all five sites, DON was the dominant form or soluble N within the soil solution, contributing between 22.5 to 82.3% of total DON in five sites. Similarly, DOS was the dominant form of soluble S within the soil solution, contributing between 81.1 to 87.1% of total dissolved S across the five sites (Table 4.1). This is in line with our findings in Chapter 3 (Table 3.1). The concentration of DOS was of the same order magnitude in all five soils ranging from 27.5 to 51.0 mg kg^{-1} soil DW, while the sulphate concentrations were one order of magnitude lower, varying from 4.4 to 11.9 mg kg^{-1} soil DW. Therefore, in our selected grasslands, DON and DOS represent important reservoirs of soluble N and S in the soil. This is also in agreement with previous studies (Farrell, Hill, Farrar, *et al.*, 2011; Meena and Improvement, 2018).

The results presented here are direct evidence to support earlier work that the uptake of amino acids by soil microbial community from soil solution can be exceedingly rapid (Jones and Kielland, 2012; Farrell, Prendergast-Miller, *et al.*, 2014; Wilkinson, Paul W. Hill, *et al.*, 2014; Carswell *et al.*, 2016). Within only 10 minutes after substrate addition, over 80% of added ^{14}C-Cys or Met had been removed from soil solution, with the exact amount varying depending on soil conditions and substrate concentrations. By the end of our 60-minute incubation experiment, less than 5% of the added amino acid-^{14}C were retained in soil solution. Moreover, given that we removed living roots in this study (and associated symbionts), it is possible that substrate depletion could occur even more rapidly *in situ* than those measured here.

Calculated half-lives of both Cys and Met depletion indicated that both the substrate itself and soil properties are strong factors influencing the rates of amino acid turnover in soil solution. On the one hand, Cys removal from soils collected from lower altitude, more

productive sites (e.g. site 1) was twice as fast as that from high altitude, less productive sites (e.g. site 5), with the same true for Met. This is supported by previous work showing that amino acids and peptide turnover rates were much faster in higher productivity grassland than in low productivity grasslands (Farrell, Macdonald, *et al.*, 2014; Wilkinson, Paul W. Hill, *et al.*, 2014). On the other hand, the substrate itself is also an important factor affecting amino acid turnover rate. For all five soils, the half-life of Met depletion was greater than that of Cys depending on soil conditions. This is consistent with previous studies indicating that, Met, a "resistant" amino acid, was degraded slower than Cys in soil (Greenwood and Lees, 1960).

The range of Cys and Met concentrations employed here was chosen to reflect those likely to be found in soils. Lower concentrations may be expected to occur after dilution of soil native water with rainfall (0.01 - 0.1 mM; (Werdin-Pfisterer, Kielland and Boone, 2009)), while higher concentrations may occur upon lysis of root cell in the rhizosphere or the addition of amino acid-based fertilisers (1 - 100 mM). Under all concentrations, the depletion rate of Cys and Met from soil solution was extremely high. Our results suggested that under low Cys and Met concentrations (0.01, 0.05 and 0.1 mM), soil microbial uptake of amino acids occurred via a single carrier system, while a second uptake pathway became important when substrate was added at higher concentrations (1 mM, 10 mM, 100 mM). The concentration of low molecular weight S-containing amino acid at which the secondary transport system becomes important here (1 mM) is higher than that found in a previous study (Paul W. Hill, Farrar and Jones, 2008), in which 0.01 and 0.1 mM was found to be the point where the second transport system became important in the microbial uptake of glucose. It is not clear whether these differences resulted from different experimental conditions or soil conditions.

4.4.2 Dynamics of Cys and Met mineralization by soil microbial community

As Cys or Met are weakly adsorbed in the grassland soil used here (data shown in Fig. 3.2, chapter 3), and very little $^{14}CO_2$ evolution was observed in the autoclaved treatment within 48 h during our experiment time-period (Fig. 4.5), we assume that amino acid mineralization from soil solution occurred entirely due to microbial activity. It is likely that mineralization of biological sulphur to inorganic sulphate under the action of microbial community is the rate determining step in the soil sulphur cycle (Meena and Improvement, 2018; Udayana *et al.*, 2021). It is striking that within only one hour of substrate addition (0.01 mM) to soil, soil microbes mineralized >10% of added ^{14}C-Cys and > 2% of added ^{14}C-Met, suggesting that soil microbes are severely carbon limited. Overall, the mineralization rates of Cys and Met by the soil microbial community were extremely rapid, but much slower than substrate uptake from soil solution, indicating that the uptake and mineralization of cysteine and methionine were decoupled in time. We ascribe this lag in mineralization to the need to strip the N and S from the amino acids prior to incorporation of the carbon skeletons into respiratory cycles. Across most sites, $^{14}CO_2$ evolution showed a biphasic pattern, which coincides with those reported previously for other low MW substrates (Paul W. Hill, Farrar and Jones, 2008; Wilkinson, Paul W. Hill, *et al.*, 2014). However, with a similar pool size in general, ^{14}C allocated to the rapid mineralization phase was respired much faster than that of the secondary slower pool (by two orders of magnitude). This supports the model that the second pool is due to the slow turnover of ^{14}C incorporated into the microbial biomass (e.g. from meosfaunal grazing).

Across all five grassland sites, when added at 0.01 mM the proportion of added ^{14}C-Cys recovered as $^{14}CO_2$ was higher than 50%, while the reverse was true for ^{14}C-Met. These results suggested that Cys is a more readily available substrate for soil microbes (i.e. anabolic processes), hence more Cys was broken down in the initial phase, while more Met was allocated to anabolic pools. When added at 100 mM, the proportion of added ^{14}C-Cys released

as $^{14}CO_2$ following rapid substrate mineralization was low (3.5%, Table 4.5), the remainder was cycled through microbial biomass before mineralization, emphasizing the importance of microbial biomass cycle in the process of microbial substrate mineralization.

4.5 Conclusions

In all five grassland soils, the microbial depletion of added ^{14}C-Cys and Met (added at a concentration of 0.01 mM) from soil solution was extremely rapid. However, although our experiments were carried out on soil shortly after collection from the field (less than 48h), due to the fact that we had removed plant roots, which have been proved to contribute to nutrient cycling (Cheng and Kuzyakov, 2005; Azcón-Aguilar and Barea, 2015), the actual turnover rates of Cys and Met in situ could be even faster. Therefore, although Cys and Met may represent only a minor component of DON and DOS pools in soil, the importance of Cys and Met for soil microbes and plant nutrition may have been underestimated due to its very fast turnover and replenishment rates in soil. We assume it's the cleavage of proteins into LMW DOS compounds rather than the uptake of amino acids from soil that limits the soil organic S cycle.

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4.7 References

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Chapter 5

Competition for S-containing amino acids (cysteine and methionine) between soil microorganisms and maize roots in the rhizosphere

Abstract

The direct uptake of amino acids from soil solution has been proposed as a potential route by which plants can acquire nitrogen (N), particularly in nutrient-limiting ecosystems. The factors regulating the potential acquisition of sulphur-containing amino acids by roots, however, remains poorly understood. Using a ^{14}C-labelling approach, we independently investigated the short-term (24 h) uptake of two S-containing amino acids, cysteine and methionine, by the soil microbial community and maize plants (*Zea mays* L.). We then studied their relative ability to capture amino acids when in competition with each other. Our results show that individually, both soil microbes and maize plants have a high ability to utilize cysteine and methionine (added at a concentration of 0.1 mM). Soil microbes captured cysteine and methionine at a rate of 413 ± 2 and 408 ± 2 nmol (kg soil)$^{-1}$ h^{-1} respectively, while uptake rates of individual amino acids by maize roots were 203 ± 36 nmol (g root DW)$^{-1}$ h^{-1} for cysteine, and 192 ± 31 nmol (g root DW)$^{-1}$ h^{-1} for methionine. When in competition with each other, the capture of free cysteine and methionine by the maize plants was very low compared to soil microbes, with only 8.8 ± 1.6 of the added amino acid captured by the plant (8.8 ± 1.9% or the total), compared to the rhizosphere microbial community (76.9 ± 8.9%). In addition, to examine the significance of cysteine and methionine for plant sulphur nutrition in the rhizosphere, we also examined how maize plants and soil microbes vary in their ability to take up and compete for organic (^{35}S-cysteine, ^{35}S-methionine) and inorganic sulphur ($Na_2{}^{35}SO_4$) over a short time scale (24 h) using ^{35}S tracer techniques. Our results showed that soil microbes could capture 16.9% of added inorganic sulphur, with 30.7% captured by plant roots; while maize plants could take up less than 10% of added amino acid-^{35}S, over 50% was recovered in the microbial biomass. These results, however, indicate that all three experimentally added sulphur forms contributed available sulphur for plant uptake, although rhizosphere microbial community showed higher capacity for capture of free cysteine and methionine in the

rhizosphere than maize plants. We suggest that this is a result of relatively high availability of inorganic nutrient in soil solution and the lack of transmembrane for amino acids on plant root cells, as well as the rapid turnover of amino acids by soil microbes in the rhizosphere. Our results also show that the addition of inorganic N and S, not C, significantly reduced the uptake of cysteine and methionine by maize plants in the rhizosphere, indicating that amino acid utilization by maize plants in our study could be regulated by inorganic N and S availability. Overall, our results imply that (1) cysteine and methionine are sources of available N and S for maize plants, contributing to total plant N and S demand, (2) amino acid mineralization rates underestimate N and S supply rates to plants, (3) plants may compete better with soil microbes when concentrations of free cysteine and methionine in soil are high.

Keywords: Cysteine; Methionine; Dissolved organic sulphur (DOS); Plant-microbial competition; Rhizosphere.

5.1 Introduction

Sulphur (S) is an essential nutrient for plants, being required for the biosynthesis of essential amino acids (i.e. cysteine and methionine; Wirtz and Droux, 2005), oligopeptides (e.g. glutathione and phytochelatins; Na and Salt, 2011), vitamins (e.g. biotin, thiamine), enzyme co-factors (e.g. Fe-S sulphur clusters), and a variety of secondary metabolites (e.g. glucosinolates and alliins; Ravilious and Jez, 2012). An adequate supply of S is therefore essential to ensure optimal crop growth, however, there is increasing evidence that S deficiency is becoming more widespread in agricultural systems (Aula *et al.*, 2019). Although S fertilisers are produced relatively cheaply as a by-product from petrochemical processing, S use efficiency in agriculture is typically very low (<25%) (Eriksen, 2009b). There is therefore a need to better understand plant-soil-microbial S cycling to enable us to devise more sustainable agricultural systems. It is generally assumed that plants can only acquire S in an inorganic form

(SO_4^2; Prasad and Shivay, 2018). However, there is growing evidence that plants can also take up a range of nutrients (e.g. N, P, Fe) held in low molecular weight (MW) organic forms, whether this applies to S remains largely unknown.

Generally, more than 90% of S held in soil is present in an insoluble organic form (Johnson, 1984). Of this, a large proportion is present in amino acids (e.g. cysteine, methionine) which are contained in proteinaceous material which makes up ca. 20-30% of soil organic matter (SOM; Stevenson, 1982). Soil microorganisms (including bacteria, archaea and fungi) play a major role in releasing plant-available sulphate through the oxidation of organic S (McLaren and Swift, 1977; Michael A. Kertesz and Mirleau, 2004a). Specifically, the release of microbially-derived sulfatases can release SO_4^{2-} from outward facing S groups in SOM. In addition, the microbial community can release exo- and endo-peptidases to release S-containing amino acids and oligopeptides into soil solution. In contrast, S embedded within SOM particles or bound to mineral surfaces may not be readily available for plants due to chemical or physical protection (Scherer, 2001).

Observations in Arctic and sub-Antarctic ecosystems have shown that the supply of inorganic N is often insufficient to meet the annual N requirement of many plant species (Smith and Steenkamp, 1992; Kielland, 1994). This has raised awareness that free amino acids and oligopeptides in soil solution, may represent a readily available source of N and C for plants as well as soil microorganisms (Geisseler *et al.*, 2010). Quantitative estimates of the contribution of this type of organic S to plant nutrition, however, remain unknown. There is very good evidence to show that plant roots can take up exogenous amino acids and oligopeptides intact (Abuzinadah et al., 1986; Yamagata and Ae, 1999; Nishizawa and Mori, 2001; Weigelt et al., 2005; Tegeder and Rentsch, 2010; Hill et al., 2011). Traditionally, however, plant roots have been considered poor competitors with soil microbes for organic nutrients in soil (Kuzyakov and Jones, 2006; Lœrkedal *et al.*, 2008). Despite this, roots possess a wide range of H^+-ATPase

fuelled amino acid and sugar co-transporters which appear to be constitutively expressed (Rentsch, Schmidt and Tegeder, 2007; D L Jones, Nguyen and Finlay, 2009). These have the capacity to take up a selected range of low MW solutes (e.g. amino acids, sugars, peptides, polyamines, urea) but not others (e.g. amino sugars, tricarboxylic acids; Jones et al., 2009; Warren, 2016). It is assumed that the selective uptake and incorporation of N, and presumably S, from amino acids requires less energy in comparison to the assimilation of NO_3^-, NH_4^+ of SO_4^{2-} (Yamagata and Ae, 1999). Others have hypothesized that uptake of amino acids from the rhizosphere is an important way to recapture C lost in root exudation to prevent excessive microbial growth in the rhizosphere and simultaneously reduce nutrient loss to the soil (Jones et al., 2009). Alternatively, these root transporters may be used in root signal transduction cascades and used to regulate developmental processes (Walch-Liu *et al.*, 2006).

Here we focus on the uptake of the two main S-containing amino acids in plants, cysteine and methionine (Noji and Saito, 2003). These compounds represent 70% of the S contained in plant tissues, although they are often only present in low abundance relative to other amino acids (Kumar *et al.*, 2019). The potential importance to crop plants of taking up S-containing amino acids from soil is likely to depend on the level of competition between the root and the rhizosphere microbial community (Cheng and Bledsoe, 2004; Xu *et al.*, 2008). Based on studies of other organic-N containing solutes, we expect that this competition will be particularly strong when concentrations of organic S are low in the soil solution (A. G. Owen and Jones, 2001). This competition may also be influenced by a range of abiotic factors, including the relative availability of inorganic N and S. Our primary aim was therefore to investigate the capacity of maize (*Zea mays* L.) to take up cysteine (Cys) and methionine (Met) and to directly compare these rates to those of the soil microbial community. As S uptake and metabolism has been shown to be influenced by intracellular levels of S-containing amino acids in plants (García et al., 2015; Miranda et al., 2001a; Smith et al., 1997), it also suggests that

there could be a close interplay between inorganic and organic S availability in the rhizosphere and root transport processes. Our secondary aim was therefore to manipulate the availability of C, N and S in the rhizosphere to determine its impact on Met and Cys capture. We hypothesized that in the short term, elevated C availability would promote increased plant capture of Cys and Met due to the provision of an alternative C supply to the microbial community. Further, we predicted that N and S addition would have no effect on Cys and Met uptake if the root amino acid transporters are constitutively expressed.

5.2 Materials and methods

5.2.1 Soil sampling and chemical characterisation

An agricultural soil (Eutric Cambisol) was collected from the Ah horizon (0-10 cm depth) of a temperate *Lolium perenne* L. dominated grassland site located at Abergwyngregyn, Gwynedd, North Wales (53°14'N, 4°01'W). This lowland grassland site is unfertilized, free draining, and lightly grazed by sheep (Fig. 5.1). Properties of the soil are listed in Table 5.1. Approximately 1 kg of soil was collected from three randomly positioned replicate plots, located 2 m apart, within our study site (5 × 5 m^2). In terms of N availability, the site is characterised as being spatially homogenous (Shaw et al., 2016). The soil was then placed in gas permeable plastic bags and transferred immediately to the laboratory. These samples represented the three independent replicates for all experiments. On return to the laboratory, the crumb structured, sandy clay loam textured soil was sieved to pass 2 mm before being stored at 4 °C until required for experimentation.

Soil moisture content was assessed by oven drying soil at 80 °C. Soil pH and electrical conductivity were analysed in a 1:5 (w/v) soil: deionised water suspension using standard electrodes. To determine the levels of available N, fresh soil (5 g) was shaken (200 rev min^{-1}, 15 min) with 0.5 M K_2SO_4 (25 ml), the suspension centrifuged (10,000 *g*, 15 min) and the

supernatant retained for analysis. The concentration of NH_4^+ in the extracts was determined colorimetrically on a Synergy MX micro-plate reader using the salicylic acid method of Mulvaney (1996), while NO_3^- was determined colorimetrically using the vanadate procedure of Miranda et al. (2001b). Total free amino acids in the extracts were determined fluorometrically using the *o*-phthaldialdehyde/β-mercaptoethanol procedure of Jones et al. (2002), while total protein was determined colorimetrically according to Jones and Kielland (2002). To determine the levels of available S, soil was shaken (200 rev min^{-1}, 15 min) with distilled water (1:5 w/v). All extracts were centrifuged (8000 *g*, 10 min), 0.45 µm syringe filtered and frozen at -20°C prior to analysis. The concentration of sulphate and other major anions in the extract was determined by ion chromatography (IC; Dionex corporation, ICS 2100, USA; Zhao and McGrath, 1994) according to ISO 10304-1:2009. Total dissolved S (DOS plus inorganic sulphate) and other major cations were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES; Varian 710ES, Agilent Technologies, USA) according to ISO 11885:2016. Dissolved organic S (DOS) concentrations were calculated from the difference between TDS and inorganic sulphate (David and Mitchell, 1985).

Table 5.1 Summary of the main properties of the soil used in the experiments. Values represent means ± SEM (*n* = 3). Data are presented on a dry soil weight basis.

Parameter	
Altitude (m)	32
Water content[a] (%)	17.11 ± 0.17
pH[b]	5.92 ± 0.02
Electrical conductivity[b] (µS cm^{-1})	73.50 ± 3.55
NH_4^+-N[c] (mg kg^{-1})	1.14 ± 0.14
NO_3^--N[c] (mg kg^{-1})	17.05 ± 0.33
Amino acids-N[c] (mg N kg^{-1})	0.61 ± 0.15
Protein-N[c] (mg N kg^{-1})	2.95 ± 1.37
Sulphate-S[d] (mg S kg^{-1})	4.37 ± 0.79
Total dissolved S[e] (mg S kg^{-1})	31.62 ± 2.14

[a]Measured by drying at 105 °C overnight and expressed on a wet weight basis.
[b]Measured in a 1:5 (v/v) soil-to-distilled water extract.
[c]Measured in a 1:5 (w/v) 0.5 M K_2SO_4 extract followed by colorimetry analysis via a Synergy-MX microplate reader.
[d]Measured in 1:5 w/v soil: distilled water extracts using an ICS-2011 ion chromatograph.
[e]Measured in 1:5 w/v soil: 0.01 M $Ca(H_2PO_4)_2$ extracts using a Varian 710ES ICP-OES.

Fig. 5.1 Detailed photographs of the area of our study.

5.2.2 Rhizosphere microbial uptake and mineralization of ^{14}C-Cys and Met

Maize plants were grown in soil-filled microcosms (rhizotubes) as described in Ström et al. (2002). The rhizotubes were constructed from nylon tube (250 mm long, 9 mm dia.) which expanded over a 0.5 cm span to a 50 mm long, 20 mm diameter section which was used to hold the seed. Holes (0.5 mm diameter) were pierced at 10 mm intervals down the length of the main rhizotube to ensure aeration. Before the addition of the pre-germinated plant, the microcosms were filled 15 ± 0.5 g of soil to a bulk density of 1.16 g cm^{-3} to reflect that in the field. After 7 d of plant growth, all of the soil volume contains primary and secondary roots and was effectively classed as rhizosphere soil. The microcosms were then harvested, the roots removed and the rhizosphere soil retained for further experimentation.

To determine the microbial turnover of Cys and Met in the rhizosphere soil, 0.5 ml of uniformly radiolabelled ^{14}C-Cys or Met (0.1, 1 or 10 mM; PerkinElmer Inc, Waltham, MA) was added to 5 g of soil contained in a 50 cm^3 sterile polypropylene tube. This range was chosen to reflect the free amino acid concentrations naturally present in rhizosphere soil solution or which arise from the lysis of a root or microbial cell (Dietz *et al.*, 1990; Jones, Shannon, *et al.*, 2005). After accounting for dilution in the native soil solution, this resulted in an actual final concentration of 0.03, 0.3 or 3 mM in soil solution.

To assess whether the breakdown of these two amino acids by the microbial biomass was affected by the availability of C, inorganic N or inorganic S, a very high concentration of either glucose (360 mg C kg^{-1} soil), NH_4Cl (30 mg N kg^{-1} soil) or Na_2SO_4 (30 mg S kg^{-1} soil) was added to the soil surface together with the ^{14}C-labelled amino acid solution. These levels were chosen based on a ca. 10-fold excess of glucose relative to Met and Cys at their highest addition rate, a C use efficiency of 50% and a microbial C:N:S ratio of 6:1. A control treatment was included where labelled Cys or Met was applied in distilled water alone.

After addition of either ^{14}C-labelled Cys or Met to the soil surface, 1 M NaOH traps (1 ml) were suspended above the soil to catch any respired $^{14}CO_2$. The tubes were then hermetically sealed and incubated at room temperature (20 ± 1 °C) in the dark. There were three replicates of each treatment. The NaOH traps were replaced after 0.5, 1, 2, 3, 12 and 24 h. The efficiency of the NaOH traps was > 98% (as determined by collecting $^{14}CO_2$ generated from adding excess 0.1 M HCl to 0.001 M Na$H^{14}CO_3$). Alongside the collection of $^{14}CO_2$, the amount of ^{14}C label remaining in soil solution was determined by destructive harvesting of replicate tubes and extraction with 25 ml of 0.5 M K_2SO_4 (200 rev min^{-1}, 15 min; Rousk and Jones, 2010). After shaking, the K_2SO_4 extracts were centrifuged (14,000 *g*, 5 min) and the supernatant recovered for analysis. The amount of ^{14}C in the K_2SO_4 extracts and NaOH traps was measured with HiSafe 3® scintillation cocktail (PerkinElmer, Waltham, MA) and a Wallac 1404 liquid scintillation counter (Wallac EG&G, UK) with automated quench correction. The amount of ^{14}C immobilised in the soil microbial biomass was calculated by the difference between ^{14}C microbial respiration plus that recovered in soil solution and the total amount of ^{14}C added to the soil (Glanville et al., 2016).

5.2.3 Rhizosphere microbial oxidation of Cys and Met in soil using ^{35}S labelling

A ^{35}S labelling approach was used to study the oxidation of Cys and Met to sulphate by the rhizosphere microbial community. As described above, 5 g of rhizosphere soil was placed in a 50 cm^3 polypropylene tube, except that 0.5 ml of either a ^{35}S-labelled Cys or Met solution was added uniformly to the soil surface at three concentrations (0.1, 1 or 10 mM; PerkinElmer Inc.). The samples were then incubated in the dark at room temperature.

After periods of 0.5, 1, 2, 3, 12 and 24 h, the soil was extracted with 25 ml of 0.01 M $CaCl_2$. After centrifugation (10,000 *g*, 15 min), the supernatant was divided into two portions. On one half, the total ^{35}S content was measured by liquid scintillation counting as described

above. This fraction contains any ^{35}S-Met or -Cys remaining in solution, any ^{35}S-intermediates and $^{35}SO_4^{2-}$. The remaining half was shaken with an equal volume of 0.1 M $BaCl_2$ (200 rev min^{-1}, 10 min) and centrifuged (14,000 *g*, 15 min) to remove any inorganic S (as a $Ba^{35}SO_4$ precipitate). The ^{35}S content of the supernatant was then determined (i.e. this fraction contains any ^{35}S-Met or -Cys remaining, any ^{35}S-intermediate products minus $^{35}SO_4^{2-}$). It was assumed that any ^{35}S not recovered from the soil was retained in the soil microbial biomass. All ^{35}S counts were decay-corrected to the start of the incubation (half-life 87.4 d). This approach relies on the low amount of SO_4^{2-} sorbed to the solid phase in this soil (Data shown in Fig. 3.1, Chapter 3).

5.2.4 Amino acid uptake by plants under sterile hydroponic conditions (^{14}C and ^{35}S)

Maize seedlings were grown under aseptic conditions in hydroponic culture using a 10% strength Long Ashton nutrient solution (Hewitt, 1952; Smith, Johnston and Cornforth, 1983). After 7 d, the maize plants were transferred to sealed plastic containers containing either ^{14}C-labelled or ^{35}S labelled S-containing amino acid (Cys or Met; 0.1 mM; (Jones and Darrah, 1992)).

For ^{14}C treaments, $^{14}CO_2$ respired from the plant was trapped using a 1 M NaOH trap (5 ml) while the amount of ^{14}C amino acid remaining in the nutrient solution was measured by removing small aliquots over time. After being exposed to the ^{14}C-amino acid for 24 h, the maize roots were rinsed with unlabelled nutrient solution and oven-dried (80 ℃ for 24 h). The ^{14}C content of the maize shoot and root were then measured with an OX-400 Biological Sample Oxidizer (RJ Harvey Instrument Corp., Hillsdale, NJ). ^{14}C in all the samples was measured by liquid scintillation counting as described above.

For the parallel ^{35}S treatments, after 24 h, the maize tissues were rinsed with unlabelled nutrient solution and oven-dried (80 ℃ for 24 h). The amount of ^{35}S in the plant tissues was

then determined by dissolving the plant tissues in Soluene-350 (40 °C, 4 h; PerkinElmer Inc.) followed by liquid scintillation counting as described above.

5.2.5 Competition for Cys and Met between plant and soil microorganisms in the rhizosphere

Maize seeds were soaked for 12 h in ultrapure water and then distributed evenly on moistened filter paper for 48 h to allow them to germinate at room temperature. Just after germination, uniform seedlings (n = 54) were transplanted into individual mesocosms (rhizotubes) as described above. When the maize roots were ca. 12 cm long (7 d after transplantation), a solution containing a ^{14}C- or ^{35}S-labelled solution of Cys or Met (0.1 mM; 0.25 ml) was injected at four different points along the rhizotubes through the aeration holes. This enabled a uniform labelling of the soil along the root length.

To determine the influence of nutrient addition on plant capture of free cysteine and methionine from soil solution, an excess of C, N or S (as glucose, NH_4Cl or Na_2SO_4) was added simultaneously with the ^{14}C-Met or Cys as described above. Again, the control treatment consisted of Cys or Met applied alone in distilled water. In addition, two higher amino acid concentrations (1 and 10 mM) were chosen to study the influence of free amino acid concentration on plant-microbial competition.

5.2.5.1 Competition for ^{14}C- Cys and Met in the rhizosphere

Individual mesocosms were then transferred to transparent gas-tight polypropylene containers (11×8 cm base and 27 cm high; Lock & Lock Ltd., Seoul, Republic of Korea; Fig. 5.2) with removable caps. Each container cap had a hole drilled in it (ca. 0.5 cm diameter), to allow physical separation of the plant shoot and root compartments. The shoot was sealed in the hole using non-phytotoxic silicon paste (Swinnen, 1994; Kuzyakov and Siniakina, 2001).

To recover $^{14}CO_2$ from the root and shoot compartments, 1 M NaOH traps (5 ml) were placed in each compartment.

24 h after injection of the amino acids, all the rhizotubes were destructively harvested by splitting the rhizo-tubes vertically with a razor blade, allowing the separation of the root, shoot, and soil components. The roots were rinsed with 0.01 M $CaCl_2$ for 30 s before being further washed with distilled water to visually remove all adhering soil. The ^{14}C or ^{35}S content of the washings was also determined by liquid scintillation counting and added to the soil solution fraction. After harvesting, the rhizosphere soil was divided into two parts: one part was shaken with 0.5 M K_2SO_4 (20 min, 200 rev min^{-1}), centrifuged (3800 *g*, 5 min) and the ^{14}C content of the supernatant measured as above. The remaining portion was used to determine the ^{14}C contained in the soil microbial biomass using the $CHCl_3$ fumigation-K_2SO_4 extraction method and an extraction efficiency correction factor of 0.35 (Voroney et al., 2008). Plant shoots and roots were oven-dried (80 °C for 24 h), after which their ^{14}C content was measured as described above.

5.2.5.2 Competition for ^{35}S-Cys and Met in the rhizosphere

For the ^{35}S labelling experiments, the amount of ^{35}S in the oven-dried plant tissues was determined by dissolving the plant tissues in Soluene-350 (40 °C, 4 h; PerkinElmer Inc.) followed by liquid scintillation counting as described above. ^{35}S was extracted from the fumigated and unfumigated soil using 0.01 M $CaCl_2$ (1:5 w/v) followed by liquid scintillation counting. Differences between the unfumigated and fumigated fraction were assumed to be retained in the soil microbial biomass (after correction using an extraction efficiency value of 0.35).

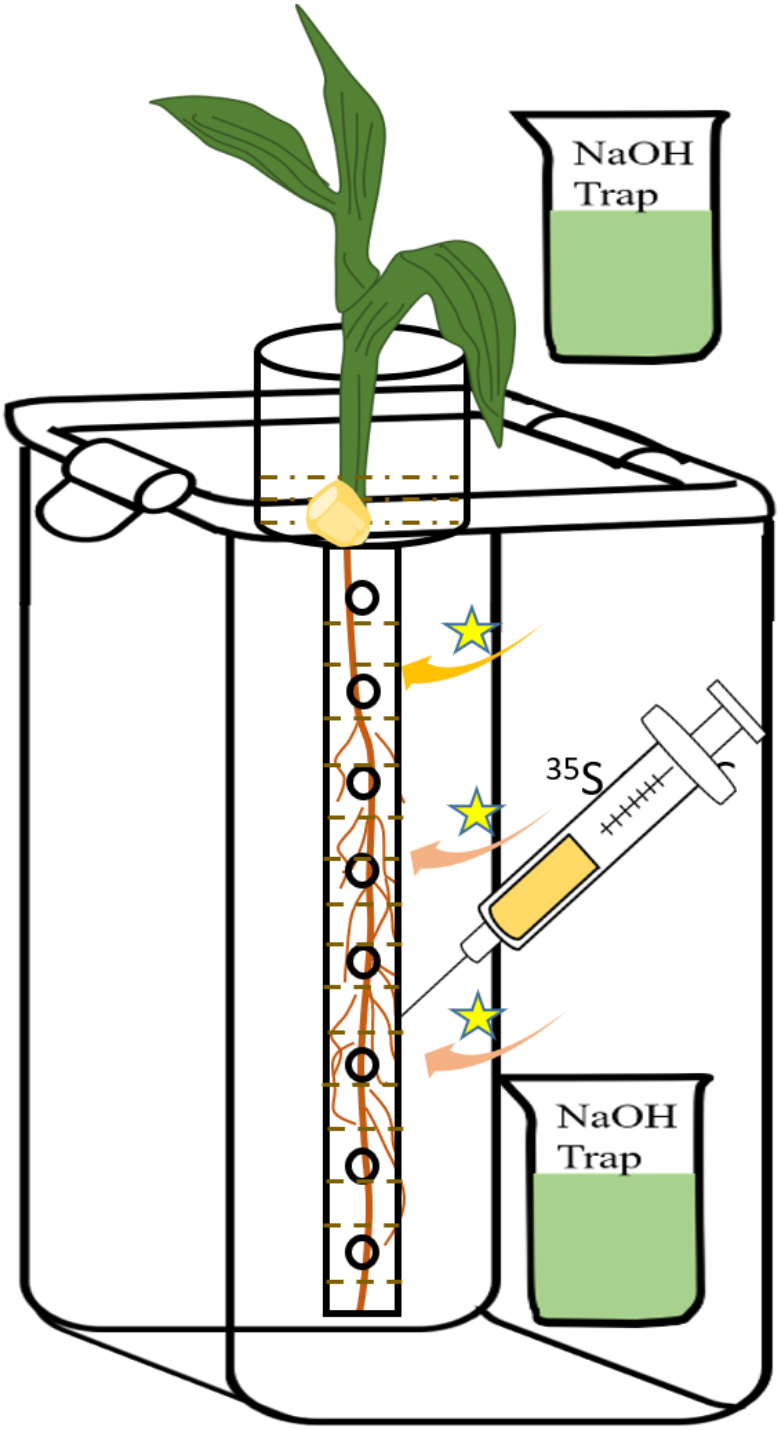

Fig. 5.2 Schematic representation of the experimental apparatus showing three-leaf stage maize plant growing down a rhizotube containing grassland soil in which ^{14}C or ^{35}S labelled amino acid is injected through pre-drilled holes. The uptake of amino acid into either plant biomass, respiration, or soil microbial biomass and respiration was determined over a 24 h chase period.

5.2.6 Statistics and data analysis

To determine the half time ($t_{½}$) of cysteine and methionine depletion in soil solution, a single first order exponential decay model was fitted to the experimental data for the 0.1 mM and 1 mM treatments according to:

$$S = y_0 + a_1 \times \exp^{(-b_1 \times t)} \quad \text{(Eqn. 1)}$$

Where S is the ^{14}C remaining in soil solution, b_1 is the exponential coefficient describing the rate of depletion of ^{14}C from soil solution by the soil microorganisms, a_1 describes the pool size and t refers to time. The half time $t_{½}$ of cysteine/methionine soil solution pools can be defined as:

$$t_{½} = \ln(2)/b_1 \quad \text{(Eqn. 2)}$$

For the 10 mM treatment, the depletion of ^{14}C-Cys or Met from the soil solution was better described by a double first order exponential decay equation (3).

$$S = a_1 \times \exp^{(-b_1 \times t)} + a_2 \times \exp^{(-b_2 \times t)} \quad \text{(Eqn. 3)}$$

The two exponential parts of the equation were assumed to represent independent transport systems (high and low affinity). The half times of each pools were calculated following Equation 2. A double first order kinetic decay equation was also fitted to the $^{14}CO_2$ evolution data.

$$C = c_1 \times \exp^{(-d_1 \times t)} + c_2 \times \exp^{(-d_2 \times t)} \quad \text{(Eqn. 4)}$$

Where c_1 describes the amount of ^{14}C allocated to the first mineralization pool and d_1 is the rate constant for a_1, c_2 is the proportion partitioned into the second slower pool described by rate constant d_2. Half time of two mineralization pools c_1 and c_2 can be calculated as described above. Further details of the modelling approach are provided in (H. C. Glanville *et al.*, 2016).

All statistical analyses were carried out in in IBM SPSS statistics v25.0 (IBM UK ltd., Portsmouth, UK). Graphs and curve fitting were produced using SigmaPlot 13.0 (Systat

software Inc., London). To assess the effects of carbon and nutrient amendment on Cys or Met mineralization and depletion rates from soil solution, Pearson's correlations were carried out against the components of the first order exponential decay equation, and half times were derived from these. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to identify group differences, with the significance level of the P-value being set at $p \leq 0.05$. All results are presented in figures and tables as mean ± the standard error.

5.3 Results

5.3.1 Use of isotopes for tracing Cys and Met utilization by microorganisms in the rhizosphere

Although mineralization of N from low molecular weight compounds such as amino acids has been studied extensively (Barraclough, 1997; O'Dowd, Barraclough and Hopkins, 1999; Jones and Kielland, 2012; Zhang *et al.*, 2015; Fujii *et al.*, 2018), few attempts have been made to study S mineralization from amino acids in soils. Therefore, we investigated the biodegradation and release of C and S moieties from cysteine and methionine by soil microorganisms using uniformly ^{14}C- or ^{35}S-labelled amino acids. We chose cysteine and methionine as model substrates for this study as the majority of the organic S in plants and microorganisms occur in S-containing amino acids (Giovanelli, Mudd and Datko, 1980; Scott, Bick and Anderson, 1981; Prasad and Shivay, 2018). In addition, cysteine and methionine are likely to be important bioavailable S sources due to their high turnover rates in soils and freshwaters (Andrew and Island, 1984; Fitzgerald, Hale and Swank, 1988; Brailsford *et al.*, 2020). Here, a ^{35}S labelling technique was applied in this study to track the fate of the ^{35}S moiety from amino acids in rhizosphere soil, enabling us to determine the rate of formation of ^{35}S-sulphate and incorporation of ^{35}S into microbial biomass, while the ^{14}C labelling technique

allowed us to determine substrate uptake into soil microbial biomass and its subsequent mineralization (CO_2).

5.3.1.1 ^{14}C-amino acid depletion from soil solution

^{14}C-amino acid removal rates under all three concentrations from soil solution were extremely rapid (Fig. 5.3). For example, the microbial community removed 93.6 ± 0.7% and 92.9 ± 0.7% of added Cys and Met from soil solution separately within 30 mins when added at 0.1 mM. This fell to 74.9 ± 4.4 and 74.8 ± 1.9% at an intermediate amino acid concentration (1 mM), and to 48.2 ± 2.1% and 35.4 ± 3.6% at the highest amino acid concentration (10 mM). The amount of ^{14}C remaining in soil solution after 30 mins indicated that cysteine-^{14}C was taken up by the microbial community at a similar rate to methionine-^{14}C.

However, on a C basis, methionine-C was taken up at a faster rate than cysteine-C due to the greater number of C atoms present. In absolute terms (i.e. µmol ^{14}C kg^{-1} soil), the actual amount of cysteine or methionine removed from soil solution increased with amino acid concentration: at the lowest amino acid concentration (0.1 mM), cysteine and methionine-^{14}C uptake rates were 28.1 ± 0.2 and 46.5 ± 0.3 µmol C (kg^{-1} soil) respectively within 30 mins of substrate addition. This increased to 224.9 ± 13.4, 373.9 ± 9.5 µmol C (kg^{-1} soil), respectively, when added at 1 mM, and to 1446.8 ± 62.2, 1771.7 ± 183.1 µmol C (kg^{-1} soil) when added at 10 mM.

The temporal dynamics of amino acid-^{14}C depletion from the soil solution were dependent upon the concentration of amino acid supplied. This is also in agreement with what we found in Chapter 4 (4.3.1.2). The depletion of ^{14}C-amino acids from soil solution at 0.1 mM and 1 mM was best described by a single first order exponential decay model ($R^2 > 0.98$ in all cases; Eqn. 1, Table 5.2 and 5.3). We estimated the time necessary for the initial amount of ^{14}C-amino acids to decrease by 50% (half time) using the exponential decay model. Using this

approach, we estimated the half-life to be 6.6 ± 0.2 min for Cys and 5.9 ± 0.3 min for Met when added at 0.1 mM. This increased to 12.6 ± 1.7 min for Cys and 10.9 ± 0.6 min for Met when added at 1 mM. When added at the highest concentration (10 mM), the depletion of ^{14}C- amino acids from the soil solution was better described by a double first order exponential decay model ($R^2 > 0.99$ in all cases; Eqn. 3, Table 5.2 and 5.3), implying that ^{14}C-Cys and Met were depleted from soil solution in two phases under higher concentrations. Approximately 40% of added Met-^{14}C (10 mM) was depleted in an initial rapid phase ($t_{½} < 10$ mins), and the remaining 60% was depleted more slowly, with half times up to around 3 d. ^{14}C-Cys depletion from soil solution (10 mM) also occurred in two phases, with 57% of Cys turning over rapidly ($t_{½} = 13$ minutess), and the remaining formed a pool that turned over much slower ($t_{½} = 24.7$ h).

The concentration of S-containing amino acid at which the secondary transport system becomes important in this chapter (10 mM) is higher than that found in Chapter 4 (4.3.1.2), in which 1mM was found to be the point where the second transport system became important in the microbial uptake of Cys and Met.

At all three amino acid concentrations, exogenous addition of N as NH_4Cl, S as Na_2SO_4 or C as glucose exerted a significant influence on the depletion rate of methionine ($p < 0.05$). When added at higher concentration (10 mM), addition of C and S, not N, increased the half-time for both the rapid and slow uptake phases ($p < 0.05$), indicating microbial uptake of cysteine is driven by the microbial demand for organic C to provide energy, as well as for organic sulphur to maintain microbial growth.

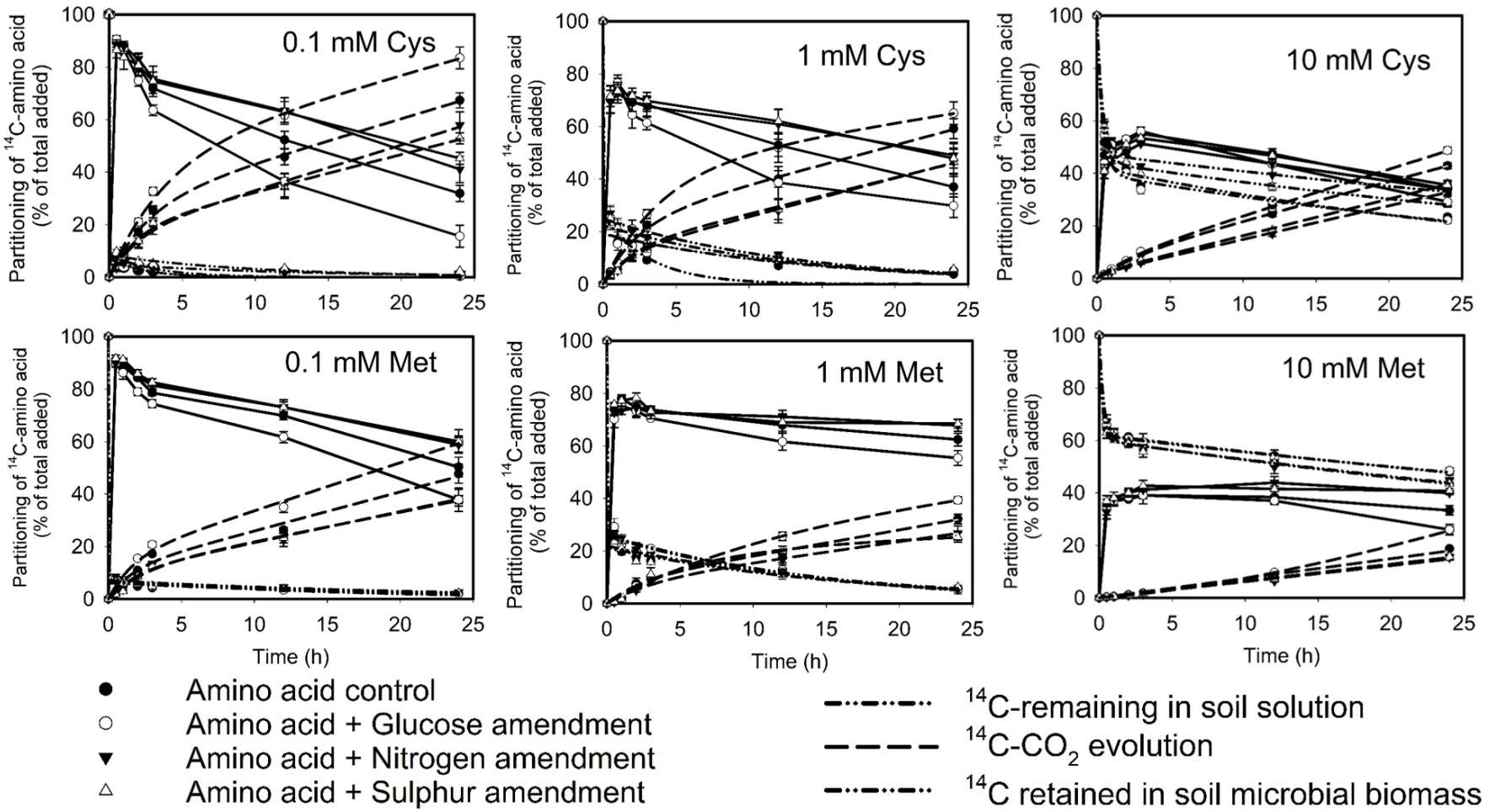

Fig. 5.3 Depletion of ^{14}C-labelled cysteine or methionine and subsequent incorporation of the ^{14}C amino acid-C into microbial biomass or respiration ($^{14}CO_2$) as affected by the addition of excess C, N and S over a 24 h-incubation period at three amino acid concentrations (0.1 mM, 1 mM, 10 mM). Values represent means ± SEM, *n* = 3.

Table 5.2 Kinetic coefficients of single (0.1 mM and 1 mM) and double (10 mM) first order curves describing the depletion of ^{14}C-Cys from soil solution over a 24 h-incubation period in the presence of an excess of C (+ Glucose), inorganic N (+ N) or sulphate (+ S). Values represent means ± SEM (n = 3). a_1 and a_2 are estimated pool sizes for fast and slow substrate uptake routes, b_1 and b_2 are the rate constants for the fast and slow uptake routes, respectively. Half-time values for each pool are calculated according to Eqn. 2.

Substrate		Cysteine						
		y_0	c_1	d_1	c_2	d_2	c_1 $t_{½}$ (min)	c_2 $t_{½}$ (h)
0.1 mM	None	2.2 ± 0.5	97.7 ± 1.1	6.3 ± 0.5			6.6 ± 0.2b	
	+Glucose	2.9 ± 0.6	97.0 ± 1.6	7.6 ± 1.5			5.4 ± 0.2c	
	+N	3.1 ± 0.8	96.8 ± 1.9	5.8 ± 0.7			7.1 ± 0.2ab	
	+S	4.4 ± 1.0	95.5 ± 2.4	5.8 ± 0.9			7.1 ± 0.4a	
1 mM	None	9.1 ± 2.1	90.7 ± 4.7	3.3 ± 0.5			12.6 ± 1.7	
	+Glucose	10.6 ± 1.7	89.3 ± 4.0	3.5 ± 0.5			11.9 ± 1.6	
	+N	14.3 ± 3.2	85.5 ± 7.6	3.5 ± 1.0			11.8 ± 1.2	
	+S	12.7 ± 2.6	87.1 ± 6.2	3.5 ± 0.8			11.6 ± 1.2	
10 mM	None		57.6 ± 4.4	3.2 ± 0.7	42.2 ± 2.8	0.03 ± 0.01	12.9 ± 1.7b	24.7 ± 1.7c
	+Glucose		59.9 ± 3.3	2.5 ± 0.3	32.8 ± 2.2	0.02 ± 0.01	16.3 ± 1.5a	27.7 ± 1.9c
	+N		52.4 ± 2.9	2.7 ± 0.3	47.4 ± 1.8	0.01 ± 0.01	15.1 ± 1.3ab	46.3 ± 5.9a
	+S		55.5 ± 2.3	2.6 ± 0.3	44.3 ± 1.5	0.02 ± 0.01	15.9 ± 2.6a	36.8 ± 5.4b

Table 5.3 Kinetic coefficients of single (0.1 mM and 1 mM) and double (10 mM) first order curves describing the depletion of ^{14}C labelled Met from soil solution over a 24 h incubation period in the presence of an excess of C (+ Glucose), inorganic N (+ N) or sulphate (+ S). Values represent means ± SEM (n = 3). a_1 and a_2 are estimated pool sizes for fast and slow substrate uptake routes, b_1 and b_2 are the rate constants for the fast and slow uptake routes, respectively. Half-time values for each pool are calculated according to equation 2. Values represent means ± standard deviation, n = 3.

Substrate		Methionine						
		y_0	c_1	d_1	c_2	d_2	c_1 $t_{½}$ (min)	c_2 $t_{½}$ (h)
0.1 mM	None	4.1 ± 0.6	95.8 ± 1.6	6.9 ± 1.2			5.9 ± 0.3	
	+ G	4.5 ± 0.8	95.4 ± 2.1	6.5 ± 1.2			6.3 ± 0.4	
	+ N	4.7 ± 0.6	95.1 ± 1.5	6.4 ± 0.7			6.4 ± 0.4	
	+ S	3.1 ± 0.8	95.6 ± 1.9	5.8 ± 0.7			5.4 ± 0.3	
1 mM	None	13.3 ± 2.4	86.3 ± 7.4	3.8 ± 0.9			10.9 ± 0.6	
	+ G	15.4 ± 3.2	84.3 ± 7.4	3.4 ± 0.9			12.3 ± 1.2	
	+ N	15.4 ± 3.4	84.3 ± 7.9	3.6 ± 1.1			11.5 ± 0.2	
	+ S	134 ± 2.4	86.5 ± 5.7	3.9 ± 0.9			10.5 ± 0.3	
10 mM	None		37.7 ± 0.9	5.2 ± 0.7	62.6 ± 0.6	0.01 ± 0.01	7.2 ± 4.8	64.7 ± 10.8
	+ G		32.8 ± 1.1	4.1 ±0.4	61.7 ± 0.6	0.01 ± 0.01	8.6 ± 6.8	68.2 ± 11.8
	+ N		39.8 ± 1.5	3.2 ± 0.3	60.2 ± 0.9	0.01 ± 0.01	12.3 ± 3.4	53.3 ± 6.7
	+ S		39.7 ± 2.0	4.9 ± 1.1	60.2 ± 1.1	0.01 ± 0.01	8.3 ± 3.5	52.0 ± 7.7

5.3.1.2 ^{14}C-Amino acid mineralization in soil

The mineralization of ^{14}C-labeled Cys and Met to $^{14}CO_2$ is shown in Fig. 5.1. Following addition of ^{14}C-labeled amino acids to the soil, there was an initial rapid phase of $^{14}CO_2$ evolution followed by a secondary slower phase of evolution (Fig. 5.3). ^{14}C-CO_2 evolution data fitted well to a first order double exponential model when added at all three concentrations (0.1 mM, 1 mM and 10 mM; $R^2 > 0.99$; Eqn. 4; Fig.5.3). The biphasic exponential dynamics of $^{14}CO_2$ evolution from cysteine and methionine were similar to those previously reported for other amino acids (Wilkinson, Paul W. Hill, *et al.*, 2014). The exponential coefficients and half-times ($t_{½}$) of amino acid mineralization are presented in Table 5.4 and 5.5.

Less than 40% of the added amino acid-^{14}C was allocated to the initial rapid mineralization pool (c_1) among all treatments, with the half-times for the turnover of this pool ranging from ca. 1.1 to 38.7 h. In contrast, the slow turnover pool (c_2) accounted for 51.4 – 91.1% of the added ^{14}C and had a much slower half time, ranging from ca. 0.5 to 1.8 d following amino acid addition. For all three amino acid concentrations used in this study, the addition of excess C as glucose significantly enhanced the partitioning of amino acid-C into catabolic and anabolic processes ($p < 0.05$).

Table 5.4 Coefficients of double (0.1 and 1 mM) and single (10 mM) first order curve fits to depletion of ^{14}C-cysteine from soil by microbial mineralization over a 24 h-incubation period in the presence of an excess of C (+ Glucose), inorganic N (+ N) or sulphate (+ S). c_1 and c_2 are estimated pool sizes for fast and slow phases of mineralization, d_1 and d_2 are the rate constants for the fast and slow phases of mineralization, respectively. Half-time values for each pool are calculated according to equation 2. Values represent means ± standard deviation, n = 3.

Substrate		c_1	d_1	c_2	d_2	c_1 t$t_{½}$ (h)	c_2 t$t_{½}$ (d)
0.1 mM	None	19.8 ± 1.2	0.5 ± 0.1	81.3 ± 1.4	0.04 ± 0.003	1.3 ± 0.1	0.8 ± 0.1
Cysteine	+ G	27.2 ± 5.7	0.4 ± 0.1	74.7 ± 5.4	0.06 ± 0.01	1.7 ± 0.3	0.5 ± 0.1
	+ N	13.6 ± 4.4	0.7 ± 0.2	87.3 ± 4.6	0.03 ± 0.004	1.1 ± 0.4	1.0 ± 0.1
	+ S	15.6 ± 6.5	0.7 ± 0.2	85.0 ± 6.8	0.02 ± 0.003	1.1 ± 0.3	1.2 ± 0.2
1 mM	None	18.3 ± 1.5	0.6 ± 0.1	82.5 ± 1.5	0.03 ± 0.003	1.3 ± 0.1	1.0 ± 0.1
Cysteine	+ G	40.0 ± 10.0	0.3 ± 0.1	61.3 ± 10.1	0.02 ± 0.003	2.3 ± 0.5	1.3 ± 0.2
	+ N	9.0 ± 2.9	0.7 ± 0.3	91.1 ± 3.1	0.02 ± 0.003	1.0 ± 0.4	1.4 ± 0.2
	+ S	12.0 ± 5.1	0.7 ± 0.3	88.6 ± 4.8	0.02 ± 0.004	1.2 ± 0.5	1.5 ± 0.3
10 mM	None	3.5 ± 1.2	0.7 ± 0.2	96.8 ± 1.1	0.02 ± 0.001	1.1 ± 0.4	1.3 ± 0.1
Cysteine	+ G	14.4 ± 23.0	0.6 ± 0.4	85.8 ± 23.4	0.03 ± 0.001	6.8 ± 12.0	1.1 ± 0.05
	+ N	36.4 ± 22.7	0.2 ± 0.4	63.2 ± 23.0	0.02 ± 0.001	32.4 ± 21.1	1.8 ± 0.1
	+ S	47.9 ± 0.2	0.02 ± 0.001	51.4 ± 0.1	0.02 ± 0.001	38.7 ± 0.9	1.6 ± 0.04

Table 5.5 Coefficients of double (0.1 and 1 mM) and single (10 mM) first order curve fits to depletion of ^{14}C-methionine from soil by microbial mineralization over a 24 h incubation period in the presence of an excess of C (+ Glucose), inorganic N (+ N) or sulphate (+ S). c_1 and c_2 are estimated pool sizes for fast and slow phases of mineralization, d_1 and d_2 are the rate constants for the fast and slow phases of mineralization, respectively. Half-time values for each pool are calculated according to equation 2. Values represent means ± standard deviation, n = 3.

Substrate		c_1	d_1	c_2	d_2	c_1 $t_{½}$ (h)	c_2 $t_{½}$ (d)
0.1 mM	None	10.4 ± 0.7	0.8 ± 0.1	90.6 ± 0.7	0.02 ± 0.003	0.9 ± 0.1	1.4 ± 0.2
Methionine	+ G	13.1 ± 0.8	0.8 ± 0.1	88.3 ± 0.7	0.03 ± 0.003	0.9 ± 0.1	0.9 ± 0.1
	+ N	9.6 ± 1.7	0.6 ± 0.1	91.4 ± 1.9	0.02 ± 0.002	1.1 ± 0.2	1.8 ± 0.2
	+ S	9.9 ± 2.2	0.5 ± 0.1	91.0 ± 2.1	0.02 ± 0.001	1.3 ± 0.2	1.9 ± 0.1
1 mM	None	8.0 ± 0.8	0.6 ± 0.1	92.9 ± 0.6	0.01 ± 0.001	1.3 ± 0.2	2.3 ± 0.1
Methionine	+ G	13.8 ± 2.8	0.2 ± 0.04	86.8 ± 2.8	0.02 ± 0.002	4.2 ± 1.1	1.9 ± 0.2
	+ N	7.9 ± 4.6	0.4 ± 0.2	92.9 ± 4.4	0.01 ± 0.003	1.6 ± 0.6	3.2 ± 1.3
	+ S	19.0 ± 7.7	0.2 ± 0.04	82.2 ± 7.4	0.004 ± 0.005	2.9 ± 0.6	7.8 ± 6.5
10 mM	None	47.3 ± 0.3	0.008 ± 0.0005	53.2 ± 0.2	0.008 ± 0.0005	82.9 ± 2.2	3.5 ± 0.1
Methionine	+ G	48.4 ± 0.4	0.01 ± 0.0008	52.6 ± 0.2	0.01 ± 0.0008	58.7 ± 3.6	2.4 ± 0.1
	+ N	46.8 ± 0.4	0.006 ± 0.0005	53.6 ± 0.3	0.006 ± 0.0005	102.5 ± 6.3	4.3 ± 0.3
	+ S	46.5 ± 0.3	0.007 ± 0.0005	53.8 ± 0.2	0.007 ± 0.0005	98.4 ± 5.7	4.1 ± 0.2

5.3.1.3 Incorporation of ^{14}C-amino acids into microbial biomass

Within three hours of substrate addition, the majority of C from cysteine and methionine was taken up into the microbial biomass among all amendments, indicating that soil microorganisms can rapidly assimilate amino acids, even in the presence of readily available C, N and S (Fig. 5.3). The amount of amino acid-C taken up by the soil microbial biomass declined rapidly as the substrate was depleted from soil solution. By the end of our incubation, 9.5 ± 0.9, 25.1 ± 1.9 µmol ^{14}C kg^{-1} soil (0.1 mM), 111 ± 12, 312 ± 12 µmol ^{14}C kg^{-1} soil (1 mM), 1014 ± 32, 1665 ± 89 µmol ^{14}C kg^{-1} soil (10 mM) of Cys and Met remained in the soil microbial biomass.

When added at lower concentrations (0.1 mM, 1mM), more Met was recovered in the microbial biomass than Cys by the end of incubation period ($p < 0.05$). This difference proved non-significant when added at a higher concentration (10 mM). It is also worth noting that for all three concentrations we used in the study, the addition of excess C as glucose generally reduced the amount of amino acid-C retained by the soil microbial biomass ($p < 0.05$), while the addition of N as NH_4Cl or S as Na_2SO_4 generally increased C allocation to this pool ($p < 0.05$) (except for 1 mM Cys in the presence of C and for 10 mM Cys in the presence of N or S as these proved non-significant, $p > 0.05$).

5.3.2 Amino acid-^{35}S utilization by soil microorganisms in the rhizosphere

Microbial biomass sulphur is an important pool in soil (Heinze *et al.*, 2021). Fig. 5.4 shows rapid accumulation of ^{35}S into the microbial biomass and mineralization of ^{35}S from amino acids due to the action of the microbial community. When added at a concentration of 0.1 mM, 73.2%, 85.9% of the ^{35}S derived from Cys or Met was incorporated into the microbial biomass by the end of incubation. This decreased to 66.8%, 75.8% when added at 1 mM, and to 44.6%, 40.1% when added at 10 mM. It could be highly likely that cysteine and methionine

were taken up intact by the microbial biomass as a closer amount of ^{14}C and ^{35}S was recovered in the microbial biomass after 30 mins. These results indicate that mineralization of cysteine and methionine was biotically mediated, which is controlled by the microbial requirement for energy and C skeletons for the maintenance and growth of microorganisms, with sulphate released as a by-product of S-amino acid turnover inside the cell.

Once taken up into the microbial biomass, excess S is rapidly released from the internal transformation of cysteine and methionine as SO_4^{2-}. When added at a concentration of 0.1 mM, ca. 12.4%, 7.2% of the total added ^{35}S-amino acid (Cys, Met) was released as $^{35}SO_4^{2-}$ after only 30 mins. Under all three concentrations, even higher amount of ^{35}S were retained in the microbial biomass from methionine. The amount of SO_4^{2-} released from methionine was lower than for cysteine, indicating that cysteine serves as a more important source of sulphate than methionine in the short term. This is in line with previous studies that cysteine showed a higher mineralization potential in soil in comparison to methionine (J. W. Fitzgerald, Hale and Swank, 1988).

In addition, some of the newly released sulphate was converted into soil organic sulphur through subsequent immobilization by soil microbial biomass. When added at 0.1 mM, around 4.8%, 1.7% of the generated $^{35}SO_4^{2-}$ from Cys or Met was incorporated into the microbial biomass or organic sulphur separately by the end of the incubation. A similar observation was made when amino acids were added at 1 mM, where the incorporation of released sulphate corresponded to approximate 14.5% and 13.4% of the total ^{35}S-Cys and Met added, respectively. However, when added at a higher concentration (10 mM), an accumulation of generated $^{35}SO_4^{2-}$ in soil solution was found with incubation time, with around 26.5% and 22.3% of total ^{35}S-Cys and Met recovered as $^{35}SO_4^{2-}$ at the end of the incubation period.

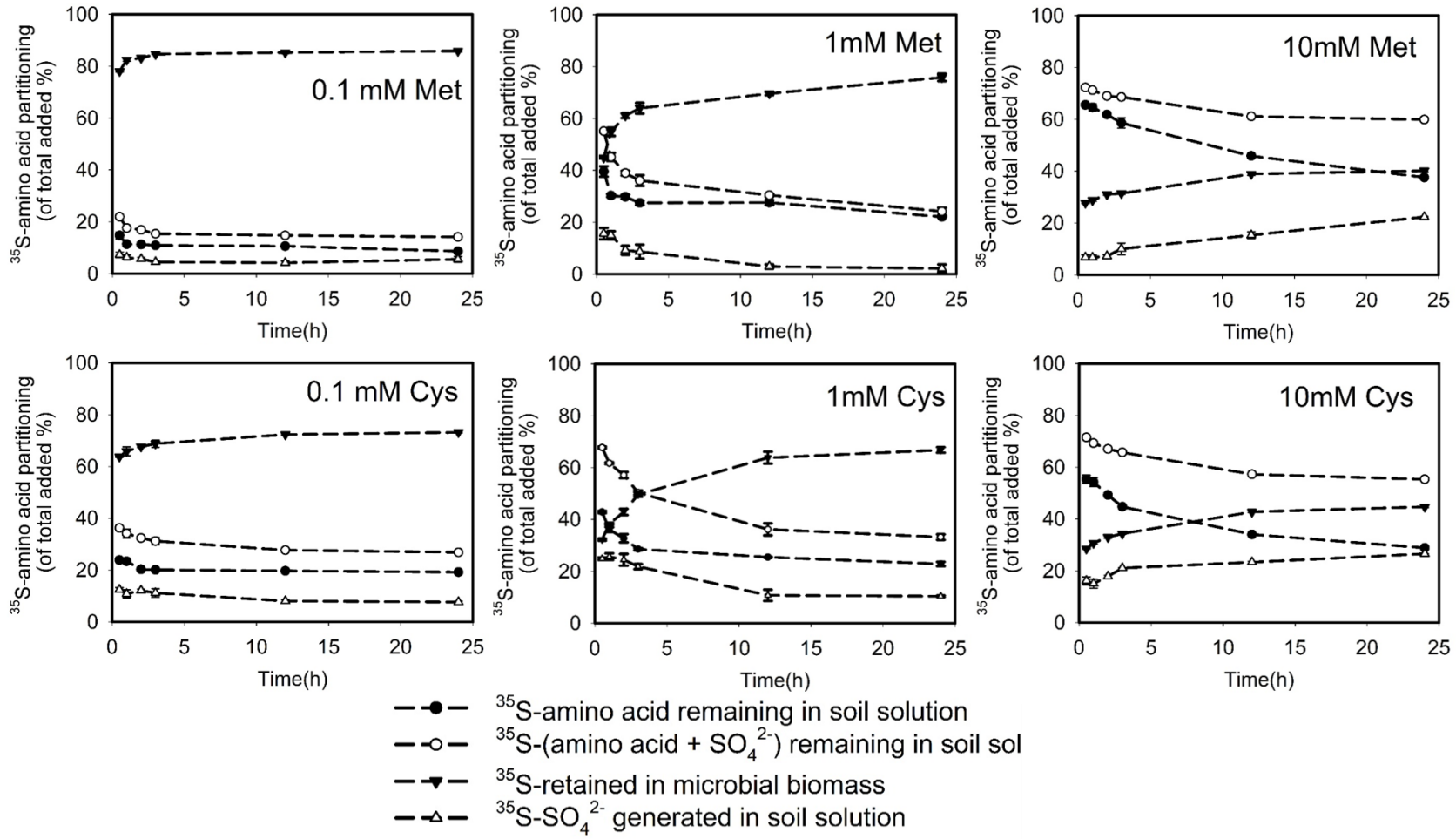

Fig. 5.4 Depletion of ^{35}S-labelled cysteine or methionine in soil solution, the formation of extractable ^{35}S-sulphate in soil and the subsequent incorporation of the ^{35}S amino acid-S into microbial biomass over a 24 h-incubation period at three added amino acid concentrations (0.1 mM, 1 mM, 10 mM). Values represent means ± SEM, n = 3.

5.3.3 Amino acid uptake by maize plants under hydroponic conditions

Results of amino acid uptake by maize plants in isolation under hydroponic conditions indicated that sterile maize roots were also capable of taking up both amino acids (Table 5.6). In total, 12.6 ± 0.8% and 14.5 ± 0.4% of the added ^{14}C-Cys and Met were taken up by the maize roots after 24 h, after which ^{14}C was incorporated into both new cell biomass and utilised for respiration. The addition of inorganic sulphate (Na_2SO_4) did not affect this partitioning process significantly ($p > 0.05$). Overall, the two S-containing amino acids behaved differently with regards to their internal partitioning in the plant. Based on the results presented in Table 5.4, 46.4 ± 3.4% and 25.5 ± 1.5 % of the added ^{14}C-Cys and Met taken up were respired by the maize plants, with the rest immobilised in the plant biomass. In addition, a similar amount of ^{35}S-cysteine and methionine was recovered in the plant by the end of the 24 h incubation, suggesting that amino acid could have been taken up intact by the maize roots.

In total, the amino acid uptake rate by the maize plant roots over the 24 h period was 0.87 nmol-^{14}C (cm^{-1} root) h^{-1} for Cys, and for 1.00 nmol-^{14}C (cm^{-1} root) h^{-1} Met when added at 0.1 mM. This compares to uptake rates into soil microorganisms for Cys of 0.41 nmol (g soil)$^{-1}$ h^{-1} and for Met of 0.40 nmol (g soil)$^{-1}$ h^{-1} when added at the same concentration. Overall, this indicates a comparable capacity for taking up externally applied S-containing amino acids from the environment (more details are shown in chapter 8).

Table 5.6 Carbon and S yields for rhizosphere microorganisms and axenic maize plants after growth on two S-containing amino acids (Cys and Met, 0.1 mM) over a 24 h-period separately. Values represent means ± SEM (n = 3). NA indicates not applicable.

Partitioning (nmol-amino acid cm^{-1} root)		^{14}C-Cys	^{35}S-Cys	^{14}C-Met	^{35}S-Met
Plant	Respiration	9.8 ± 1.3	NA	6.2 ± 0.3	NA
	Plant biomass	11.2 ± 0.4	36.5 ± 3.6	18.0 ± 0.8	39.1 ± 4.4
Soil microorganisms	Microbial respiration	6.7 ± 0.3	NA	4.8 ± 0.4	NA
	Microbial biomass	3.2 ± 0.3	7.3 ± 0.02	5.0 ± 0.3	8.6 ± 0.03

5.3.4 Competition for ^{14}C from Cys and Met between plants and soil microorganisms in the rhizosphere as affected by nutrient amendment

In accordance with results from the soil-only experiments, a rapid use of Cys and Met was seen in the microcosms containing both soil microorganisms and plant roots, with <10% of the ^{14}C-amino acid recovered from soil solution after 24 h (Table 5.7 and 5.8). The competition results indicated that plant roots were poor competitors for free Cys and Met in soil solution, with only 2.1 ± 0.2% and 8.3 ± 0.6% of ^{14}C-Cys and Met recovered in the plant biomass. A further 4.9 ± 0.3% and 2.1 ± 0.3% was also released through shoot respiration. Overall, the total plant capture of ^{14}C-Cys and Met amounted to 12.6 ± 0.8% and 14.5 ± 0.4% for Cys and Met, respectively. In contrast, a large proportion of ^{14}C-Cys and Met was recovered in the soil microbial biomass (36.7 ± 5.1%, 32.8 ± 5.9%, respectively), along with 46.8 ± 0.4, 37.4 ± 0.5% evolved in microbial respiration. The total uptake of Cys and Met by the microbial community was therefore estimated to be 83.5 ± 5.1% and 70.2 ± 6.3%, respectively, of the total amino acids added to the microcosm. A caveat to this is that we could not account for root respiration, however, we predict this to only contribute a small amount to soil $^{14}CO_2$ evolution based on the sterile, plant-only ^{14}C partitioning results.

The addition of N or S did not significantly influence the accumulation of ^{14}C-Cys and Met into maize plant root tissues, but significantly reduced translocation of ^{14}C to the plant shoot and thus the proportion of $^{14}CO_2$ respired by the shoot ($p < 0.05$). In total, the addition of N and S led to a significant reduction of total capture of ^{14}C-Cys and Met by the maize plants, while the addition of glucose-C to the soil did not seem to have a significant influence on plant capture of either amino acid. On the other hand, addition of glucose-C resulted in a significant increase in the recovery of ^{14}C-Cys and Met as ^{14}C-CO_2 from the soil ($p < 0.05$), whilst addition of N and S gave caused a significant reduction in the amount of ^{14}C-Cys and Met respired as CO_2 from the soil ($p < 0.05$).

Table 5.7 Percentage partitioning of ^{14}C label after the introduction of 0.1 mM ^{14}C-Cys into the maize rhizosphere over a 24 h incubation period in the presence of an excess of C (+ Glucose), inorganic N (+ N) or sulphate (+ S). Values represent means ± SEM (n = 3). *Different letters of each row indicate significant differences ($p < 0.05$) between treatments.

		^{14}C-Cys	^{14}C-Cys + N	^{14}C-Cys + S	^{14}C-Cys + G
Plant	Shoot respiration	4.9 ± 0.3^{a}	2.0 ± 0.4^{b}	2.6 ± 0.7^{b}	5.7 ± 0.7^{a}
	Shoot	1.6 ± 0.1^{a}	1.2 ± 0.02^{b}	1.0 ± 0.1^{b}	1.1 ± 0.2^{b}
	Root	0.5 ± 0.2^{ab}	0.4 ± 0.1^{b}	0.4 ± 0.02^{b}	0.6 ± 0.1^{a}
	Total	7.4 ± 0.6^{a}	3.6 ± 0.5^{b}	3.9 ± 0.8^{b}	7.3 ± 0.8^{a}
Soil	Microbial biomass	36.8 ± 5.1^{b}	33.1 ± 1.8^{bc}	49.3 ± 7.8^{a}	26.8 ± 1.3^{c}
	Microbial respiration	46.8 ± 0.4^{b}	40.6 ± 1.1^{c}	38.9 ± 0.7^{c}	57.6 ± 2.1^{a}
	Remaining in solution	2.3 ± 0.9	5.5 ± 3.7	6.1 ± 2.9	2.2 ± 0.5
	Total	83.6 ± 5.1^{c}	73.7 ± 1.3^{b}	88.2 ± 7.3^{ac}	84.4 ± 2.9^{c}
Total recovery		90.6 ± 5.6	77.3 ± 3.6	92.2 ± 8.1	91.7 ± 3.4

Table 5.8 Percentage partitioning of ^{14}C label after the introduction of 0.1 mM ^{14}C-Met into maize rhizosphere over a 24 h incubation period in the presence of an excess of C (+ Glucose), inorganic N (+ N) or sulphate (+ S). Values represent means ± SEM (n = 3). *Different letters of each row indicate significant differences ($p < 0.05$) between treatments.

		^{14}C-Met	^{14}C-Met + N	^{14}C-Met + S	^{14}C-Met + G
Plant	Shoot respiration	2.1 ± 0.3a	1.4 ± 0.1b	1.4 ± 0.2b	1.9 ± 0.1a
	Shoot	5.4 ± 0.3a	3.4 ± 0.5b	3.4 ± 0.4b	5.77 ± 1.5a
	Root	3.0 ± 0.4	2.7 ± 0.2	3.0 ± 0.5	2.5 ± 0.5
	Total	10.4 ± 0.4a	7.5 ± 0.6b	7.8 ± 0.5b	10.2 ± 1.1a
Soil	Microbial biomass	32.8 ± 5.9	42.4 ± 9.0	31.7 ± 3.6	29.83 ± 8.4
	Microbial respiration	37.4 ± 0.5b	32.6 ± 0.4c	31.1 ± 0.6c	41.7 ± 2.1a
	Remaining in solution	6.4 ± 0.8	6.9 ± 1.1	9.4 ± 1.9	9.3 ± 2.6
	Total	76.6 ± 7.1	81.9 ± 9.0	72.2 ± 4.3	80.80 ± 10.1
Total recovery		80.7 ± 6.3	82.4 ± 8.9	70.5 ± 3.7	81.7 ± 7.3

Table 5.9 Partitioning of ^{14}C label after the introduction of ^{14}C-labelled cysteine or methionine into the maize rhizosphere for 24 h at three different concentrations. Values represent means ± SEM (n = 3). Average plant shoot weight is 86.5 mg (n = 18), and average plant root weight is 56.9 mg (n = 18).

Percentage (%)	^{14}C-Cys			^{14}C-Met		
	0.1 mM	1 mM	10 mM	0.1 mM	1 mM	10 mM
Plant shoot respiration	4.9 ± 0.3	4.2 ± 0.5	2.2 ± 0.2	2.1 ± 0.3	1.6 ± 0.1	0.4 ± 0.1
Microbial respiration	46.8 ± 0.3	38.1 ± 3.1	20.1 ± 2.8	37.4 ± 0.5	32.1 ± 4.8	15.4 ± 1.4
Plant shoot uptake	1.6 ± 0.1	1.1 ± 0.3	0.7 ± 0.1	5.4 ± 0.3	4.5 ± 0.6	1.4 ± 0.1
Plant root uptake	0.5 ± 0.2	0.3 ± 0.04	0.1 ± 0.02	3.0 ± 0.4	0.7 ± 0.1	0.5 ± 0.1
Remaining in soil solution	2.3 ± 0.9	9. 5 ± 2.9	29.2 ± 3.5	6.4 ± 0.8	19.2 ± 2.9	38.4 ± 4.9
Microbial storage	36.7 ± 5.1	31.2 ± 3.9	19.2 ± 2.4	32.8 ± 5.9	31.9 ± 1.1	24.2 ± 3.9
Plant capture	7.4 ± 0.5	5.6 ± 0.4	3.1 ± 0.3	10.4 ± 0.4	6.8 ± 0.7	2.3 ± 0.2
Microbial capture	83.6 ± 5.1	69.3 ± 3.7	39.5 ± 3.2	76.6 ± 7.1	64.0 ± 6.1	39.6 ± 3.7
Total recovered	90.6 ± 5.6	84.4 ± 6.1	71.5 ± 3.3	80.7 ± 6.3	90.2 ± 2.8	90.3 ± 5.1

Table 5.10 Percentage partitioning of ^{35}S-Cys, Met or $Na_2{}^{35}SO_4$ (0.1 mM) between maize tissues and soil microorganisms in the rhizosphere over a 24 h-incubation period. Values represent means ± SEM (n = 3). *Different letters of each row indicate significant differences ($p < 0.05$) among treatments.

		^{35}S-Cys	^{35}S-Cys + Na_2SO_4	^{35}S-Met	^{35}S-Met + Na_2SO_4	^{35}S-Na_2SO_4
Plant	Shoot	6.8 ± 0.9b	7.2 ± 1.1b	3.5 ± 0.8b	2.8 ±0.4b	29.3 ± 7.6a
	Root	2.8 ± 0.6b	2.6 ± 0.4b	2.4 ± 0.5b	1.7 ±0.2b	8.7 ± 1.2a
	Total	9.6 ± 1.1b	9.8 ± 1.5b	5.9 ± 1.2b	4.6 ± 0.5b	30.7 ± 7.5a
Soil	Microbial biomass	51.7 ± 1.3ab	41.2 ± 14.8b	61.5 ± 2.5a	52.6 ± 7.3ab	16.9 ± 7.6c
	Remaining in solution	18.0 ± 0.5a	16.7 ± 1.4a	5.9 ± 1.0c	5.1 ± 0.6c	13.7 ± 1.0b
	Total	69.8 ± 1.3a	57.8 ± 13.7a	67.5 ± 3.6a	57.7 ± 7.2a	30.7 ± 7.5b
Total recovery		79.3 ± 0.2	67.7 ± 15.1	73.4 ± 3.9	62.3 ± 7.7	68.7 ± 6.7

Our observations are consistent with other studies on plant uptake of amino acids (Persson and Näsholm, 2001; Kielland, McFarland and Olson, 2006; Hill and Jones, 2019), suggesting that cysteine and methionine may constitute a potentially important source of N and S for plant growth. Accordingly, it may be suggested that amino acid uptake rates are related to soil amino acids concentration, as microbial utilization of amino acids may influence the availability of amino acids to plant roots in soil solution (Lipson *et al.*, 1999) and thus the degree of competition between plant roots and the soil microbial community (Sauheitl, Glaser and Weigelt, 2009). Here we hypothesized that plant-microbial competition for Cys and Met would be diminished at high concentrations of free amino acid in soil solution. To test this hypothesis, the effect of three amino acid N (S) concentrations (0.1 mM, 1 mM, 10 mM) on the competition for Cys and Met between plants and soil microorganisms was investigated using ^{14}C labelling. Our results revealed that total plant ^{14}C-amino acid acquisition was significantly affected by amino acid concentration (Table 5.9). Overall, higher amino acid concentrations increased the availability of the amino acids allowing greater capture and reduced competition with soil microorganisms.

5.3.5 Competition for ^{35}S from Cys and Met between plants and soil microorganisms as affected by sulphate amendment

The ^{35}S results revealed that among all three S sources (added at 0.1 mM), inorganic sulphate is the preferred S source for maize plants, with around 30% of the added sulphate incorporated into plant biomass within 24 h (Table 5.10). This was higher than that of both S-containing amino acids (3 times higher than that of cysteine, 5 times higher than that of methionine). In contrast, organic amino acid-S was the preferred S source by soil microorganisms in comparison to inorganic sulphate, with more than 40% of the added ^{35}S derived from amino acids retained in soil microbial biomass, while only 16.9% of the ^{35}S-

sulphate was recovered in the soil microbial biomass. For all three S sources, more ^{35}S was transported and retained in the plant shoot, indicating that S taken up by the plant is readily mobile and distributed through the whole plant to optimize performance.

5.4 Discussion

It is widely accepted from pure culture studies that soil microbial growth can rely solely on amino acids, with inorganic N released via deamination or transamination, and C skeletons released as C sources for glucogenic or ketogenic catabolic pathways (Moe, 2013). While C and N mineralization rates of amino acid in soils have been quantified and shown to be very rapid (Jones, 1999; Jones and Kielland, 2002; Wilkinson *et al.*, 2014; Almulla, Jones and Roberts, 2018; Jones *et al.*, 2018), mineralization of organic S in soils is not well understood. This is partly due to the complexity caused by multiple pathways involved in the mineralization of soil organic S. For example, S mineralization can occur through a range of biochemical processes such as the hydrolyzation of organic S by intra- and extra-cellular sulfatases, driven by microbial S demand, while biological process is driven by microbial energy needs, with S releases as a by-product (McGill and Cole, 1981; Zhao, Wu and McGrath, 1996). Specifically, the transformation of cysteine and methionine are first dominated with deamination and the release of ammonium (Greenwood and Lees, 1956; Ruiz-Herrera and Starkey, 1969a; Sohn and Ho, 1995), followed by desulphination of substituted pyruvic acid to sulphate (Freney, 1960; Andrew and Island, 1984; Hale and Fitzgerald, 1990). Further work could explore the metabolic profile of the soil after S-amino acid addition to investigate the relative importance of these different catabolic and anabolic pathways.

From our ^{14}C-radiotracer results we concluded that the soil cysteine and methionine pool (0.1 mM) would turnover in the rhizosphere 553-685 times per day, with utilization rates and internal partitioning of Cys and Met affected by the inorganic N and S status of the soil.

However, as the concentrations of Cys and Met found *in situ* are typically lower (Autry and Fitzgerald, 1990; Werdin-Pfisterer, Kielland and Boone, 2009), actual C mineralization from cysteine and methionine in soil solution under field conditions may be even faster. These results support our hypothesis that cysteine and methionine are ubiquitously rapidly utilized by soil microorganisms and therefore there is likely to be strong competition both within the soil microbial community and with plants for this low molecular weight S resource pool. It is likely that strong inter-plant competition may also occur.

Previous studies on the relationships between S, C and N mineralization indicated that S mineralization did not occur in parallel with other elements (Homann and Harrison, 1992; Haque *et al.*, 2007). Rapid ^{35}S-cysteine and methionine incorporation into the microbial biomass indicated that the mineralization of the two S-containing amino acids in soil was biotically mediated, rather than abiotic or occurring by extracellular enzymes residual in the soil. This decoupling of elemental cycles was also confirmed by faster C mineralization (^{14}C-amino acid to $^{14}CO_2$) relative to S mineralization (^{35}S-amino acid to SO_4^{2-}). We ascribe this to the microbial requirement to strip N and S from the amino acids to produce C skeletons that can enter respiratory cycles for energy production. It would therefore be expected that C and N excretion would occur in advance of $^{14}CO_2$ production. It is also interesting to note that Cys was mineralized to a slightly greater extent than Met in all treatments. From this, we assume that Cys may serve as a more important S source for plants and soil microorganisms compared to Met due to its more rapid turnover as well as higher utilization efficiency by soil microorganisms. This probably also reflects the greater proportion of Cys relative to Met in proteins which enter the soil in plant and microbial residues.

It is a commonly held view that plants are capable of taking up amino acids and utilizing them as a N source for growth under sterile hydroponic conditions (Schmidt and Stewart, 1999; Jämtgård, Näsholm and Huss-Danell, 2008). Amino acids are transported into plant root cells

through active, proton-coupled symporters (Bush, 1993; Tegeder and Rentsch, 2010). Following uptake into the root cytoplasm, amino acids are used for the production of new cell biomass and to produce energy. When it comes to S, the observation that sulphate uptake by root cells is inhibited by organic S compounds such as cysteine and methionine implies that these compounds may also be bioavailable to root cells (Hart and Filner, 1969). Previous evidence suggests that cysteine and methionine can be rapidly taken up by tobacco cells (Parthier, Malaviya and Mothes, 1964; Harrington and Smith, 1977) and algae (Deane and O'Brien, 1981). However, little is known about potential contribution of S-containing amino acids to higher plant S nutrition such as agricultural crops. Here our results under hydroponic conditions revealed that comparable amounts of ^{14}C-Cys and Met were taken up by maize roots, followed by translocation to the shoot where it was transformed and used for respiration. When cysteine and methionine were ^{35}S labelled, incorporation of ^{35}S into the plant confirmed that maize plants possess the capacity to access amino acids from soil solution should they become available.

In short-term rhizosphere competition studies for amino acids, plants are generally considered poor competitors against soil microorganisms (Hill and Jones, 2019). Our data indicated, however, that maize plants competed quite well for Cys and Met against the microbial community, especially at high exogenous amino acid concentrations. This indicates that organic N and S mineralization to NH_4^+ and SO_4^{2-} is not a prerequisite for plant uptake of these nutrients. A significant change in the rate of plant Cys and Met uptake occurred when inorganic N and S was added to the soil (but not glucose-C), indicating that S-containing amino acids utilization by maize plants could be regulated by inorganic N and S availability. Further research is needed to reveal the distribution of organic S in specific root tissues, cells and compartments to better understand S transport and metabolism in response to the surrounding

environment. The top-down control in response to shoot S and N status on the root uptake of Cys and Met is also required (i.e. source-sink relationships).

One interesting question addressed here was whether plant-microbial competition in the rhizosphere is affected by different S forms (i.e. inorganic vs. organic). Our results showed that maize plants recovered more ^{35}S than soil microorganisms in the inorganic S treatment, while soil microorganisms recovered more ^{35}S than maize plants in the organic S (^{35}S-Cys and Met) treatments. We ascribe this to the fact that heterotrophic soil microorganisms demand C and N as well as S for their growth, whereas autotrophic plants only require N and S from soil.

5.5 Conclusions

In conclusion, experimental evidence was presented here that both maize plant roots and soil microorganisms have a similar capacity to take up free Cys and Met from the external solution when studied in isolation. However, when they are allowed to compete together, maize roots showed a lower ability to utilize both S-containing amino acids compared to the rhizosphere microbial community. Our results suggest that plants compete better for Cys and Met when their levels in soil are high. This suggests that plants may be better than microorganisms at exploiting ephemeral hotspots of organic N and S in soil. The present study greatly expands our understanding of terrestrial S cycling, as previously it was assumed that low molecular weight S compounds such as Cys and Met must be extracellularly cleaved to inorganic forms before plant uptake. Our study also points out the clear need for studies of plant acquisition of Cys and Met *in situ* where amino acid concentrations are low, in order to learn more about the contribution of low molecular weight S containing compounds for plant N and S nutrition. It would also be useful to study plants which have a greater S demand such as oilseed rape and other Brassicas (Basumatary *et al.*, 2021). In addition, DOS in soil does not just exist as free Cys and Met. It would therefore be desirable to investigate the plant and

microbial uptake of a wider variety of DOS compounds such as oligopeptides and S-containing secondary metabolites which may be released into soil. Lastly, the contribution of inorganic and organic S forms to the production of volatile organic S (VOS) would also be worthwhile as very little is known about the factors that control the production, consumption and transport of VOS in soil.

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5.7 References

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Chapter 7

Tracing the mineralization rates of C, N and S from cysteine and methionine in a grassland soil: A ^{14}C and ^{35}S dual-labelling study

Abstract

Cysteine (Cys) and Methionine (Met) represent the two main sulphur (S)-containing amino-acids found in soil solution. Although general measures of S cycling (e.g., sulfatase activity) provide valuable information concerning the cycling of labile organic S in soil, detailed information regarding the microbial transformation pathways of Cys and Met at a molecular level remain poorly characterised. Therefore, in this study a ^{14}C and ^{35}S dual-isotopic labelling approach was used to trace the fate of C and S derived from Cys and Met in an agricultural grassland soil over a 7-day incubation period. Microbial biomass C, N, and S were analysed by the $CHCl_3$ fumigation-extraction method, and CO_2 evolution along with inorganic nutrient release (NH_4^+, NO_3^- and SO_4^{2-}) were also measured. We then imposed an excess of C as glucose, or excess NPS (as NH_4NO_3, KH_2PO_4 and K_2SO_4) to investigate whether the Cys and Met mineralization process was affected by manipulating C, N and S availability in the soil solution. Our results showed that after 168 h, 2.7 – 19.5% of the ^{14}C derived from Cys and Met had been immobilised in the microbial biomass, 67.2 – 89.2% had been respired as $^{14}CO_2$ while the recovery of ^{35}S label in the soil microbial biomass ranged from 11.9 – 41.8%. Overall, our results indicated that microbial communities have a high capacity to utilize Cys and Met but that they enter multiple metabolic pathways once inside the cell. While some of the amino acids may be directly used in protein synthesis, other metabolic pathways lead to NH_4^+ and SO_4^{2-} being released back into the soil with the NH_4^+ then rapidly converted to NO_3^- by the nitrifier community. The resulting C-skeletons were dissimilated and used to produce energy, leading to the release of $^{14}CO_2$. The significant differences in C, N and S mineralization processing demonstrate a decoupling of the S and C cycles at the molecular level. Further, glucose-C addition shifted the allocation of C inside the cell to more catabolic processes and greater mineralization, while in comparison inorganic N, P and S availability had much less effect on resource partitioning.

Keywords: Biodegradation; Dissolved organic sulphur; Nutrient availability; Radioisotope tracers; ^{14}C tracer; ^{35}S tracer; Grassland soil; Turnover.

7.1 Introduction

Agricultural mineral soils have a quasi-constant mean carbon (C): nitrogen (N): phosphorous (P): sulphur (S) ratio of 10000: 833: 200: 143 on a mass basis (Kirkby *et al.*, 2011). Consequently, changes in the abundance of one element are often balanced by increased or decreases in another. Over the next few decades, it is predicted that global C and N inputs to soil will increase due to the need to improve plant productivity and food security (Liang et al., 2011; Sillen & Dieleman, 2012). Thus, terrestrial ecosystems are expected to develop a higher demand for other nutrients, including S and other micronutrients stripped from the soil system at harvest. (Jones et al., 2013). Typically, N, P, and S are seen as the major nutrients that are most likely to limit plant and microbial growth (Fermoso *et al.*, 2019), with their availability in soil a key constraint for the productivity of terrestrial ecosystems (Marschner & Rengel, 2012; Vitousek & Howarth, 1991). In most agroecosystems, plant nutrition relies in part on the decomposition and mineralization of organically bound N and S mediated by the soil microbial community (Sahrawat, 1981; Michael A. Kertesz and Mirleau, 2004a). Many of the labile organic N- and S-containing compounds in soil and are present in a low molecular weight (MW) soluble form within the dissolved organic matter (DOM) pool. This pool represents a key component of C, N and S cycling (Brailsford, 2019), and the breakdown of DOM in soil is a critical step in supplying available nutrients for plant growth (Marschner & Kalbitz, 2003).

More than 90% of the soil's S reserves are bound within soil organic matter (SOM; Eriksen, 2009) with most of this held within protein moieties. Soil microorganisms secrete large quantities of proteases into soil which facilitate the breakdown of proteins and peptides into free amino acids (AA) (Jan et al., 2009; Kandeler et al., 1999). Therefore, despite the fact that plants mainly absorb S as inorganic sulphate, organic S (carbon-bonded S and ester S) may be also be an important source of S to plants, especially at the height of the growing season,

or when there is a shortfall in the supply of inorganic sulphate (Freney et al., 1981). Acid hydrolysis has revealed that amino acids are the largest pool of organic in soil (Stevenson, 1982), and that they also represent an important energy and N source for soil organisms. Within this amino acid pool, S-containing cysteine (Cys) and methionine (Met), constitute the most important reserve of combined C, N and S in soil (Scott et al., 1981) and they also constitute the majority of organic S in plants (Allaway and Thompson, 1966). Therefore, understanding the bioavailability and fate of these S-containing amino acids in the plant-soil-microbial system is important step in understanding and modelling terrestrial N and S cycling.

Turnover rates of low MW dissolved organic C in soil have been measured by adding isotopically labelled (e.g. ^{13}C, ^{14}C) substrates to soil in laboratory incubations and measuring the evolved $^{13}CO_2/^{14}CO_2$ (Hill et al., 2008a; O'Dowd & Hopkins, 1998). The isotopic approach also provides information on the internal partitioning of the added ^{14}C in the microbial biomass. After uptake, the amino acid-C taken up by the soil microbial community is partitioned into two pools associated with either catabolic or anabolic processes (Jones et al., 2009). The catabolic production of $^{14}CO_2$ is normally characterized by an initial rapid phase (< 3 h) followed by a slower phase that continues for several weeks. The first rapid phase of $^{14}CO_2$ production is attributable to the immediate use of the substrate in catabolic processes (i.e. respiration) and typically accounts for approximately 7-16% of the added amino acid-^{14}C (Boddy et al., 2007; Wilkinson et al., 2014a). This is followed by a secondary slower phase of $^{14}CO_2$ production which is attributable to the subsequent turnover of (i.e. the ^{14}C incorporated into the anabolic pool (i.e. maintenance respiration) and ultimately accounts for 48-87% of the added amino acid-^{14}C (Boddy *et al.*, 2007; Farrell, Hill, Wanniarachchi, *et al.*, 2011).

The mineralization of amino acid-N occurs via the direct route in which organic N compounds are taken up intact by soil microorganisms, assimilated and then any excess N is released as NH_4^+ back into the soil (Barraclough et al., 2015; Finzi & Berthrong, 2005).

Alternatively, amino acid-N held in exposed amino groups can be released extracellularly through the action of deaminases, after which the NH_4^+ can be taken up and assimilated (Jansson and Persson, 2015). Similarly, based on the stoichiometric relationship between C, N and S, it is suggested that both biological and biochemical mineralisation processes are involved in the mineralisation of organic S (David et al., 1983; McGill & Cole, 1981). However, it should be noted that sulfatases cannot act on Cys and Met. Biochemical mineralization is mainly controlled by the S supply to soil microorganisms rather than their need for energy, which is responsible for the release of sulphates from ester sulphates by the arylsulphatase enzyme (Tabatabai and Bremner, 1970), while biological mineralization is driven by the energy demand of actively growing microorganisms (Wu et al., 1993) (shown in Fig. 7.1), releasing inorganic sulphate as a by-product, which is responsible for the release of sulphate from carbon-bonded S (Ghani et al., 1992). Some of the newly released inorganic N and sulphate is then converted to soil organic S through subsequent immobilization by the microbial biomass (Barraclough, 1997; Vermeiren et al., 2018).

Due to the lack of sensitive and specific methods for detection, identification and estimation of individual S compounds, the biodegradation processes of S from DOS in soils remain uncertain. Previous studies indicate that Cys is decomposed rapidly in soil to inorganic sulphate (Freney, 1960), following the sequence: cysteine → cystine → cystine disulphoxide → cysteine sulphinic → inorganic sulphate (Levine, 1982). In contrast, the most frequently reported end-product for Met decomposition is methanethiol (Ruiz-Herrera and Starkey, 1969b; Segal and Starkey, 1969), with other products reported as dimethyl disulphide (Ruiz-Herrera and Starkey, 1969c; W L Banwart and Bremner, 1976), dimethyl sulfide (W. L. Banwart and Bremner, 1975), hydrogen sulfide (Nader and Walker, 1970; Zinder and Brock, 1978), and inorganic sulphate (Fitzgerald and Andrew, 1984). Based on the multiple steps required to break down these amino acids extracellularly, it seems more energetically efficient

to take them inside the cell and assimilate them using as required based on the prevailing conditions.

From the current knowledge of S decomposition processes in soils, it is possible that microbial demand for C is the dominant driver for both biochemical and biological mineralisation of organic S (Ghani, McLaren and Swift, 1992). Therefore, the supply of a readily available C source to the soil may have a considerable effect on organic S mineralization. If available C concentrations in the soil are too low to meet microbial demand, mineralisation of organic S would take place, and during this process greater S excretion is likely. Conversely, in the presence of high amounts available C in soil, immobilisation of inorganic S is likely to occur. Similarly, since sulphatase activity is related to concentrations of its end-product (inorganic sulphate), when inorganic sulphate concentrations are too low to meet microbial demand, sulphatase enzymes are used to hydrolyse sulphate esters. Conversely, addition of sulphate to the soil may hinder the mineralisation or soil native organic S. By experimentally manipulating the amounts and the availability of C, N and S in soils, we aim to gain a better understanding of what regulates the fate of Cys and Met in soil.

Net nutrient mineralization represents the sum of gross mineralization and immobilization (Schimel and Bennett, 2004; Scherer, 2009). The mineralization and immobilization processes in soil are tightly linked to microbial activity. Soil microbial activity in soil is regulated by the supply of energy and nutrients. Generally, it is reported that the cut-off point of the C/N ratio with respect to mineralization/immobilization is around 25 (Kumar and Goh, 1999). Materials with a narrow C/N ratio (below 25) are expected to result in net mineralization, whereas materials with wider C/N ratio favour immobilization due to the abundance of C relative to N (Kumar and Goh, 2003). Therefore, experimental manipulation of C-to-nutrient ratios in soil could help differentiate between the effects of nutrient availability on the biodegradation and microbial use of the organic N and S pools in soil. It should be noted

that the C:N:S ratio for Cys (3:1:1) and Met (5:1:1) will always favour excretion of excess SO_4^{2-} and NH_4^+ when supplied as a sole C source to microbial cells (bacterial C:N:S ratio of 8:1:0.25) (Fagerbakke et al., 1996).

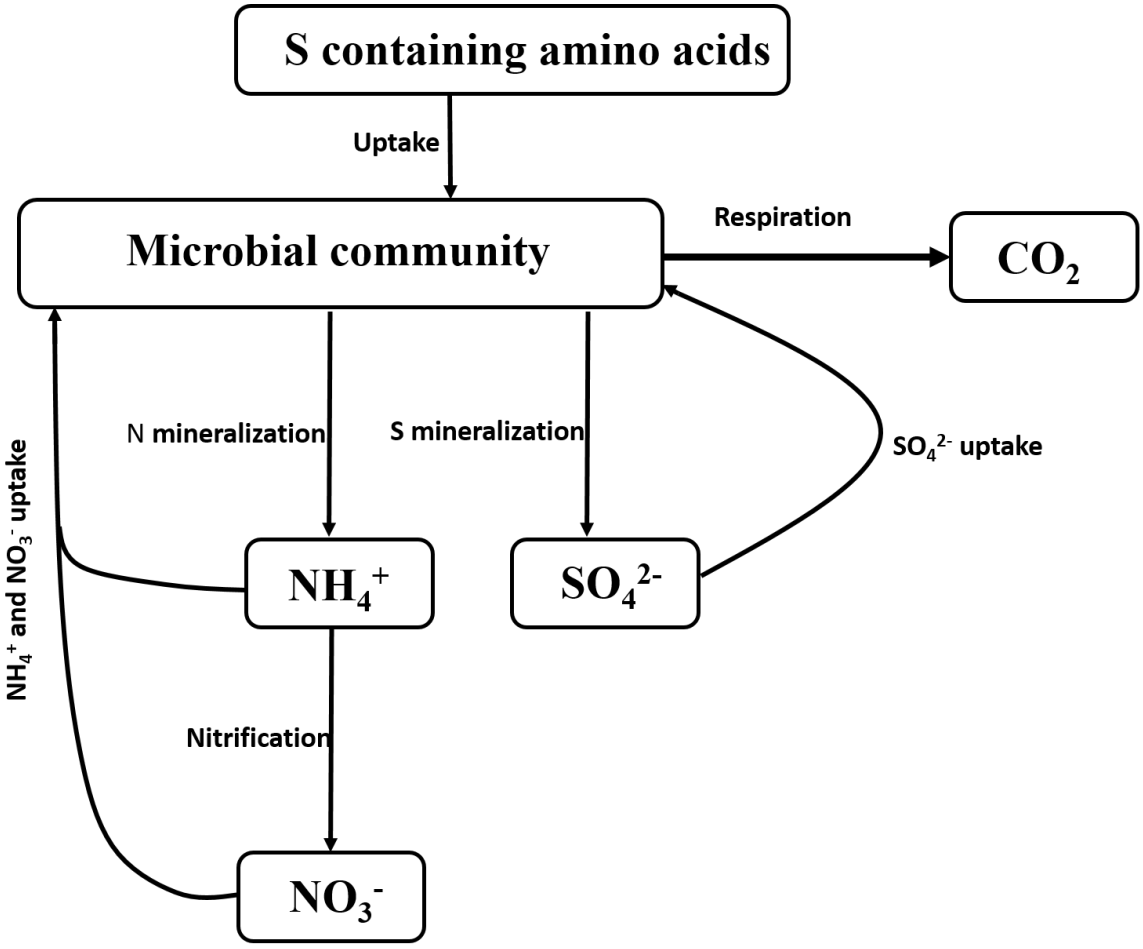

Fig. 7.1 Schematic representation of the microbial degradation of -S-containing amino acids in soil (biological mineralization process). In this study, microbial respiration and amino acid consumption were determined, as well as gross rates of inorganic N and S fluxes, namely, N mineralization, ammonium consumption, nitrification, nitrate consumption, S mineralization and sulphate consumption.

In this study, a non-fertilized grassland soil was chosen to avoid the effects of long-term fertilization on soil microbial community structure and functioning. After adding ^{14}C- or ^{35}S-labelled Cys and Met to soil, we tracked their fate in terms of immobilization in the microbial biomass, C mineralisation and inorganic nutrient release (Fig. 7.1). The specific objectives of

this study were to (1) compare short-term concurrent mineralization of C, N and S derived Cys and Met over 7 d; and (2) assess the effect of inorganic nutrient availability on microbial mineralization and utilization of Cys and Met. Such studies would provide valuable information for future modelling of S transformations and nutrient coupling in soil. Further, the information will improve our fundamental mechanistic understanding of S cycling with the aim of designing better fertiliser management regimes to address S deficiency.

7.2 Materials and methods

7.2.1 Study sites and soil sampling

Replicate batches of soil samples (n = 3) were collected in May 2018 from the Ah horizon (0 -10 cm) of an unfertilized grassland plot at Abergwyngregyn, Gwynedd, UK (53°14'N 4°11W). On return to the laboratory, the soil was sieved (< 2 mm) to remove stones, fine roots, and other plant debris, where it was stored at < 4 °C prior to further analysis. Soil pH and electrical conductivity (EC) were determined in 1:5 (w/v) soil-to-distilled water extracts using standard electrodes. Microbial biomass C and N (MBC/N) were determined by the chloroform-fumigation extraction procedure of Voroney et al. (2008). Briefly, the amount of DOC and DON was determined before and after $CHCl_3$ fumigation (48 h) with 0.5 M K_2SO_4 extracts (30 min, 200 rev min^{-1}) using K_{EC} and K_{EN} extraction factors of 0.35 and 0.5, respectively (Voroney et al., 2008). For NO_3^- and NH_4^+ analysis, the soil was extracted with 0.5 M K_2SO_4 (1:5 w/v) and NO_3^- in the extracts determined colorimetrically on a Synergy MX microplate reader using the vanadate procedure of Miranda et al. (2001), while NH_4^+ was determined colorimetrically using the salicylate procedure of Mulvaney (1996). Free amino acids and hydrolysable protein was determined by the *o*-phthaldialdehyde fluorescence method of Jones et al. (2002) using a Cary Eclipse fluorimeter. All values are reported on a dry soil weight basis (Table 7.1).

Table 7.1 Physiochemical properties of the soil used in this study. Data is presented on a soil dry weight basis. Values represent means ± SEM (n = 3).

Parameter	Values
Altitude (m)	32
Water content (%)	17.1 ± 0.2
pH	5.92 ± 0.02
Electrical conductivity (µS cm^{-1})	74 ± 4
Microbial biomass C (g kg^{-1})	0.67 ± 0.02
Microbial biomass N (mg kg^{-1})	16.8 ± 21.6
Total dissolved N (mg kg^{-1})	42.6 ± 0.7
Total dissolved C (mg kg^{-1})	35.1 ± 8.7
Total dissolved C:N of bulk soil	5.34 ± 0.19
Ammonium-N (mg kg^{-1})	1.14 ± 0.14
Nitrate-N (mg kg^{-1})	17.1 ± 0.3
Amino acids (mg N kg^{-1})	0.61 ± 0.15
Protein (mg N kg^{-1})	2.95 ± 1.37
Sulphate-S (mg S kg^{-1})	4.37 ± 0.79
Total dissolved SS (mg S kg^{-1})	31.6 ± 2.1

7.2.2 Incubation experiments

The incubation experiments lasted for 7 d, as previous studies have shown that the response of the microbial biomass was relatively fast (several hours to several days) when labile substrates are added to soil (Blagodatskaya & Kuzyakov, 2013; Blagodatskaya et al., 2007, 2009). The incubations were carried out at room temperature (21 ± 1 °C) in the dark, in the absence of plants. All treatments were conducted in triplicate. Samples for microbial biomass C, N and S determination (MBC, MBN, MBS) were taken before application of the experimental treatments. Each experimental unit comprised 5 g of field-moist soil held in individual 50 cm^3 polypropylene centrifuge tubes with a screw cap. All radiolabelled substrates were made up in 1 ml of deionized water, before being uniformly applied to the soil surface dropwise using a pipette. All centrifuge tubes were set up at the same time under the same experimental conditions. The incubation experiment consisted of two parallel experiments, one involving ^{14}C and the other using ^{35}S.

Briefly, soil in each unit was spiked with 1 ml of ^{14}C-labelled or ^{35}S-labelled Cys or Met (1 mM; 0.3 kBq ml^{-1}; PerkinElmer Inc, Waltham, MA) either in the presence or absence

of glucose-C or inorganic nutrients (N, P and S). Glucose (+G treatment) was applied to soil at a rate of 360 mg C kg^{-1} soil, whereas inorganic N, P and S (+NPS treatments) were added as 100 mg N (NH_4NO_3), 30 mg P (KH_2PO_4), 30 mg S (K_2SO_4) per kg soil. Glucose addition was equivalent to ca. 50% of the native microbial biomass C content. NPS application rates were chosen to represent common UK field fertiliser application practices (DEFRA, 2016). To measure the speed of glucose turnover, an additional treatment was used in which the ^{14}C-amino acids were replaced with ^{14}C-glucose (0.3 kBq ml^{-1}; Sigma-Aldrich Ltd., Poole, UK). We chose Cys and Met as model substrates as C-bonded S as they represent the dominant low molecular weight dissolved organic S compounds entering soil (Fitzgerald et al., 1988; Yazzie et al., 1994; Yeoh & Watson, 1982). The concentration of Cys and Met (1 mM) was chosen to reflect those likely to occur in the rhizosphere upon lysis of root cells.

7.2.3 Partitioning of the ^{14}C into different compartments

After application of the ^{14}C-labelled amino acid solution to the soil surface, ^{14}C either remained in solution, or was taken up by the microbial community. After uptake, the amino acids can be used either for microbial growth (synthesis of new cells; ^{14}C-biomass) or for production of energy ($^{14}CO_2$). Therefore, we determined their partitioning of the added ^{14}C isotope into a range of pools as follows:

- $^{14}C_O$: ^{14}C-substrate added at time zero, determined by counting the ^{14}C-substrate solution (kBq);
- $^{14}C_{uf}$: ^{14}C in the 0.5 M K_2SO_4 extract of non-fumigated soils (kBq);
- CO_2 partitioning: ($^{14}CO_2$ trapped in NaOH/$^{14}C_O$) × 100 (%);
- $^{14}C_{FE}$: (^{14}C in the 0.5 M K_2SO_4 extract of fumigated soils - $^{14}C_{uf}$) ×100/$^{14}C_O$ (%);
- $^{14}C_{MB}$: Microbial biomass ^{14}C = ($^{14}C_O$ - CO_2 - $^{14}C_{uf}$) ×100/$^{14}C_O$ (%);
- $^{14}C_{FNON-FE}$: $^{14}C_{MB}$ - $^{14}C_{FE}$ (%);

- $^{14}C_{efficiency} = {}^{14}C_{MB}/{}^{14}C_{O}$;
- K_{ec}: $({}^{14}C_{FE}/{}^{14}C_{MB})$.

7.2.3.1 Microbial mineralization of ^{14}C-labelled Cys and Met

After addition of ^{14}C labeled amino acid solution to the soil surface, a 6 ml polypropylene tube containing 1 M NaOH (1 ml) was placed inside the incubation vessels above the soil to trap respired $^{14}CO_2$. These NaOH CO_2 traps were changed hourly up to 24 h and then after 48, 72, 96, 120, 144 and 168 h after substrate addition to quantify microbial respiration rates. After removal, the amount of $^{14}CO_2$ in the 1 M NaOH traps was determined by liquid scintillation counting using a Wallac 1404 scintillation counter (Wallac EG&G, Milton Keynes, UK) and Optiphase HiSafe 3 alkali-compatible scintillation fluid (Perkin Elmer Inc., Waltham, MA).

7.2.3.2 Determination of microbial biomass ^{14}C

Microbial biomass ^{14}C was measured by the $CHCl_3$ fumigation incubation method (Voroney et al., 2008). Briefly, at nine different time points (6, 12, 24, 48, 72, 96, 120, 144 and 168 h) after substrate addition, 5 g of soil was exposed to chloroform vapour for 24 h. After removal of the fumigant, the soil was extracted with 25 ml of 0.5 M K_2SO_4 (30 min, 200 rev min^{-1}) to recover any ^{14}C-label remaining in the soil solution or held on soil exchange surfaces. The extracts were centrifuged (4000 rev min^{-1}, 5 min), and ^{14}C in the supernatant determined by liquid scintillation counting as described previously. A non-fumigated control was extracted alongside the fumigated counterparts. The ^{14}C contained in the microbial biomass was calculated as the differences of ^{14}C recovered in the fumigated and non-fumigated samples, adjusted with the extraction factor of 0.35.

7.2.4 Partitioning of the ^{35}S into different compartments

Similar to the approach taken above for ^{14}C, the partitioning of ^{35}S was determined as follows:

- $^{35}S_O$: ^{35}S -substrate added at time zero, determined by counting the ^{35}S-substrate solution (kBq);
- $^{35}S_{uf}$: ^{35}S in the 0.01 M $CaCl_2$ extract of non-fumigated soils (kBq);
- $^{35}S_{FE}$: (^{35}S in the 0.01 M $CaCl_2$ extract of fumigated soils - $^{35}S_{uf}$) ×100/$^{35}S_O$ (%);
- $^{35}S_{MB}$: Microbial biomass ^{35}S = ($^{35}S_O$ - $^{35}S_{uf}$) × 100/$^{35}S_O$ (%);
- $^{35}S_{FNON-FE}$: $^{35}S_{MB}$ - $^{35}S_{FE}$ (%);
- K_{ec}: ($^{35}S_{FE}$/$^{35}S_{MB}$).

7.2.4.1 Production of volatile sulphur compounds from Cys or Met mineralization

To determine the rate of volatile sulphur compounds produced after the addition of the ^{35}S-labelled Cys or Met to the soil, 5 g of soil was placed in a 50 cm^3 centrifuge tube, and 1 ml of 1 mM ^{35}S-labelled Cys or Met added to the soil surface as described above. A 6 ml polypropylene tubes filled with 1 ml of ethanol (> 98%; Sigma-Aldrich Ltd.) was placed inside the closed incubation vessel to trap any volatile sulphur compounds evolved. The ethanol traps were collected and changed after 6, 12, 24, 48, 72, 96, 122, 144 and 168 hours, and ^{35}S was determined by liquid scintillation counting as described previously. It should be noted that this is not a proven technology for trapping volatile S and in retrospect is unlikely to capture all forms or volatile S.

7.2.4.2 Determination of microbial biomass ^{35}S

At each sampling time, ^{35}S remaining free in the soil was extracted by adding 25 ml of 0.01 M $CaCl_2$ solution to the soil. The soils were then extracted on an end-over-end shaker (30

min, 200 rev min^{-1}), and subsequently centrifuged (5 min, 4000 rev min^{-1}). The supernatant was then analysed for total ^{35}S activity by liquid scintillation counting as described previously. To separate organic-S and sulphate-S in the soil extract, a $BaCl_2$ turbidimetric/precipitation approach was used (Combs et al., 1998). Briefly, 10 ml of the centrifuged extract was mixed with 10 ml of 1 M $BaCl_2$ on an end-over-end shaker for 10 min at 200 rev min^{-1}. Subsequently, the mixture was centrifuged (5 min, 4000 rev min^{-1}) and the supernatant analysed for organic ^{35}S (the inorganic S having precipitated as $BaSO_4$). The difference between ^{35}S activity in soil solution before and after $BaSO_4$ precipitation was considered as inorganic sulphate generated from mineralisation of the added amino acids.

To estimate microbial biomass-S, chloroform fumigation-extraction was used as described above except that 0.01 M $CaCl_2$ was used as the extractant. ^{35}S retained in the microbial biomass was calculated as the differences in $CaCl_2$ extractable ^{35}S between fumigated and non-fumigated samples, adjusted with an extraction factor of 0.35 (Voroney et al., 2008). All ^{35}S data were corrected for radioactive decay relative to the start of the incubation (half time of 87.4 d; Holtzhauer, 2006; Zoon, 1987).

7.2.5 Microbial biomass analysis

The chloroform fumigation-extraction procedure used above was used to determine microbial biomass (C_{mic}, N_{mic}, S_{mic}). In this case, total dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) in the 0.5 M K_2SO_4 extracts from both the fumigated and un-fumigated samples were analysed with a Multi N/C 2100 analyser (AnalytikJena, Jena, Germany). C_{mic} and N_{mic} were determined as the difference of TOC or TN concentrations between the fumigated and non-fumigated samples, corrected by the extraction factor 0.35 (K_{EC}) and 0.5 (K_{EN}), respectively (Joergensen et al., 1998; Vance et al., 1987; Wu et al., 1990).

For S_{mic}, the total dissolved S in the fumigated and unfumigated extracts was determined by ICP-OES.

7.2.6 Statistical and data analysis

7.2.6.1 Half-time of amino acid-^{14}C mineralization

By difference, the amount of respired $^{14}CO_2$ was used to calculate the ^{14}C remaining in soil and the microbial biomass pools (% of total added available to soil microbes). Previous studies have demonstrated that low MW substrate mineralization occurs in two distinct phases (Hill et al., 2008b; Wilkinson et al., 2014b). In this study, the mineralization of Cys and Met to $^{14}CO_2$ was described by a double first order exponential decay equation:

$$y_1 = (a_1 \times \exp^{-b_1 t}) + (a_2 \times \exp^{-b_2 t}) \quad \text{(Eqn. 1)}$$

Where y_1 the amount of ^{14}C remaining in the soil and microbial biomass, t is time, b_1 is the exponential coefficient describing the primary mineralization phase, b_2 is the exponential coefficient describing the secondary mineralization of the microbial biomass, while a_1 and a_2 represent the size of two pools. Half-times of the soil/microbial biomass pools can therefore be defined as:

$$t_{½} = \ln(2)/ b_1 \text{ or } t_{½} = \ln(2)/ b_2 \quad \text{(Eqn. 2)}$$

7.2.6.2 Half-time of amino acid-^{14}C depletion in soil

To determine the half-time ($t_{½}$) of amino acid-^{14}C depletion in the soil solution, a double first order exponential decay was fitted to the experimental data according to:

$$y_2 = (a_3 \times \exp^{-b_3 t}) + (a_4 \times \exp^{-b_4 t}) \quad \text{(Eqn. 3)}$$

Where y_2 is the ^{14}C remaining in soil solution, b_3 and b_4 the exponential coefficient describing the rate of amino acid-^{14}C depletion by soil microbial community, a_3 and a_4 describes the sizes of pools and t is time. The half-time of the soil solution pools a_3 and a_4 can therefore be

calculated according to Eqn. 2. We assumed that the two exponential parts of the equation represented uptake of substrate by two independent carrier systems.

7.2.6.3 Half-time of amino acid-^{35}S depletion in soil

Similar to the approach taken for ^{14}C above, a double first order exponential decay equation was also used to calculate the ^{35}S-amino acid depletion from soil solution as follows:

$$y_3 = (a_5 \times \exp^{-b_5 t}) + (a_6 \times \exp^{-b_6 t}) \quad \text{(Eqn. 4)}$$

Where y_3 is the ^{35}S remaining in the soil solution, b_5 and b_6 the exponential coefficient describing depletion by soil microbial community, a_5 and a_6 describes the sizes of pools and t is time. The half-time of the soil solution pools a_5 and a_6 can therefore be calculated according to Eqn. 2. We assumed that the two exponential parts of the equation represented uptake of substrate by two independent carrier systems.

7.2.6.4 Half-time of microbial biomass turnover in soil

The turnover time of C_{mic} and S_{mic} was estimated from the decrease of ^{14}C and ^{35}S in microbial biomass with time when a clear exponential decay pattern was detectable. Here, we focused solely on the production of C that was incorporated into microbial biomass, the respired C was excluded. Generally, a first order exponential decay equation fitted well to the data (based on the amount of ^{14}C or ^{35}S-labelled C_{mic} and S_{mic} at each sampling time) where:

$$A_t = A_0 \times \exp^{-kt} \quad \text{(Eqn. 5)}$$

Where A_0 and A_t are the content of $^{14}C_{mic}$ or $^{35}S_{mic}$ at times $t = 0$ and t, respectively. The initial time point of t was set at 24 h after substrate addition, as this was the time at which an approximate equilibrium was attained between exchangeable soil C and ^{14}C, soil S and ^{35}S. In addition, the $^{14}C_{mic}$ and $^{35}S_{mic}$ reached the peak and started to decline at this time. k is the decay rate, therefore the turnover time is calculated by Eqn. 6:

$$t_{½} = \ln(2)/k \quad \text{(Eqn. 6)}$$

All treatments were replicated three times. All statistical analyses were carried out using SPSS v25.0 (SPSS Inc., Chicago, IL, USA), with $p < 0.05$ used as the upper limit for statistical significance. The exponential decay curves for $^{14}C_{mic}$ and $^{35}S_{mic}$ were fitted to the experimental results using SigmaPlot v13.0 (SPSS Inc., Chicago, IL), and the adjusted R^2 for the curves as well as the significance of each parameter were calculated.

7.3 Results

7.3.1 Amino acid-^{14}C mineralization in soil

Following addition of ^{14}C-labeled Cys and Met to soil, there was an initial rapid phase of $^{14}CO_2$ evolution followed by a secondary slower phase (Fig. 7.2). This pattern was similar in all treatments. The kinetics of $^{14}CO_2$ evolution was biphasic for all treatments and the rate constants (b_1 and b_2) for these two phases are presented in Table 7.2. The loss of $^{14}CO_2$ from the soil was best described by a double first order exponential equation (Eq (1); Fig. 7.2; $r^2 > 0.99$), which is in line with our findings in Chapter 4, 5 and previous studies (Boddy et al., 2008; Glanville et al., 2012b; Mariano et al., 2016; Scow et al., 1986). The recovery of $^{14}CO_2$ after the addition of ^{14}C-glucose to the soil was significantly smaller than that recovered after the addition of ^{14}C-amino acids ($p < 0.05$).

Overall, the mineralization of added amino acid-^{14}C by the microbial community was extremely rapid, suggesting that soil microbes are severely C-limited. Generally, the addition of glucose-C or nutrients did not affect the two-phase microbial respiration pattern of Cys or Met, yet noticeable differences in the rate constants for the two pools were found. Overall, addition of glucose-C increased the allocation of ^{14}C to the fast pool, whereas the addition of NPS decreased allocation to this pool.

Table 7.2 Kinetic coefficients of double first order exponential decay models describing the depletion of ^{14}C-Cys or Met from soil by mineralization over a 168 h-incubation period in the absence (control) or presence of either glucose (+G) or nutrients (+NPS). The corresponding mineralization of ^{14}C-glucose in the presence of Cys and Met is also presented. a_1 and a_2 are estimated pool sizes for the fast and slow phases of mineralization, and b_1 and b_2 are the rate constants for the fast and slow phases of mineralization, respectively. $t_{½}$ values are the half-times for pool a_1 and a_2 determined from b_1 and b_2. Values represent means ± SEM (n = 3). The R^2 represents the goodness of fit to the experimental data.

Substrate	Treatments	a_1	b_1	a_2	b_2	a_1 $t_{½}$ (h)	a_2 $t_{½}$ (d)	R^2
Cys	+G	65.1 ± 2.7	0.07 ± 0.003	30.4 ± 2.9	0.004 ± 0.0010	12.8 ± 5.3	7.3 ± 3.3	0.99
	Control	62.7 ± 0.9	0.08 ± 0.002	34.8 ± 1.1	0.001 ± 0.0003	9.2 ± 8.4	23.1 ± 5.2	0.99
	+NPS	44.7 ± 2.0	0.08 ± 0.006	51.7 ± 2.2	0.003 ± 0.0003	9.5 ± 1.4	10.7 ± 1.6	0.99
Met	+G	43.0 ± 0.6	0.04 ± 0.004	42.3 ± 0.9	0.04 ± 0.004	17.3 ± 1.7	0.7 ± 0.1	0.99
	Control	72.9 ± 1.5	0.04 ± 0.001	28.4 ± 1.6	0.003 ± 0.0005	18.6 ± 5.9	29.6 ± 7.3	0.99
	+NPS	49.3 ± 2.0	0.07 ± 0.005	53.4 ± 2.2	0.003 ± 0.0001	9.3 ± 1.0	10.7 ± 3.9	0.99
Glucose	+Cys	33.7 ± 1.4	0.06 ± 0.004	67.5 ± 1.5	0.003 ± 0.0002	11.9 ± 2.4	72.4 ± 3.7	0.99
	+Met	36.2 ± 1.6	0.06 ± 0.004	65.4 ±1.7	0.007 ± 0.0001	12.9 ± 2.4	40.4 ± 6.4	0.99

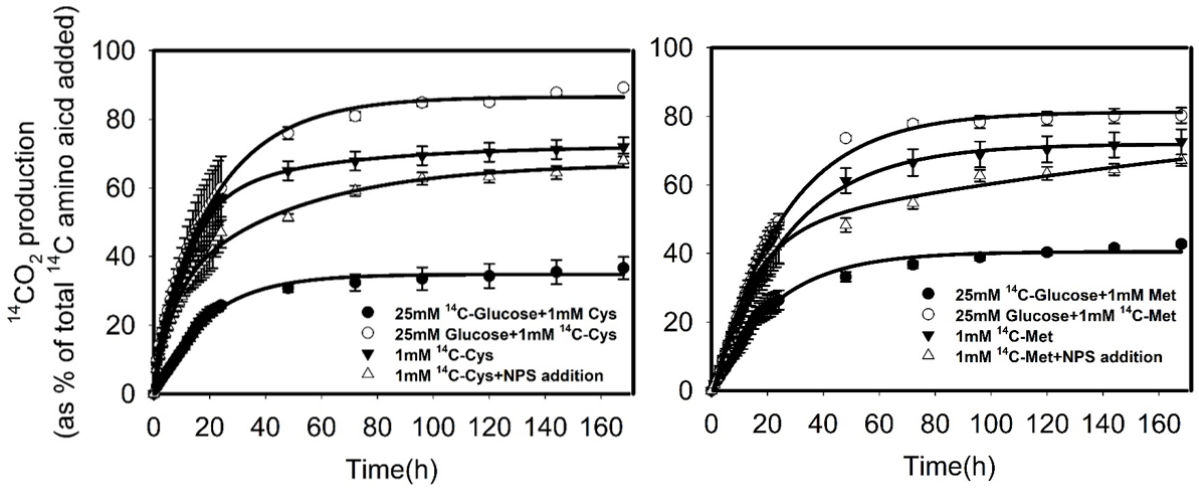

Fig. 7.2 Cumulative $^{14}CO_2$ evolution from a grassland soil after the addition of ^{14}C-labelled Cys or Met (1 mM). Lines represent fits of a double exponential first kinetic equation to the experimental data. Values represent means ± SEM (n = 3).

There was a close similarity between the dynamics of $^{14}CO_2$ evolution from Cys and Met throughout the 7-d period. Within the first 48 h, the proportion of Cys-^{14}C mineralized was higher than that of Met. However, due to Met being more enriched in C on a molar basis, the actual rate of mineralization of Met-^{14}C started to exceed that of Cys-^{14}C 10 h after addition. According to our calculation, the mineralization rate for Met for the first 10 h after addition remained relatively constant, ranging from 292 ± 31 to 309 ± 12 μg ^{14}C-Cys DW soil kg^{-1} h^{-1}, which is lower than that for Cys, ranging from 297 ± 71 to 699 ± 79 μg ^{14}C-Met DW soil kg^{-1} h^{-1}. From 10 h onwards, the mineralization rates of Cys decreased dramatically to slightly below that of Met.

The half-times of the ^{14}C substrates in the soil solution, calculated using the double exponential kinetic model, are presented in Table 7.2. The results showed that the turnover of both glucose and amino acids in soil solution was extremely rapid, with an average first phase half-time of 17.7 ± 4.4 h. ANOVA indicated that neither substrate type nor nutrient amendment had a significant effect on the rate of loss of these compounds from soil solution (p > 0.05). In contrast to the half-time for the first phase of mineralisation (12.4 ± 2.2 h for glucose-^{14}C and

12.8 ± 4.9 h for amino acid-^{14}C), the rate constant for the second phase was significantly longer, with an average value of 56.4 ± 18.2 d for glucose-^{14}C and 16.3 ± 9.3 d for the amino acid-^{14}C.

Studies on the uptake and use of amino acids in pure microbial cultures have shown that they are transported into the cell by amino acid group (e.g. neutral, basic, acidic) and isomer-specific membrane transporters (Popova, Dietz and Golldack, 2003; Tilsner *et al.*, 2005), and are differentially metabolized (Bender, 2012). It is likely that internal metabolic control of microbial amino acid transport and synthesis pathways significantly influence amino acid mineralization rates. Previous studies have shown that bacteria, actinomycetes, and filamentous fungi are all capable of decomposing Met, with the release of ammonia, volatile S and ketobutyric acid (Segal and Starkey, 1969), while a mixed population of soil microorganisms oxidized Cys to sulphate (Freney, 1967). Our results suggested that although Cys and Met are both weakly sorbed in soil (Data shown in Fig. 3.1, Chapter 3), they exhibited very different respiration rates and microbial assimilation efficiencies (Ma) (Ma = biomass-^{14}C / (biomass-^{14}C + $^{14}CO_2$)).

7.3.2 Amino acid depletion from soil

7.3.2.1 Amino acid-^{14}C depletion from soil

Overall, the depletion of both ^{14}C-labelled Cys and Met from soil solution was extremely rapid (Fig. 7.3). For example, after 3 h, 81.2 ± 0.6% of the added Cys had been taken up by the microbial community, whereas 74.4 ± 0.5% of the Met was taken up. At the end of the 24 h incubation period, marginally more ^{14}C-Met remained in the soil than ^{14}C-Cys (p < 0.01) in all three treatments. In addition, after 24 h, the content of 0.5 M K_2SO_4-extractable ^{14}C in the soil had declined to < 10% of the applied amino acid-^{14}C, indicating that > 90% of the added amino acid-^{14}C was consumed by the microbial community. This rapid disappearance

of substrate-^{14}C is consistent with other studies (Vinolas, Vallejo and Jones, 2001; Jones *et al.*, 2018).

No significant difference ($p > 0.05$) between the amount of K_2SO_4-extractable ^{14}C was observed in the three different treatments. In contrast, the extractable ^{14}C content derived from glucose in soil for the first 12 h was higher by a factor of two than that derived from both amino acids, and smaller by a factor of three afterwards. This indicates different utilization mechanisms for glucose and the two amino acids by the microbial community.

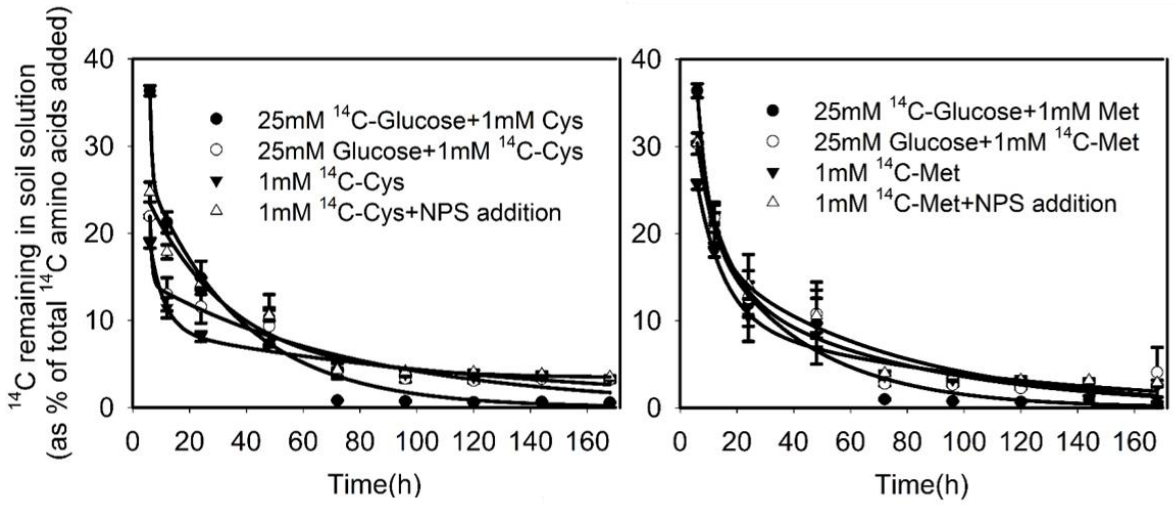

Fig. 7.3 Amount of ^{14}C-label remaining in soil solution after addition of ^{14}C-Cys or Met to a grassland soil relative to the amount added at time zero (100%). Values represent means ± standard deviation (n = 3). Lines represent fits of a double first order exponential decay equation to the experimental data ($r^2 > 0.80$ in all cases; Eq, (3)).

7.3.2.2 Amino acid-^{35}S depletion from soil

In general, ^{35}S-Cys and ^{35}S-Met depletion from the soil solution was also extremely rapid, with a much higher proportion of ^{35}S-Cys removed from the soil solution than ^{35}S- Met. After only 6 h, the total $CaCl_2$ extractable ^{35}S (SO_4^{2-}-S + org-S) derived from Cys and Met control treatments was 52.1 ± 1.2 and 25.5 ± 4.1%, respectively, suggesting around 47.9% and 74.5% of the Cys-^{35}S and Met-^{35}S was removed from soil solution by microorganisms. Three

days after substrate addition, the total extractable ^{35}S content significantly decreased, by more than half, and then remained consistently low (< 15% of added) until the end of the experiment. The addition of glucose-C or inorganic nutrients did not affect this trend significantly (p > 0.05). The depletion of ^{35}S-Cys and ^{35}S-Met from soil solution was best described by a double first order exponential decay equation (r^2 > 0.80 in all cases; Eq, (3); Fig. 7.4).

As sources of soil sulphate, our results showed that Cys is more readily mineralized than Met. By applying the precipitation method, we found that the majority of ^{35}S radioactivity detected from soil solution was recovered as inorganic sulphate, indicating that immediately after addition of ^{35}S-Cys and Met, ^{35}S was mineralised and released back to the soil as sulphate. This sulphate pool was then gradually incorporated back into the microbial biomass or was present as non-extractable organic sulphur compounds. While there was clear evidence of the oxidation of Cys and Met to sulphate, the mechanisms by which they were decomposed, and the intermediates formed during this process remain unknown.

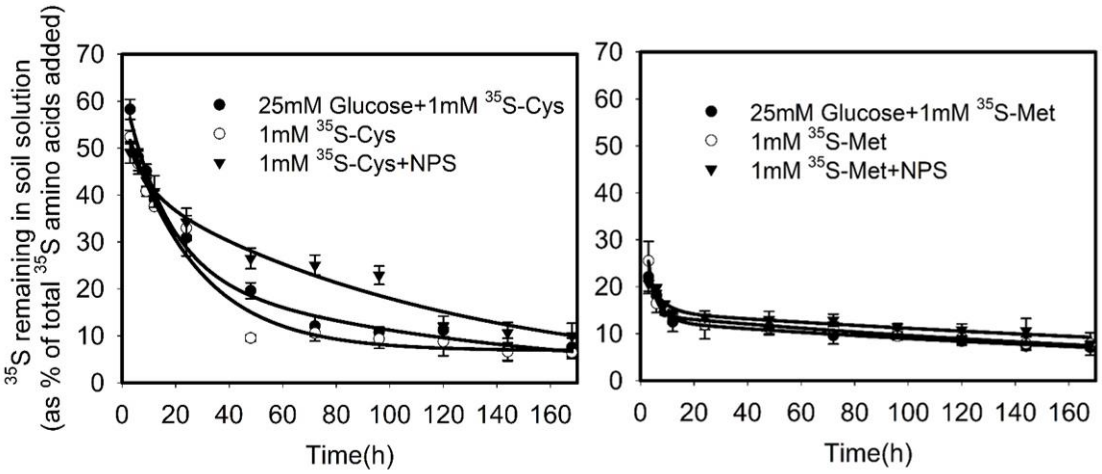

Fig. 7.4 Amount of ^{35}S-label remaining in soil solution after addition of ^{35}S-labelled Cys or Met to grassland soil relative to the amount added at time zero (100%). Values represent means ± standard deviation (n = 3). Lines represents fist of a double first order exponential decay equation to the experimental data (r^2 > 0.80 in all cases; Eq, (4)).

7.3.3 Soil microbial biomass C and N

Within 9 h of substrate addition, C_{mic} increased from 782 ± 52 mg C kg^{-1} DW soil (Control soil) to 1006 ± 20 mg C kg^{-1} soil and 917 ± 67 mg C kg^{-1} soil for Cys and Met, respectively (Fig. 7.5). Thereafter, it decreased gradually to the level seen before amino acid addition. An increase in C_{mic} concentrations due to glucose addition alone were significant ($p < 0.01$) for the whole incubation period; the extent of the increase varied from 47.6 to 76.9% with time, whereas the addition of S combined with NPS did not affect C_{mic} to a significant extent ($p > 0.05$).

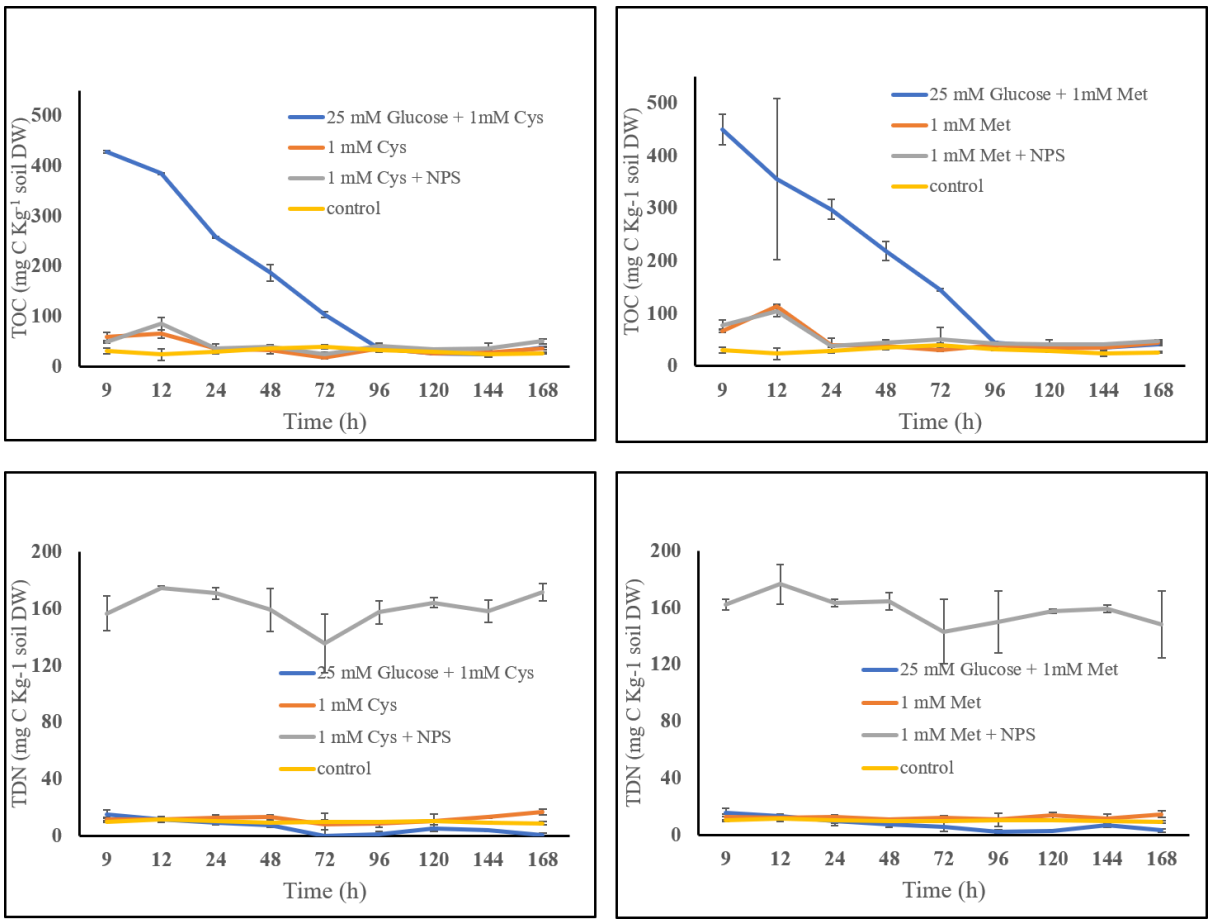

Fig. 7.5 Dissolved organic carbon (TOC; mg C kg^{-1} DW soil) and N (TDN; mg N kg^{-1} DW soil) concentrations in grassland soil solution subjected to treatments of C or NPS addition. Values represent means ± standard deviation ($n = 3$).

Similarly, N_{mic} decreased gradually after substrate addition. NPS addition did not greatly influence this trend, whereas following glucose-C addition, N_{mic} increased from 63.3 ± 13.2 (Control soil) to 69.4 ± 7.9 mg N_{mic} kg^{-1} DW soil and 66.5 ± 1.1 mg N_{mic} kg^{-1} soil within 9 h for Cys and Met individually. After 9 h, N_{mic} content decreased to a level similar to seen before substrate addition and then remained relatively stable for the rest of the incubation period.

7.3.4 Turnover time of microbial biomass ^{14}C and ^{35}S

The turnover time of ^{14}C and ^{35}S in the microbial community was estimated from the decline of radioisotope in the biomass over time. In this study, between 24-168 h was chosen to calculate this because isotope incorporation into the microbial biomass between this time-period followed a clear first order exponential decay (Table 7.3 and Table 7.4).

7.3.4.1 ^{14}C tracer incorporation into microbial biomass and turnover

During the incubation period, ^{14}C recovery in the microbial biomass increased immediately after substrate addition, then decreased afterwards. Overall, the ^{14}C recovery rate from Met in the microbial biomass was slightly higher than that from ^{14}C-Cys, indicating different amino acid-C uptake mechanisms (Fig. 7.6). After 24 h, as much as 30% of the ^{14}C-Cys was recovered in the microbial biomass. The ^{14}C recovery in the microbial biomass was close to the glucose and NPS amended soils, showing that C and nutrient addition did not significantly change the microbial carbon uptake of Cys or Met-C. The ^{14}C decline rate in MBC was best fitted with an exponential decay equation. From this, the calculated half-time for ^{14}C-Met in the biomass was 6.3 d, this is quite similar to that of ^{14}C-Cys (6.2 d). There were no significant differences among the three treatments. We estimated that the degradation of MBC was the main source of second phase microbial mineralization for both Cys and Met, which could be regulated by a range of factors.

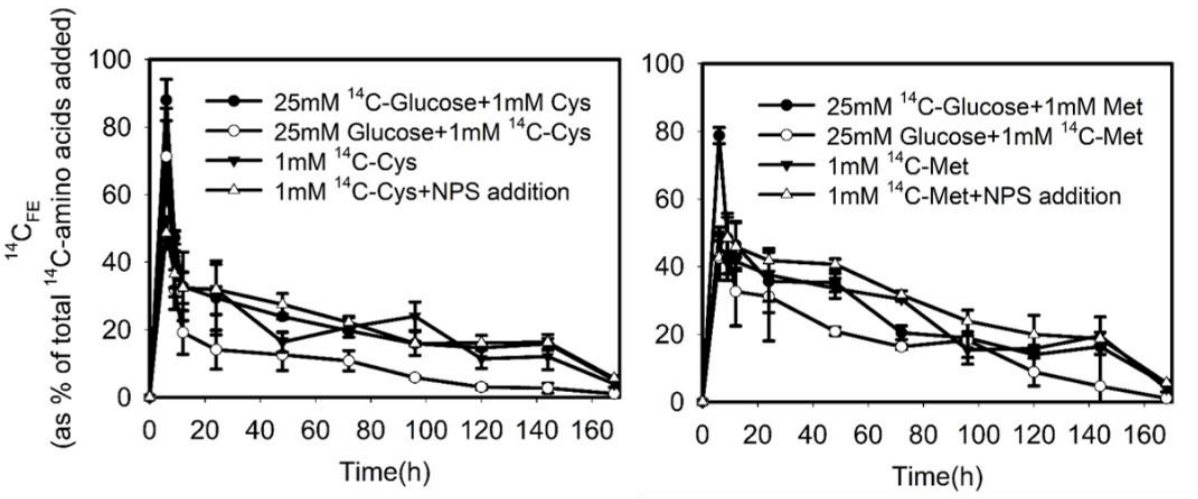

Fig. 7.6 The fraction of ^{14}C-C_{mic} in soil after following the addition of amino acids to soil in the presence or absence of glucose-C or inorganic nutrients (+NPS). Values represent means ± SEM (n = 3). Lines represent $^{14}C_{mic}$ obtained by the fumigation extraction method, adjusted with the extraction factor of 0.35.

7.3.4.2 ^{35}S tracer incorporation into microbial biomass and turnover

After amino acid addition, there was a rapid incorporation of ^{35}S into the microbial biomass for the first 12 h, after which a gradual decrease followed. A higher proportion of ^{35}S derived from Met was measured in microbial biomass than from Cys. Three hours after substrate addition, 38.2% of ^{35}S-Cys was recovered in the microbial biomass, while 67.6% was recovered from the ^{35}S-Met. This result is consistent with ^{35}S depletion from soil solution, where an opposite trend was found for the two amino acids.

The soil microbial biomass S fraction in soil is often considered to be relatively labile and the most active S pool. Clearly, ^{35}S was turning over rapidly in microbial the biomass. Specifically, the turnover time of $^{35}S_{mic}$ was longest in soil amended with NPS (10.4 and 15.6 d for Cys and Met, respectively), followed by control soil (7.4 and 10.1 d), and soil amended with glucose (3.5 and 7.8 d). Overall, the turnover of ^{35}S- Cys was faster than ^{35}S- Met (Fig. 7.7, Table 7.4).

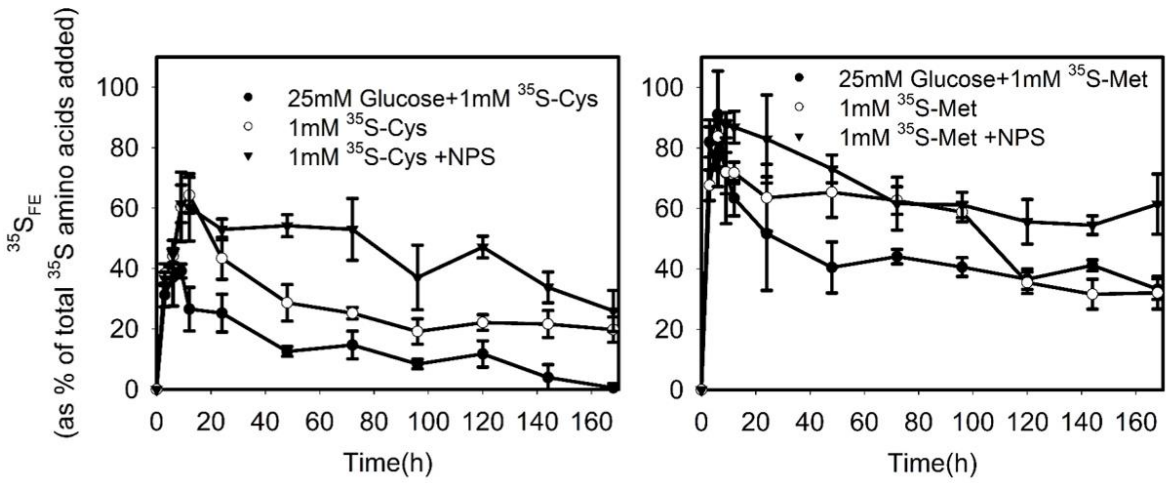

Fig. 7.7 The fraction of ^{35}S labelled S_{mic} after substrate addition. Values represent means ± SEM (n = 3). Lines represent $^{35}S_{mic}$ obtained by fumigation extraction method, adjusted with the extraction factor of 0.35.

Table 7.3 Turnover of soil microbial biomass ^{14}C-labelled Cays or Met in a grassland soil after either glucose-C (+G) or nutrient (+NPS) input. The corresponding mineralization of ^{14}C-glucose in the presence of Cys and Met is also presented. A first order exponential decay equation Eqn. 5 was fitted to the experimental data. Values represent means ± SEM (n = 3). The R^2 represents the goodness of fit to the experimental data.

Substrate	**Treatments**	***A*$_0$-^{14}C**	***K*-^{14}C**	**t½ (d)**	**R^2**
Cys	+G	21.3 ± 2.8	0.013 ± 0.002	3.7 ± 0.8	0.92
	Control	35.8 ± 7.5	0.008 ± 0.003	6.2 ± 0.5	0.69
	+NPS	39.6 ± 3.9	0.008 ± 0.001	6.5 ± 2.2	0.90
Met	+G	42.4 ± 5.8	0.013 ± 0.002	4.3 ± 0.8	0.91
	Control	49.8 ± 7.4	0.009 ± 0.002	6.3 ± 1.4	0.83
	+NPS	56.3 ± 5.9	0.009 ± 0.001	6.7 ± 1.6	0.91
Glucose	+Cys	35.3 ± 3.7	0.008 ± 0.001	6.5 ± 1.3	0.89
	+Met	47.9 ± 5.9	0.008 ± 0.002	4.8 ± 0.8	0.89

Table 7.4 Turnover of soil microbial biomass ^{35}S-Cys or Met in grassland soil after glucose-C (+G) or nutrient (+NPS) input. A first order exponential decay equation Eqn. 5 was fitted to the experimental data. Values represent means ± SEM (n = 3). The R^2 represents the goodness of fit to the experimental data.

Substrate	Treatments	*A*$_0$-^{35}S	*K*-^{35}S	t½ (d)	R^2
Cys	+G	32.2 ± 6.2	0.013 ± 0.003	3.6 ± 1.5	0.83
	Control	43.3 ± 5.5	0.006 ± 0.002	7.4 ± 2.0	0.74
	+NPS	63.5 ± 6.7	0.004 ± 0.001	10.4 ± 2.6	0.73
Met	+G	50.7 ± 3.5	0.005 ± 0.001	7.8 ± 0.6	0.80
	Control	80.8 ± 9.0	0.002 ± 0.001	10.1 ± 3.2	0.67
	+NPS	82.4 ± 5.9	0.003 ± 0.001	15.7 ± 1.4	0.71

7.3.5 Inorganic nitrogen release from cysteine and methionine decomposition

It is now well established that the breakdown of organic N compounds in soil involves a cascade of N transformations (e.g. ammonification, nitrification) (Verma, Chaudhary and Goyal, 2018; Fujii *et al.*, 2020; Ma *et al.*, 2021). In this study, NH_4^+ and NO_3^- concentrations over the 7-day incubation period was measured. Control treatments were included where substrate solution was replaced with distilled water.

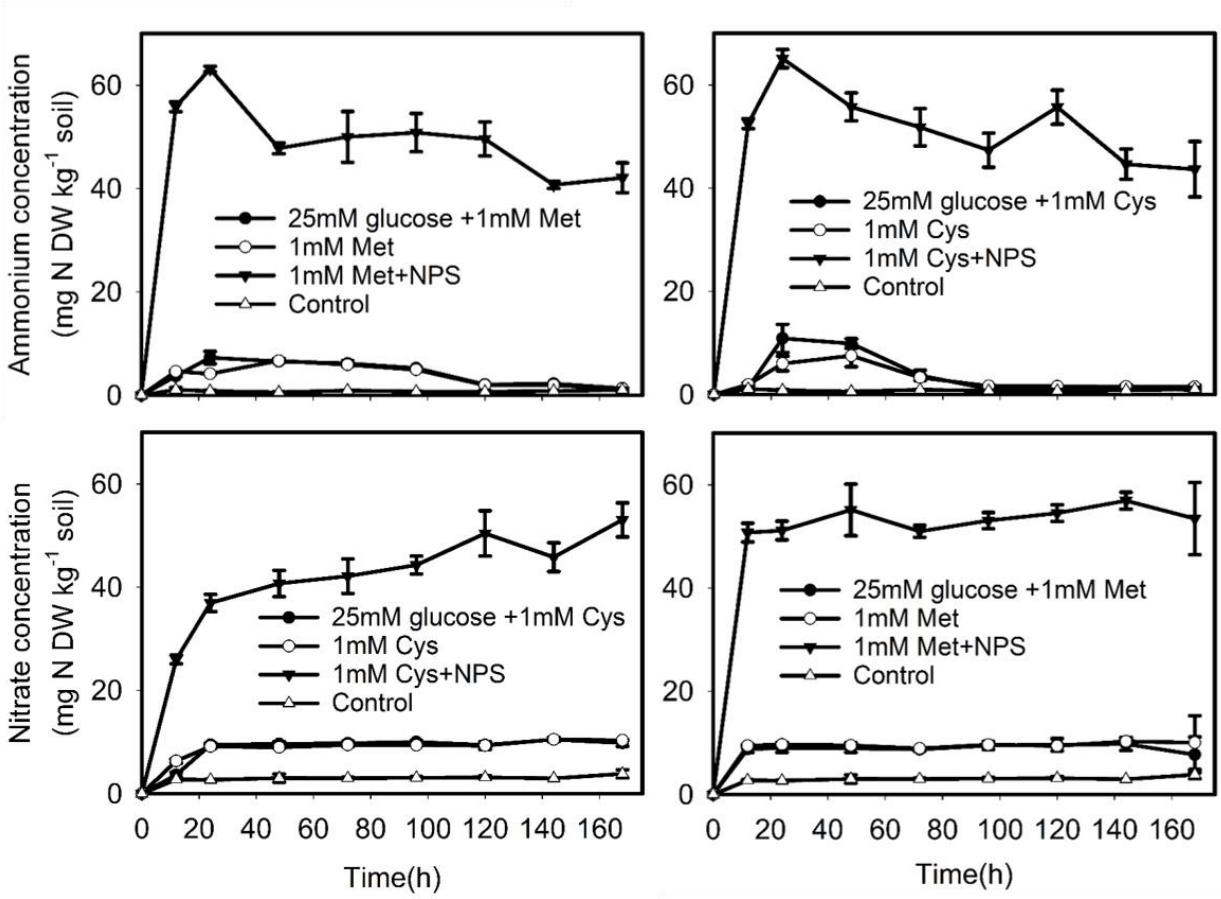

Fig. 7.8 Generation of ammonium and nitrate from added Cys and Met (1 mM) in soil solution over a 7-day period. Data expressed on a dry soil weight basis. Values represent means ± SEM (n = 3).

After a preliminary lag phase of about 24 h, the production of ammonium reached a maximum for all treatments (Fig. 7.8). During this ammonification period, there was a sharp rise in the rate of CO_2 loss from the soil, indicating an extensive breakdown of the amino acids.

After ammonium-N generation by the breakdown of amino acids, nitrification occurred, with ca. 70% of the ammonium-N being converted to nitrate-N. The course of ammonification followed by nitrification is shown in Fig. 7.8.

7.4 Discussion

Immediately after substrate addition, amino acid-^{14}C was rapidly removed from soil solution by soil microbes, with half-lives calculated to be < 3 h, suggesting that the free amino acid pool in soil turns over many times daily. This supports previous studies that have also revealed short amino acid half-lives in soil (Geisseler & Horwath, 2014; Ma et al., 2017). The results from all three treatments all conformed to a biphasic exponential dynamics of $^{14}CO_2$ evolution, similar to those reported previously (Chotte et al., 1997; Glanville et al., 2012a; Saggar et al., 1996). Overall, the mineralization of Cys by the soil microbial community was extremely rapid, with the first rapid phase of CO_2 production attributable to the immediate use of the substrate in catabolic process (i.e. respiration), and the remaining substrate being taken up and immobilized in the microbial biomass (i.e. formation of new biomass or storage polymers). The slower second phase of $^{14}CO_2$ production is then attributable to the subsequent turnover of the soil microbial community or storage polymers leading to the production of $^{14}CO_2$. Glucose addition promoted greater mineralization of both Cys and Met, while it had less of an effect on the proportion of ^{14}C incorporated in microbial biomass, resulting in a more complete utilization of both amino acids. In contrast, nutrient (NPS) addition had less effect on Cys and Met mineralization. We ascribe the lack effect of NPS addition to the low C/N ratio of these amino acids (C/N ratio = 3 for Cys and 5 for Met) which results in excess microbial NH_4^+ being excreted into the soil.

It has been demonstrated that, in the case of simple and generally weakly sorbed amino acids, soil microorganisms tend to take up intact amino acids into the cells using specific

transport proteins (Padan, 2009), instead of mineralizing the amino acids outside the cell prior to the assimilation of the remaining C skeleton. To determine the mineralization rate of amino acid-N in soil, the excretion of amino acid derived NH_4^+ and NO_3^- into the soil was measured over the 7-day incubation period. We provide clear evidence of successive stages of amino acid breakdown. A rapid loss of amino acid was associated with an increase of free ammonium in the soil solution, as well as a sharp rise in the $^{14}CO_2$ release indicating an extensive breakdown of the added amino acid. Then the ammonium-N produced by the breakdown of amino acids in soil becomes available to the nitrifier community leading to NO_3^- production.

Sulphur mineralization from organic-S together with immobilization of new released inorganic sulphate regulates S availability to plants (Li et al., 2001). In this study, S derived from both Cys and Met was rapidly converted to sulphate. Results indicated that conversion of Cys and Met to sulphate was almost complete 24 h after amino acid addition. This is in line with previous studies (Meena and Improvement, 2018). A similar trend for the distribution of amino acid-S between sulphate release and microbial biomass S incorporation was found for Cys and Met. Amount of inorganic sulphate decreased gradually throughout the incubation period, indicating a re-capture of sulphate by microorganisms. The proportion of amino acid-S incorporated into the microbial biomass showed a similar trend through the incubation, although lagged behind inorganic sulphate production: $^{14}C_{mic}$ reached a maximum before our first sampling (3 h after substrate addition), while $^{35}S_{mic}$ reached maximum with a slight lag ($^{35}S_{mic}$ from Cys peaked around 9 h after substrate addition, while $^{35}S_{mic}$ from Met reached peak after 6 h).

It is also interesting to note that by the end of the incubation there was incomplete recovery of the total ^{35}S added in both amino acids (total recovery < 100%). It is possible that this may be associated with analytical errors, including incomplete mixing of soil sampled. But some of this 'missing' S could also be due to volatile S emissions from soil (Banwart and

Bremner, 1975; Brown *et al.*, 2021), or incorporation into organic matter or absorbed by mineral colloids (Gustafsson, Akram and Tiberg, 2015; Heinze *et al.*, 2021) and therefore not extractable by 0.01 M $CaCl_2$. In this study, we set up ethanol traps to capture any volatile sulphur compounds released, yet these all proved to have minimal ^{35}S activity. This does not necessarily mean that volatile sulphur compounds were not produced by microbial decomposition of Cys and Met, just that we used the wrong approach to capture them. Previous studies have shown that some soils have a substantial capacity for sorption of volatile sulphur suggesting that a heating step may be needed to induce their desorption from soil prior to capture (Banwart and Bremner, 1975; Ko and Chu, 2005; Ko, Chu and Tseng, 2006). Further work is required to investigate the microbial use of other dissolved organic sulphur (DOS) compounds and also compare microbial preference for S forms when they are exposed to multiple sources of DOS compounds, as would occur under field conditions.

7.5 Conclusions and outlook

In conclusion, our results indicate that decomposition of S-containing amino acids (Cys and Met) to CO_2, NH_4^+, NO_3^-, and SO_4^{2-} in soil mainly occurs as a result of biological oxidation (Fig. 7.9). This is also in agreement with previous studies on the breakdown or low molecular weight organic acids in soil (Roy and White, 2013; Tanikawa *et al.*, 2013; Creamer *et al.*, 2014). Due to the low C/N ratio of Cys and Met, short-term addition of inorganic NPS to soil had no detectable effect on amino acid mineralization rates, while the addition of a readily available C source, such as glucose, led to a high and rapid rate of sulphate incorporation into soil organic fractions.

Understanding S transformations in soils is necessary for predicting the S supply from the soil organic matter to crops. The evidence is conclusive that there is rapid interconversion of organic and inorganic S forms in the soil, such as mineralization (the transformation of

organic S into inorganic sulphate) and immobilization (incorporation of sulphate into soil organic compounds or soil microbial biomass). This cycling is microbiologically mediated. Yet little is still known about the specific microbial species or genera that play important roles in the soil S transformation cycle.

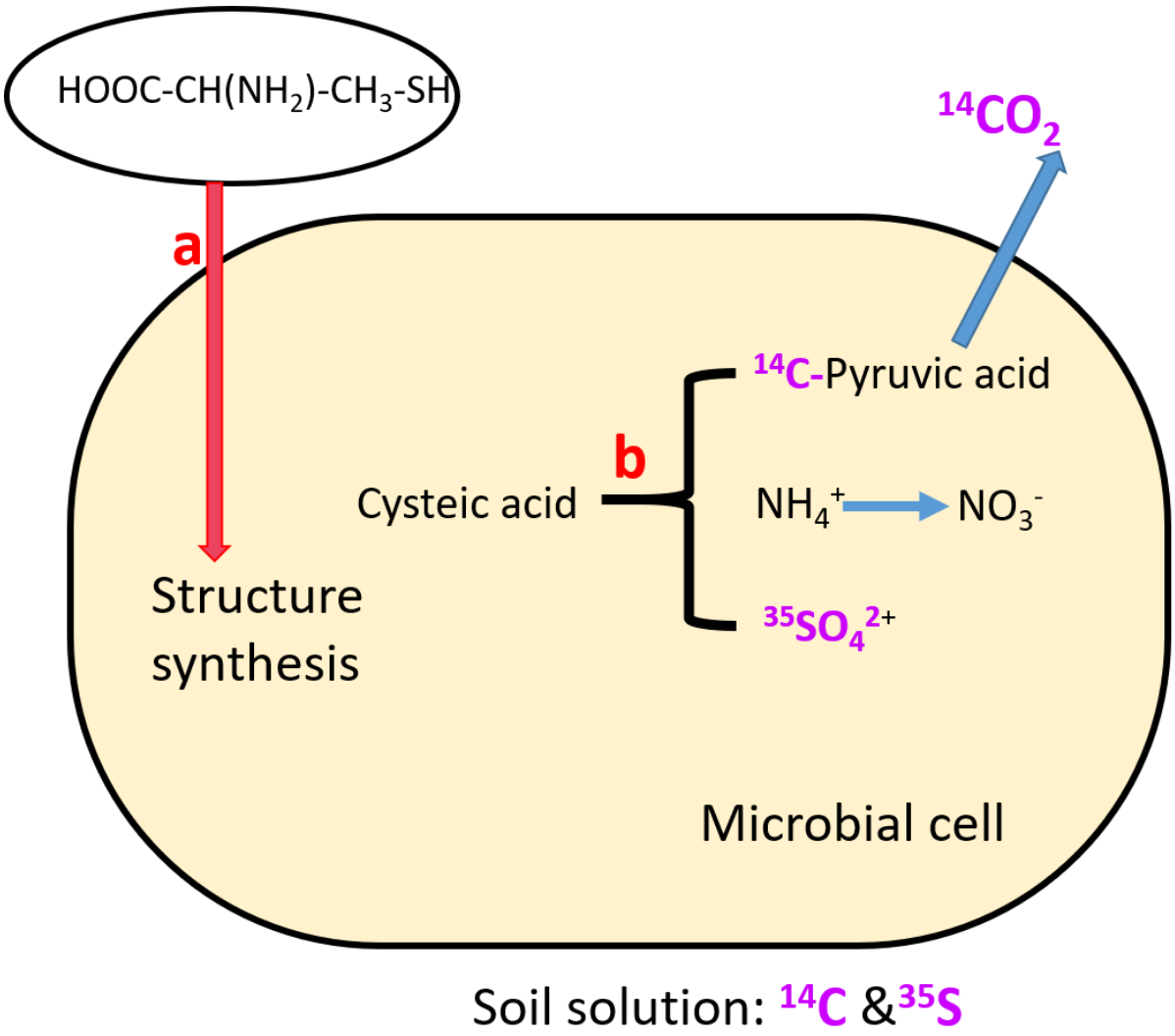

Fig. 7.9 Schematic presentation of carbon, nitrogen and sulphur mineralization from the S-containing amino acid-cysteine in grassland soils. a: labelled amino acid uptake into soil microbial biomass; b: subsequent microbial mineralization.

7.6 Acknowledgements

I would like to thank Joseph Cotton and Mark Hughes for soil sample collection, and Jonathan Roberts for assistance with isotope analysis in the laboratory. This work was performed during a studentship funded by Bangor University and the China Scholarship Council (201606510012).

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Chapter 8

Short-term uptake and efflux of sulphur containing amino acids and inorganic sulphate by maize roots under sterile hydroponic conditions

Abstract

A variety of plants possess the ability to utilize organic nitrogen (N) compounds such as amino acids, however, this has not been thoroughly studied for sulphur (S)-containing amino acids. A critical question is whether plants can acquire dissolved organic S (DOS) without assistance from soil microorganisms, and if so, how does this compare with that of inorganic sulphate uptake? To address this, we measured the uptake of three S compounds ((cysteine, methionine and sulphate) by maize (*Zea mays* L.). Plants were labelled in hydroponic culture with either ^{14}C or ^{35}S at ecologically relevant concentrations (100 µM) over short time periods (24 h). Efflux of ^{35}S-compounds was also estimated by monitoring the increase of ^{35}S content in the root bathing solution after pre-feeding the plants with each compound. The uptake results showed that sulphate was the preferred S source by maize, with a two-fold greater S accumulation compared to that of Cys or Met. However, sulphate uptake was inhibited by the presence of the organic S sources. In addition, we estimated that at least 66% of Cys and 73% of Met was rapidly taken up intact by the roots, even when inorganic sulphate was available. This indicates that low molecular weight S-containing amino acids could theoretically constitute a significant proportion of the plant's S supply under certain conditions. The uptake of Cys and Met was, however, sensitive to the presence of each other, indicating their uptake occurred via a common membrane transport system. In addition, we present evidence to show that once taken up by the roots, S transitorily accumulates in the root cell vacuoles where it is more susceptible to root efflux through passive diffusion. However, a large proportion of the S taken up is rapidly transformed and translocated to the shoot preventing efflux. In conclusion, we present direct evidence for the uptake and assimilation of dissolved organic S compounds and that maize plants are very effective in retaining and cycling S at the whole-plant level.

Keywords: Cysteine; Methionine; Sulphate; Uptake; Root exudation; Radiotracer; ^{14}C; ^{35}S; Maize.

8.1 Introduction

It is generally thought that sulphur (S) is predominantly taken up by plant roots in an inorganic form (i.e. sulphate; Moss, 1978). In actively growing plants, the S is then transported in the xylem via a selective distribution/redistribution system to the expanding leaves (Adiputra and Anderson, 1992, 1993), where assimilation into organic S takes place in the light (Leustek, 2002; Takahashi, 2010; Takahashi *et al.*, 2011). It should be noted, however, that in the case of N, many plants are opportunistic, being capable of taking up a range of organic or inorganic-N forms depending on the prevailing conditions in the soil (Moreau et al., 2019). Further, it is now clear that plants can take up significant quantities of organic nitrogen (N), especially simple forms such as amino acids and oligopeptides and when inorganic N levels in soil are low (Falkengren-Grerup, Månsson and Olsson, 2000; Hawkins, Johansen and George, 2000; Henry and Jefferies, 2003; Weigelt, Bol and Bardgett, 2005; Gallet-Budynek *et al.*, 2009; Ge *et al.*, 2009; Czaban *et al.*, 2016; Song *et al.*, 2016), yet only a few studies have focussed on S-containing amino acids.

It has been reported that over 90% of soil S is present in an organic form, and that a large proportion of this is present as carbon (C)-bonded S (e.g. cysteine, methionine; Cys and Met) (Scott, Bick and Anderson, 1981). Therefore, direct evidence is required to determine if S-containing amino acids are taken up intact by plant roots, as well as their quantitative contribution to plant S demand compared to inorganic sulphate. In previous studies, nutrient uptake in plants is often studied in simplified systems, such as excised roots in hydroponic culture (Ferrari and Renosto, 1972; Soldal and Nissen, 1978). This technique has been used extensively and has provided valuable information about specific uptake rates of nutrients at the molecular and cellular level. Some researchers, however, have argued that excised roots may artificially increase the loss of nutrients from roots and thereby inhibit net uptake (Lucash *et al.*, 2007), resulting in an unrealistic estimation of root uptake. Excising roots also alters the

source-sink relationships within the plant. This may feedback on root membrane transport systems and repress uptake if the above-ground sink is removed. It is therefore essential to study nutrient uptake in intact plants.

Cysteine (Cys) and methionine (Met) are two essential S-containing amino acids required for primary and secondary metabolism in plants (Droux, 2004). Cys is the first reduced S product resulting from the sulphate assimilation pathway (Saito, 2000), while both Cys and Met play a major role in the growth and development of plant cells (Ravanel *et al.*, 1998; Saito, 2000; Wirtz and Droux, 2005). Evidence has been presented that shows that Cys can be actively transported into cultured tobacco cells (Harrington and Smith, 1977), where it can be rapidly metabolized, the products being pyruvate, ammonium and S-sulfocysteine (Tishel and Mazelis, 1966). In addition, the transport of Met into excised plant roots has also been studied(Wright, 1962; Persson and Näsholm, 2001), with observations suggesting that both Cys and Met can be taken up by the same membrane transport system as other free amino acids.

It is known that most nutrients taken up by roots are transported to the shoot via the xylem. These can then be returned in reduced forms to the root via the phloem (Scott and Brewer, 1980; Duarte and Larsson, 1993; Atkins and Smith, 2007). This translocation of organic compounds from leaves, and release of root exudates such as sugars, amino acids and organic acids by roots, are particularly important when plants are growing in nutrient deficient soils or when plant species have a very low capacity for reducing nutrients in their roots (Carvalhais *et al.*, 2011). Therefore, root systems of plants can not only import water and nutrients from soil solution, but also release low and high molecular-weight-compounds into the environment (Smith, 1969; Dakora and Phillips, 2002). Amino acids are generally considered to be the second most abundant class in terms of the total amount exuded by plant root systems, after sugars (Smith, 1976; Jaeger *et al.*, 1999). Depending on the cause and mechanisms, amino acid release from roots may include active transport (Badri *et al.*, 2009;

Lesuffleur and Cliquet, 2010) or passive diffusion (Brophy and Heichel, 1989; Rroço, Kosegarten and Mengel, 2002; Vives-Peris *et al.*, 2020). Passive diffusion of amino acids is caused by the large concentration gradient between the cytoplasm of root cells (e.g. 1 – 10 mM) and the outside soil solution (0.1 – 10 µM) (Phillips *et al.*, 2004), while active transport of amino acids is mediated by proteins located in the root plasma membrane and can release amino acids against the electrochemical potential gradient (Okumoto *et al.*, 2004).

In previous studies, dual labelled (^{13}C, ^{15}N) compounds have been used to estimate amino acid uptake into plants (Näsholm, Huss-Danell and Högberg, 2000; Streeter, Bol and Bardgett, 2000; Wei, Chen and Yu, 2015). In these experiments, intact uptake is implied if the slope of the correlation of ^{13}C to ^{15}N excess in the plant tissue is the same as in the parent amino acid compounds fed to the plants. In this present study, dual isotope labelling: ^{14}C (Biernath, Fischer and Kuzyakov, 2008; Rasmussen *et al.*, 2010) and ^{35}S were used to estimate intact uptake of amino acids. ^{14}C labelling has been used in studies of hydroponic (soil free, nutrient water) systems (Pratelli, Boyd and Pilot, 2016; Oburger and Jones, 2018). A ^{14}C tracer was chosen in this study to eliminate the problem of ^{13}C dilution by the high ^{12}C content in plant tissues, as ^{13}C isotope can be strongly diluted in plant tissues making it difficult to detect ^{13}C in bulk plant tissues. A ^{14}C tracer approach allows estimation of the incorporation of amino acid-^{14}C into plant tissues, as well as the amino acid-^{14}C loss in the form of $^{14}CO_2$ produced during deamination and breakdown of the C skeleton in the TCA cycle (Näsholm and Persson, 2001), or in processes relating to photorespiration (Bauwe, Hagemann and Fernie, 2010). In this study, the intact uptake of amino acids can be implied if the slope of the correlation of ^{14}C to ^{35}S excess in the plant tissue is the same as in the intact amino acid (e.g. 3 ^{14}C : 1 ^{35}S in the case of added ^{14}C, ^{35}S-Cys, 5 ^{14}C : 1 ^{35}S in the case of added ^{14}C, ^{35}S-Met).

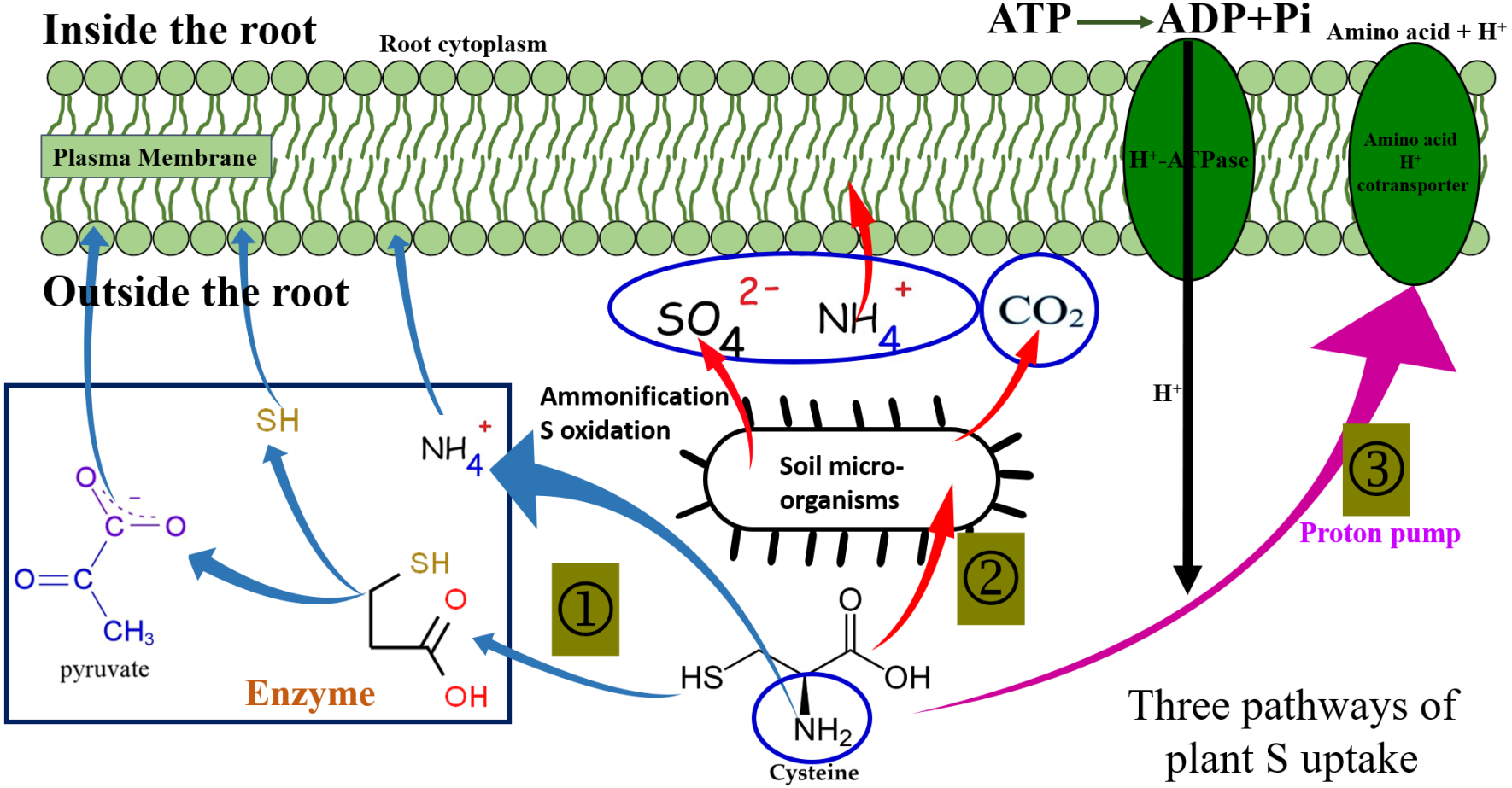

Fig. 8.1 Potential routes for root uptake of carbon, nitrogen and sulphur from cysteine added to the soil solution.

Although a large number of studies have focused on inorganic sulphate metabolism, data on the regulation of S-containing amino acids uptake, transport and efflux in plants are relatively scarce. Therefore, the primary objective of this study was to quantify the importance of intact uptake of Cys and Met relative to inorganic sulphate uptake by maize under sterile hydroponic conditions using isotope-based methods; the secondary objective was to assess S translocation and efflux from roots (i.e. root exudation). We chose maize as the model plant as it is known to possess a high demand for both N and S (Rasheed, Ali and Mahmood, 2004; Sutar, 2017), and is capable of taking up exogenously applied amino acids (Salmenkallio and Sopanen, 1989; Moran-Zuloaga *et al.*, 2015).

8.2 Materials and methods

8.2.1 Plant material and nutrient solution

Maize (*Zea mays* L.) seeds were sterilised in 2% sodium hypochlorite (1 min) and rinsed twice with sterile water (Cuero, Smith and Lacey, 1986; Sauer and Burroughs, 1986). The seeds were then soaked for 24 h in sterile deionized water and allowed to germinate on moist filter paper at room temperature (ca. 20 °C) under sterile conditions. After 48 h, each seedling was transferred into individual microcosms. Each microcosm consisted of 25 ml polypropylene containers filled with 20 ml of full-strength S-free Long Ashton nutrient solution (Hewitt, 1952; Smith, Johnston and Cornforth, 1983). The composition of the full-strength nutrient solution was as follows (g 10 L^{-1}) $MgCl_2 \cdot 6H_2O$, 3.05; KCl, 1.49; $CaCl_2 \cdot 2H_2O$, 5.88; $NaH_2PO_4.2H_2O$, 2.92; $Na_2HPO_4.12H_2O$, 0.47; H_3BO_3, 0.86; $MnCl_2.H_2O$, 0.30; $ZnCl_2$, 0.03; $CuCl_2.2H_2O$, 0.06; $Na_2MoO_4.2H_2O$, 0.005; FeEDTA, 0.33; MES buffer, 0.19; $NaNO_3$, 3.40; NH_4Cl, 2.14. After the addition of an individual seedling, the microcosms were placed in a climate-controlled cabinet with 16-h photoperiod maintained at 25 ± 0.5 °C. All sulphate nutrient salts were replaced with chloride salts in this study, so plants growing in our nutrient

solution were therefore expected to be S deficient once seed reserved are exhausted. Seven days after transplanting, plants were transferred to 10%-strength S-free Long Ashton solution for a further 3 d. All experiments were conducted on 10-d-old plants in 10%-strength hydroponic solution, with three fully expanded leaves on the main shoot.

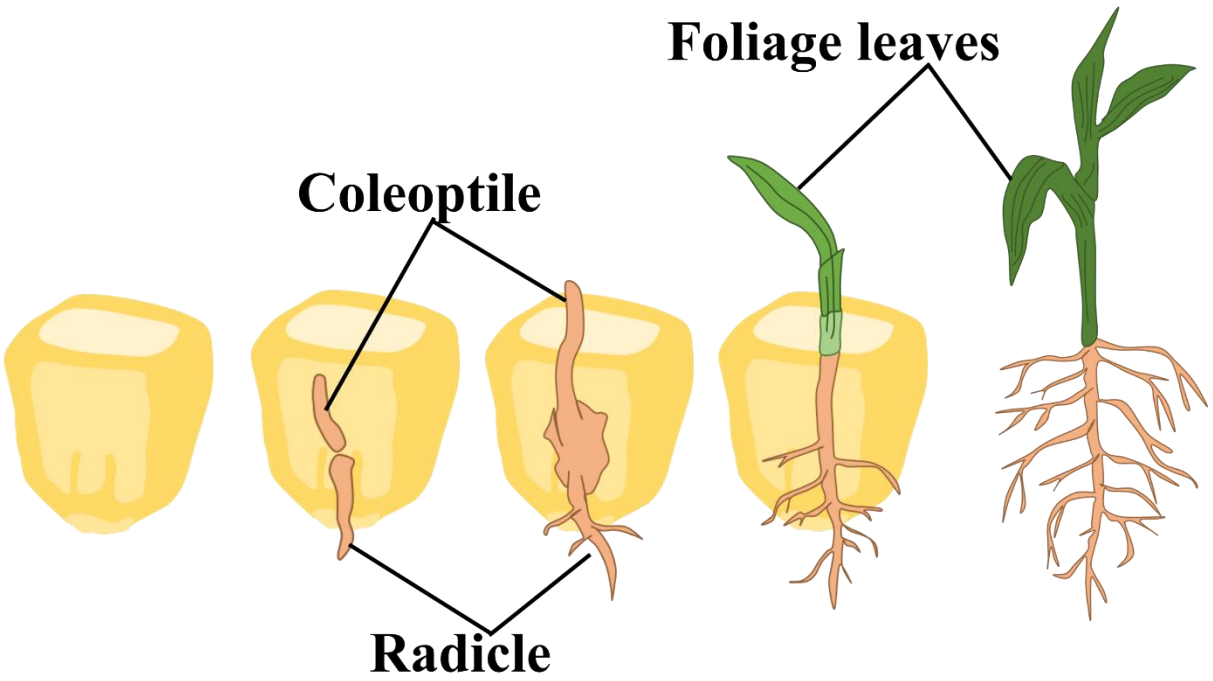

Fig. 8.2 Growth stages of maize plants (*Zea mays* L.) from seedling to the three-leave stage.

At the three-leaf stage, there are three elongated leaves and the tip of the fourth leaf appears at the centre of the leaf whorl (Fig. 8.2). This stage is a pivot point of maize growth from heterotrophic growth (i.e. growth relying on seed reserves) to autotrophic growth (i.e. growth relying on photosynthesis after the exhaustion of seed reserves) (Cooper and MacDonald, 1970; Hanway, 1966). All three compounds were chosen to reflect possible organic (Cys, Met) and inorganic (Na_2SO_4) S compounds typically released and exposed to plant roots during the breakdown of soil organic matter.

To ensure and maintain sterile conditions, all nutrient stock solutions, deionized water and containers (polypropylene vials, syringe, pipette tips etc) used in the experiments were autoclaved. In addition, roots were rinsed with sterile 10%-strength Long Ashton nutrient solution (to remove any exoenzymes and exudates) prior to transfer to the $^{35}S/^{14}C$-labelled solution. Light was excluded from the nutrient solution by tightly wrapping the root compartment with aluminium foil. Experiments were carried out during daylight hours with plants exposed to the same light intensity and temperatures as described above.

8.2.2 Experiment 1: Plant uptake and partitioning of ^{14}C-Cys and Met under hydroponic conditions

Briefly, maize plants were rinsed with sterile 10%-strength Long Ashton nutrient solution before being placed into individual 25 ml polypropylene containers filled with 20 ml of 10%-strength S-free Long Ashton nutrient solution. Experiment one included six treatments:

(a) ^{14}C-Cys

(b) ^{14}C-Cys + Met

(c) ^{14}C-Cys + Na_2SO_4

(d) ^{14}C-Met

(e) ^{14}C- Met + Cys

(f) ^{14}C- Met + Na_2SO_4

The concentration of each S compound in the final nutrient solution was 100 µM (i.e. in treatment b, the concentration of Cys and Met are 100 µM separately), which was in the range of previously reported amino acid concentrations in soil solution (Jones and Darrah, 1994; Johnson and Pregitzer, 2007). After injection of labelled material(s) into the nutrient solution, the plant-solution system was sealed and the $^{14}CO_2$ efflux from plant tissues was trapped by placing a container of 1 M NaOH solution (10 ml) inside the container (Fig. 8.3). After 24 h,

^{14}C activity in each compartment was determined separately. Plant roots and shoots were destructively harvested. Plant materials were first rinsed with 0.01 M $CaCl_2$ for 30 s to remove any isotope adhered to the plant surface, then rinsed with unlabelled 10%-strength Long Ashton nutrient solution. Plant shoots and roots were then oven dried (80 °C, 24 h), weighed and ground to a powder separately. ^{14}C activity in the plant tissues (shoots and roots) was determined with an OX-400 Biological Sample Oxidizer (RJ Harvey Instrument Corp., Hillsdale, NJ). The liberated $^{14}CO_2$ was collected in Oxosol scintillation fluid (National Diagnostics, Hessle, UK). ^{14}C was then quantified by liquid scintillation counting using a Wallac 1404 scintillation counter with automated quench correction (Wallac EG&G, Milton Keynes, UK). The amount of ^{14}C-amino acid remaining in the nutrient solution alongside the amount of plant respiration was also determined.

8.2.3 Experiment 2: Plant uptake and distribution of ^{35}S-Cys, Met and sulphate under hydroponic conditions

Maize germination, transplanting and nutrient provision were the same as in Experiment 1 except that ^{14}C was replaced with a ^{35}S tracer and no NaOH traps was required (Fig. 8.4). The concentration of each ^{35}S-labelled compound in the final nutrient solution was 100 µM and the chase period was 24 h at which time the incorporation of ^{35}S into plant tissues was determined. Experiment 2 included nine treatments:

(a) ^{35}S-Cys

(b) ^{35}S-Cys + Met

(c) ^{35}S-Cys + Na_2SO_4

(d) ^{35}S-Met

(e) ^{35}S-Met + Cys

(f) ^{35}S-Met + Na_2SO_4

(g) ^{35}S-$Na_2{}^{35}SO_4$

(h) ^{35}S-$Na_2{}^{35}SO_4$ + Cys

(i) ^{35}S-$Na_2{}^{35}SO_4$ + Met

To determine the liberation of inorganic S in nutrient solution during the course of experiment 2 (e.g., from exudation or exoenzyme activity), the nutrient solution at the end of the experiment was divided equally into two parts. Half of the nutrient solution was used for ^{35}S quantification (i.e. inorganic sulphate mineralized from Cys or Met, plus organic ^{35}S) directly by liquid scintillation counting. The remaining half was shaken (200 rev min^{-1}; 5 min) with the same volume of 0.1 M $BaCl_2$ (10 ml) and centrifuged (4000 rev min^{-1}; 5 min) to precipitate and remove any inorganic sulphate present in the Cys or Met treatments (i.e. as $Ba^{35}SO_4$). In both cases the amount of ^{35}S in the resultant solutions was determined by liquid scintillation counting as described above.

The amount of ^{35}S incorporated into the plant materials was also determined. Plant materials were first rinsed with 0.01 M $CaCl_2$ for 30 s to remove any isotope adhered to the plant surface, then rinsed with unlabelled 10%-strength Long Ashton nutrient solution. Plant material was then divided into roots and shoots, weighed and dried at 80 ℃ in the oven prior to further measurements. To determine the total amount of ^{35}S incorporated into plant tissues, aliquots of 40 mg of powdered dried samples were placed in glass vials and 1 ml of Soluene-350 (PerkinElmer Life Sciences, Inc) added. The vials were then capped and incubated (40℃, 4 h) until the samples were fully digested and an almost colourless. This prevents the presence of pigments, chlorophyll in our case, causing inaccurate readings on the scintillation counter (Gibson, 1980; Smith and Lang, 1987; Thomson and Temple, 2020). The amount of ^{35}S was then determined by liquid scintillation counting as described above.

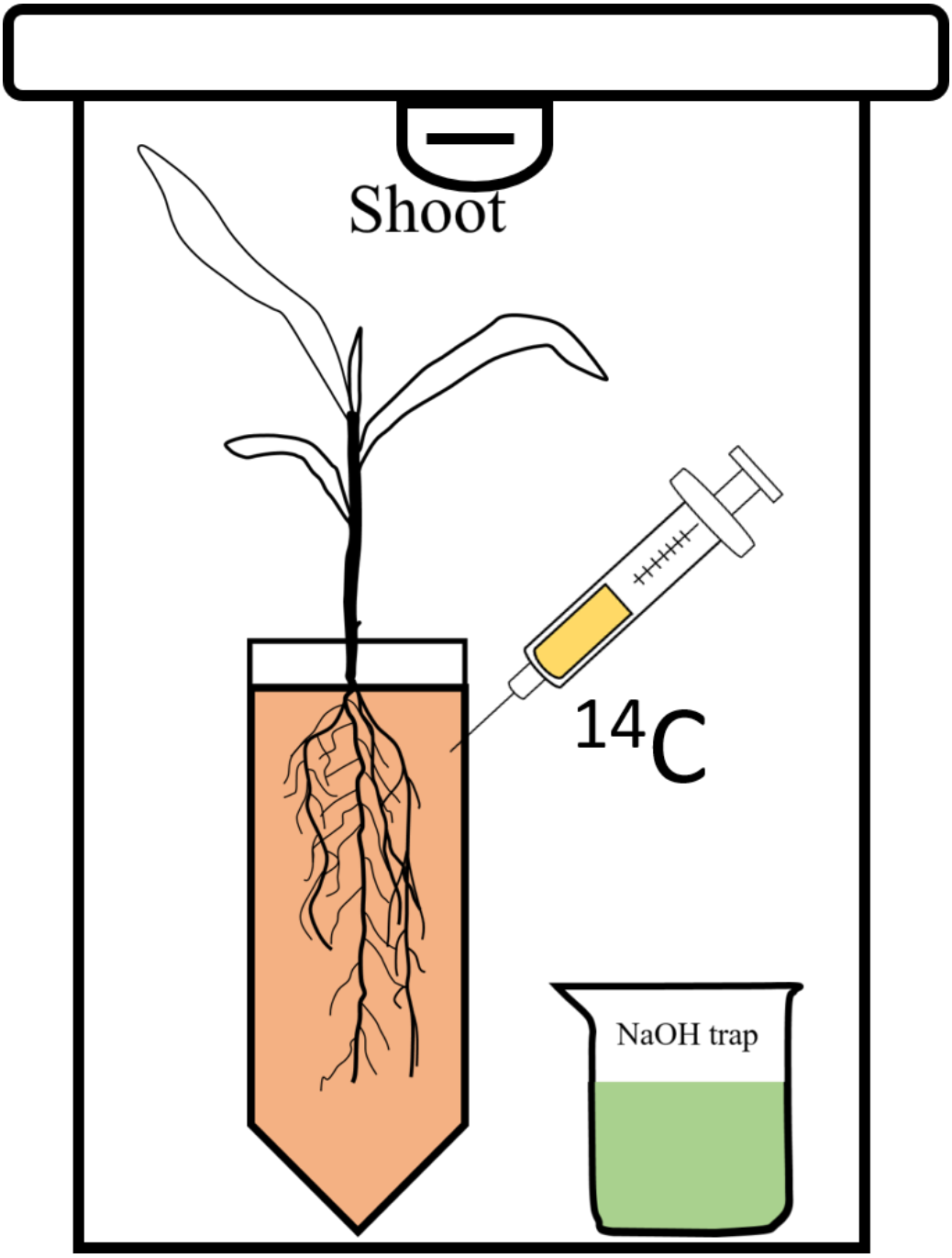

Fig. 8.3 Schematic representation of the maize microcosm into which ^{14}C-labelled amino acid (Cys or Met) was injected. Each polypropylene tube was filled with 20 ml of 10%-strength Long Ashton nutrient solution. The final concentration of the two sulphur-containing amino acids in nutrient solution were brought to 100 µM separately. The ^{14}C-radioisotope was then injected into the 10%-strength Long Ashton nutrient solution in the polypropylene tubes. The ^{14}C-amino acid movement and incorporation into different root and shoot sections was measured after 24 h after the injection. $^{14}CO_2$ evolved from shoot compartment was trapped in 10 ml of 1 M NaOH.

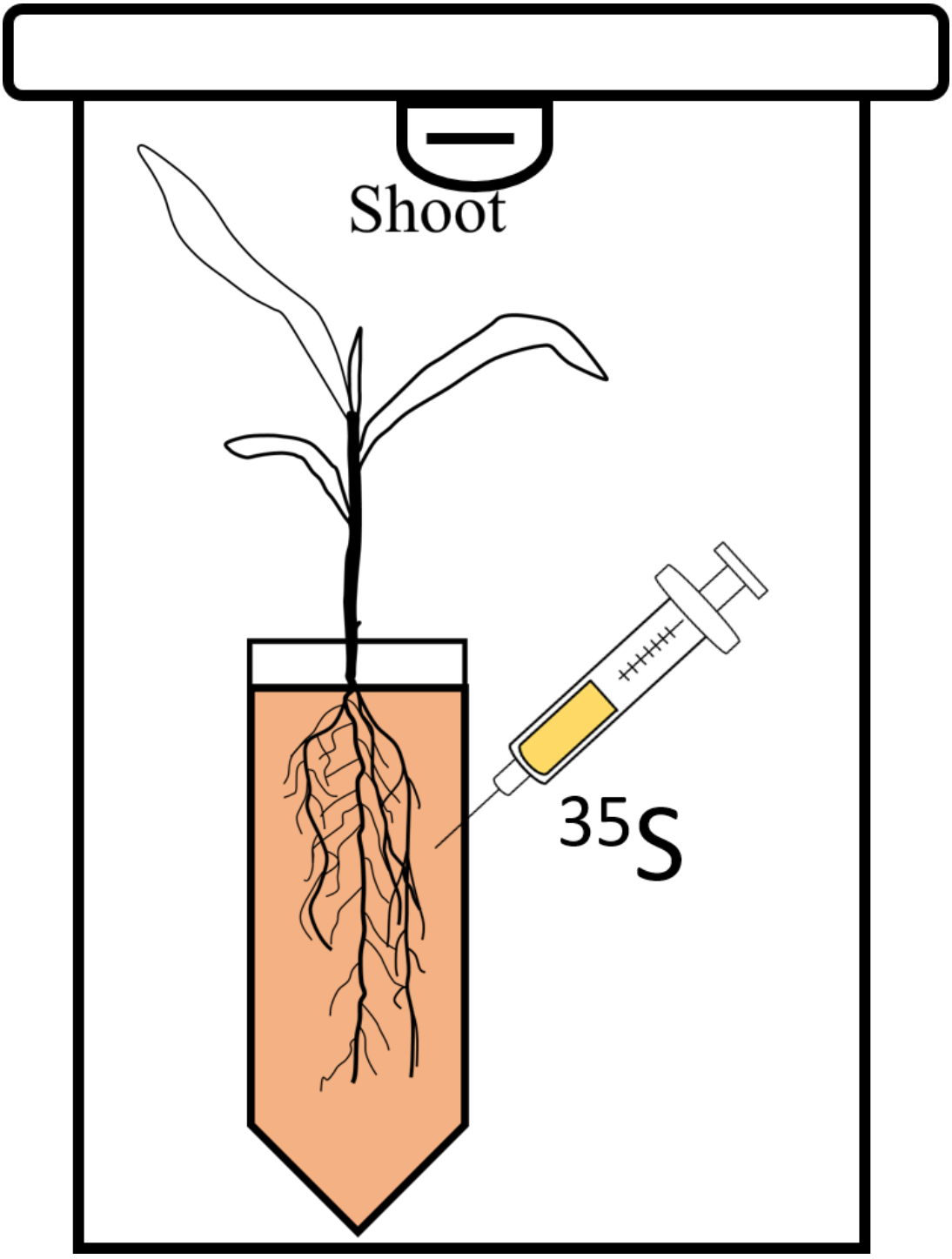

Fig. 8.4 Schematic representation of the maize microcosm into which ^{35}S-labelled Cys, Met or inorganic sulphate was injected. The same experimental set up as experiment 1 was adopted here, except that NaOH traps were not placed inside Lock & Lock plastic containers. The final concentration of Cys, Met or inorganic sulphate in nutrient solution were brought to 100 µM separately. The ^{35}S-radioisotope was then injected into the 10%-strength Long Ashton nutrient solution in the polypropylene tubes. The ^{35}S depletion from nutrient solution as well as the incorporation into plant root and shoot sections were measured after 24 h after the injection.

8.2.4 Experiment 3: Efflux of ^{35}S-labelled Cys, Met and SO_4^{2-} from maize roots

Nine uniform sterile maize plants were removed from 10%-strength Long Ashton nutrient solution and transferred into the open barrel of individual 25 ml polypropylene syringes with a two-way stopcock connected at the bottom (Fig. 8.5). Each syringe was filled with 20 ml of one isotopically labelled S compound (i.e. ^{35}S-Na_2SO_4, ^{35}S-Cys or ^{35}S-Met; 100 µM) in 10%-strength Long Ashton S-free nutrient solution. This simple axenic system facilitated collection of root-derived ^{35}S efflux and minimizes root damage and overestimation of efflux (Ayers and Thornton, 1968).

After being transferred to the new nutrient solution, maize plants were supplied with each ^{35}S compound for 1 h in the external root bathing medium. After 1 h, the labelled nutrient solution was removed by opening the valve at the bottom of each syringe and the plants rinsed with sterile deionized water to remove any isotope adhering to the roots. The syringe was then refilled with 20 ml of non-^{35}S-labelled nutrient solution. This root bathing solution was collected and replaced every 10 mins over an 80 min period. The amount of ^{35}S label present in the collected solutions in either an organic or inorganic form was determined using the 0.1 M $BaCl_2$ precipitation procedure described above. This enabled the efflux of both sulphate and organic S from maize roots to be determined. ^{35}S efflux rate from roots (S_{efflux}) was expressed as µmol g^{-1} DW root h^{-1} as the increase of ^{35}S in the bathing solution between the start (T_0) and the end (T_t) of the sampling period, where R is dry root biomass and T denotes sampling time.

$$S_{efflux} = (T_t - T_0) / (R \times T) \quad \text{(Eqn. 1)}$$

Many studies have investigated the efflux of low MW organic solutes and ions (e.g. K^+, Cl^-, sugars, etc.) after pre-loading the roots. This efflux process typically involves three distinct root compartments, namely, the apoplast, cytoplasm and vacuole (Thoiron *et al.*, 1981; Saftner, Daie and Wyse, 1983). In this study, we can discount the fast-exchanging (<1 min) apoplastic compartment as we washed the roots prior to monitoring efflux. Here we fitted a mathematical

model to the experimental efflux data (Rauser, 1987) in which the leakage of S compounds to the outer bathing solution was considered as the sum of two diffusional processes from the cytoplasm and vacuole to the root bathing medium:

$$y = a \times (1 - \exp^{(-c \times t)}) + b \times (1 - \exp^{(-d \times t)}) \quad \text{(Eqn. 2)}$$

Where *y* is the accumulated ^{35}S washed out of plant roots, *a* and *b* is the size of the S storage pool in the cytoplasm and vacuole respectively, *c* and *d* are the exponential coefficient describing the rate of ^{35}S release from these pools into the external root bathing solution. The half time ($t_{½}$) of each pool can then be calculated as:

$$t_{½} = \ln(2)/c \text{ or } t_{½} = \ln(2)/d \quad \text{(Eqn. 3)}$$

The initial volume of different S compounds in the two compartments A and B can be calculated by:

$$A = a - c / d \times a; B = b + c / d \times a \quad \text{(Eqn. 4)}$$

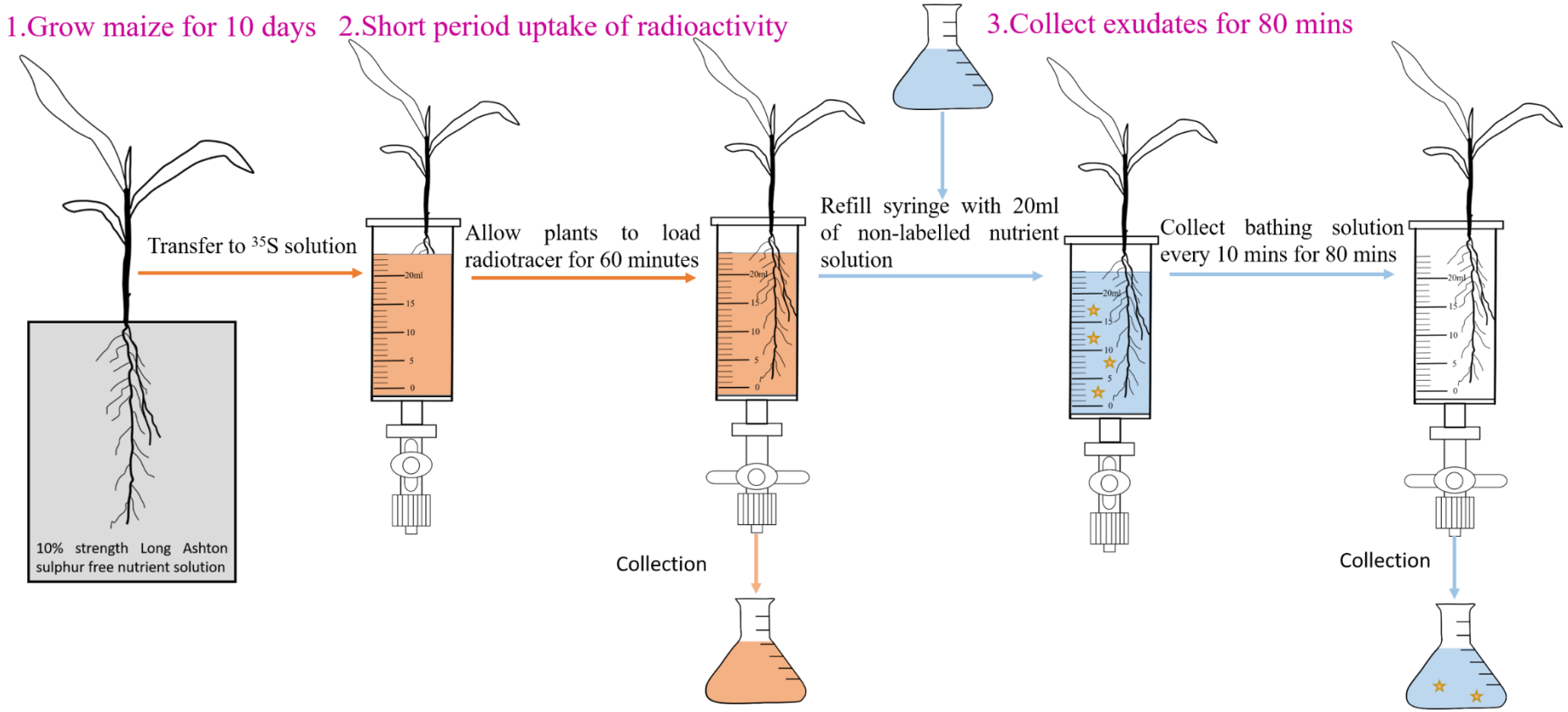

Fig. 8.5 Schematic illustrating how ^{35}S labelling were used to obtain an estimate of sulphur efflux by maize plants (*Zea mays* L.).

8.2.5 Experiment 4: A split root system-cycling of amino acid S and other nutrients between shoots and roots in young maize plants

In the fourth experiment, fifteen uniform maize plants were selected and the roots of each maize plant split approximately equally between two separate containers of nutrient solution. At the start of the experiment, one of the root compartments was exposed to a radioisotope solution (i.e. either ^{35}S-Cys, ^{14}C-Cys, ^{35}S-Met, ^{14}C-Met or ^{35}S-Na_2SO_4; 100 µM). Roots in this compartment were termed 'donor' roots; while the other root compartment was immersed into unlabelled nutrient solution, and roots in this compartment were termed 'receiver' roots. The 'donor root' compartment was used for the labelling and 'receiver root' compartment was used for determining the internal cycling and subsequent release of radioisotope.

Each experiment unit was placed inside a 2 L translucent sealable plastic container (Lock & Lock; Really Useful Products Ltd, West Yorkshire, UK; Fig. 8.6). After 24 h, the shoots were removed and the roots harvested and rinsed with 0.01 M $CaCl_2$ for 30 s to remove any surface isotope contamination. Nutrient solution from both compartments was also collected to determine the amount of isotope depletion by the donor root and exudation from the receiver root. Radioactivity in the plants and solutions were determined by liquid scintillation counting as described above.

For ^{14}C treatments, 1 M NaOH trap (10 ml) were placed inside the 2 L plastic container beside maize plant to catch any $^{14}CO_2$ evolved from the plant. For ^{35}S treatments, no NaOH traps were placed.

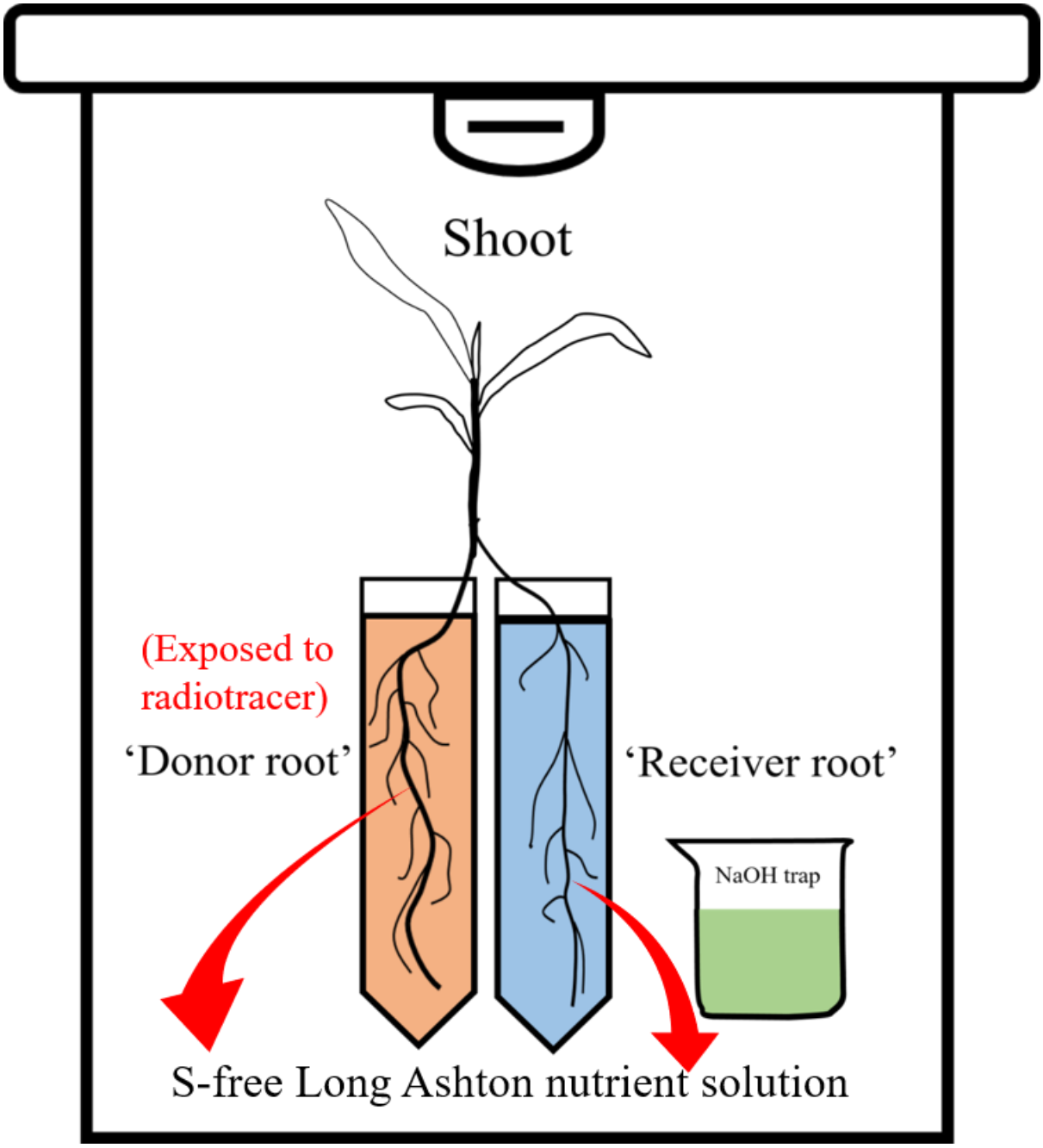

Fig. 8.6 Schematic representation of the experimental apparatus showing the maize plant growing in a split root system with only 'donor root' exposed to radioisotope. This system was allowed to develop over a 24 h-period.

8.2.6 Statistics and data analysis

All experiments had three replicates per treatment and used plants with similar shoot heights (ca. 12 cm) and root lengths (ca. 9 cm). When calculating root S influx, it was assumed that efflux of the S compounds was minimal during the exposure period. Similarly, for calculation of S efflux, it was assumed that S uptake was minimal during the exposure period. All data analysis was carried out in in IBM SPSS Statistics v25 (IBM UK Ltd., Portsmouth, UK). One way ANOVA followed by Tukey's post-hoc test was used to identify treatment differences, with the significance level set at $p < 0.05$. Graphs and curve fitting were produced using SigmaPlot v13.0 (Systat software Inc., London). The results are presented as means ± SEM ($n = 3$) and only significant differences are discussed ($p < 0.05$).

8.3 Results

8.3.1 Plant uptake and distribution of ^{14}C-labelled Cys and Met under sterile hydroponic conditions

In experiment 1, the plant uptake of ^{14}C-labelled Cys and Met over time was measured under sterile hydroponic conditions. The results indicated that sterile maize roots rapidly took up both Cys and Met, after which the amino acid-C was incorporated into both new cell biomass and utilised for respiration (Fig. 8.7). A similar amino acid incorporation rate was recorded for both amino acids: 203.4 ± 35.7 nmol ^{14}C (g root DW)$^{-1}$ h^{-1} for Cys, and 191.9 ± 30.7 nmol ^{35}S (g root DW)$^{-1}$ h^{-1} for Met. However, the partitioning of ^{14}C among the different plant compartments varied for the two amino acids. Overall, a higher proportion of Cys was partitioned into plant respiration while a higher proportion of Met was partitioned into plant biomass ($p < 0.05$). Based on our calculation, 5.8 ± 0.7 % and 3.7 ± 0.2 % of the added Cys and Met were respired by the maize plants, respectively, whereas 6.7 ± 0.3% and 10.8 ± 0.5% were incorporated into plant biomass (shoot plus root tissues), respectively.

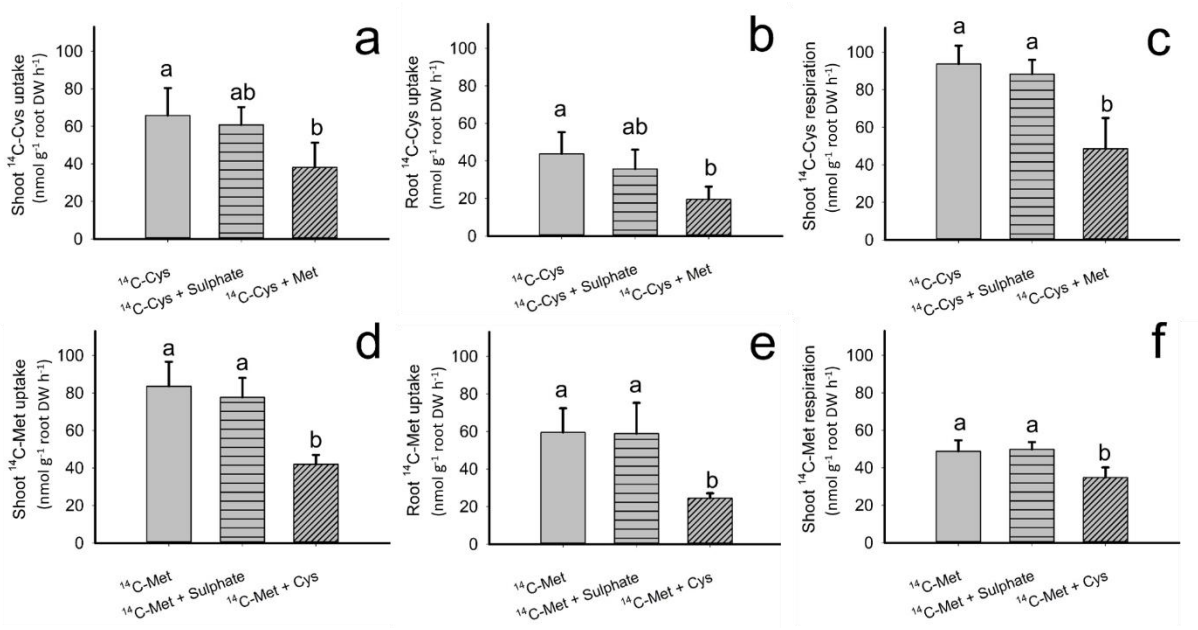

Fig. 8.7 Distribution of ^{14}C-labelled Met and Cys in maize plants after 24 h of a) ^{14}C-Cys recovered in the shoots; b) ^{14}C-Cys recovered in the roots; c) ^{14}C-Cys respiration from the maize plant; d) ^{14}C-Met recovered in the shoots; e) ^{14}C- Met recovered in the roots; f) ^{14}C-Met respiration from the maize plant. Bars and lines represent mean ± SEM (n = 3). Common lowercase letters denote a lack of significant difference between treatments (One way ANOVA: LSD test, $p < 0.05$).

Overall, the total percentage of ^{14}C recovered in plant shoots, roots, solution and CO_2 evolution from root-solution system exceeded 80% for all treatments. The highest amount of ^{14}C recovered in the ^{14}C-Cys among all three compartments (shoots, roots, and respiration) was for CO_2 evolution, constituting 5.8 ± 0.7% of ^{14}C-Cys input. Only a small fraction of the ^{14}C derived from Cys was retained in plant roots after uptake (2.6 ± 0.1%), while a larger proportion (4.1 ± 0.2%) was transported to the shoots. Total plant utilization of ^{14}C derived from Met (14.5 ± 0.4%) was similar to that of Cys (12.6 ± 0.8%), however, the highest ^{14}C content for Met was found in the shoots (6.3 ± 0.3%), followed by the roots (4.5 ± 0.5%) and respiration (3.7 ± 0.2%).

Plant uptake of Cys and Met decreased in the presence of each other. This implies that competition between Cys and Met occurred for entry into the root cells, probably due to a

common transport carrier. Cys supply led to a decrease in Met partitioning into respiration, shoot tissue, and root tissue by 29.3 ± 10.3%, 49.3 ± 13.1%, and 57.9 ± 10.6% respectively, while Met supply led to a decrease in Cys incorporation by 48.3 ± 12.1%, 42.1 ± 10.1%, and 55.3 ± 6.7%, respectively. In contrast, plant uptake of Cys and Met was not markedly affected by the presence of inorganic sulphate ($p < 0.05$) (Fig. 8.7). This implies that Cys and Met may be a more favourable source of S even under situations of high access to inorganic sulphate. It should be noted that the lower concentrations of Cys and Met in soil solutions *in situ* may limit the actual contribution of these compounds to plant nutrition.

8.3.2 Uptake and distribution of ^{35}S-labelled Met, Cys and sulphate by maize plant roots under sterile hydroponic conditions

In experiment two, the uptake of ^{35}S-labelled Cys, Met and sulphate was assessed over 24 h. The uptake period was chosen to ensure that sterility was maintained as well as ensuring that all the S in the external medium was not depleted during the incubation period (Barber and Gunn, 1974; Gaume, Mächler and Frossard, 2001). The uptake of all three S forms were similar in that the labelled S taken up was not retained in root tissues, but was rapidly transported to the shoots. However, there were striking differences in the ability of the maize plants to utilize the different S compounds. Cys and Met uptake by the whole plant was 347 ± 15 and 390 ± 54 nmol ^{35}S g^{-1} root DW h^{-1}, respectively over the 24-h period. However, there was a much greater accumulation of sulphate, being 2.2- and 1.9-fold higher than Cys and Met, respectively. The higher uptake could be explained by faster ^{35}S-sulphate transport from root to shoot tissue, as similar values for root retention of all three S sources were obtained. Overall, 10.3 ± 1.4%, 10.1 ± 0.8%, and 11.5 ± 1.1% of the added ^{35}S was recovered in the plant roots from Cys, Met, and sulphate respectively. However, in terms of transportation to shoot tissue, sulphate was more mobile than both amino acids as only 11.6 ± 0.9 % and 13.3 ± 1.9 % of Cys and Met was

detected in shoot tissues, whereas a significantly greater proportion (34.1 ± 2.2 %) of the added sulphate ($p < 0.05$) was found in the shoots.

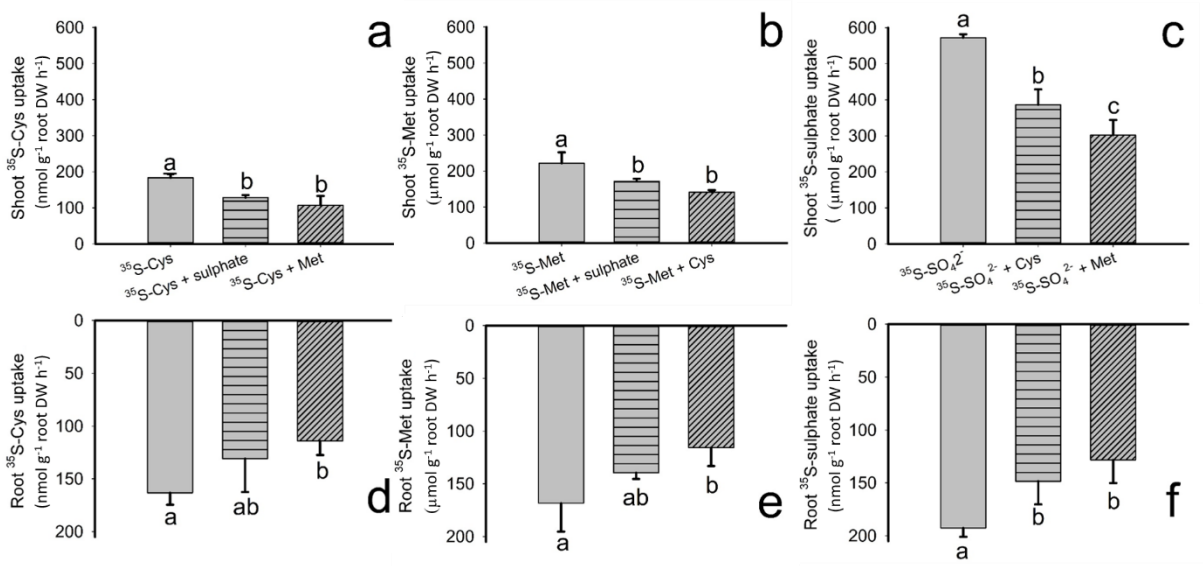

Fig. 8.8 Partitioning of ^{35}S label after the introduction of radio isotope into hydroponic maize plants for 24 h. a) ^{35}S-Cys recovered in the shoots; b) ^{35}S-Met recovered in the shoots; c) ^{35}S-SO_4^{2-} recovered in the shoots; d) ^{35}S-Cys recovered in the roots; e) ^{35}S-Met recovered in the roots; f) ^{35}S-SO_4^{2-} recovered in the roots. Values represent means ± SEM ($n = 3$).

The presence of other S sources on the root uptake of each S compound (i.e. competition) was also examined (Fig. 8.8). The root uptake of both amino acids was unaffected by the presence of sulphate ($p > 0.05$), but transportation of ^{35}S-Cys and ^{35}S-Met to the shoot was markedly decreased in the presence of sulphate by 29.8 and 21.3 % ($p < 0.05$), respectively. This resulted in a significant inhibition of Cys uptake when sulphate was present ($p < 0.05$), although it proved non-significant for Met. In contrast, root ^{35}S-sulphate uptake and transportation to shoots was markedly decreased by 32.6% and 47.3% in the presence of Cys, and by 22.6 % and 33.5 % in the presence of Met respectively, indicating that there was a downregulation of inorganic S uptake by organic S compounds. In addition, Cys and Met resulted in significantly decreased levels of both root uptake and shoot transportation of each

other ($p < 0.05$). Cys supply effectively decreased root uptake and shoot transportation of Met by 35.2% and 29.7%, respectively ($p < 0.05$), while Met supply effectively decreased root uptake and transportation of Cys by 42.1% and 30.1%, respectively ($p < 0.05$).

8.3.3 Intact uptake of cysteine and methionine

To determine whether Cys and Met were taken up as intact molecules or as inorganic compounds after enzymatic or microbial degradation, results of the uptake techniques (i.e. via ^{14}C and ^{35}S labelling) were combined (Fig. 8.9). The co-location of ^{14}C and ^{35}S appears a reasonable measure of intact uptake by plants roots under sterile hydroponic conditions. Our results clearly show that a large proportion of the supplied tracer may have been taken up intact. This was illustrated by plots of total ^{14}C vs total ^{35}S in maize roots ($R^2 = 0.68$ for Cys, $R^2 = 0.83$ for Met).

A higher proportion of ^{35}S (experiment two) than ^{14}C (experiment one) derived from both amino acids in plant material was detected in the plant tissue. The discrepancy between expected and measured ratios of ^{14}C to ^{35}S may be explained by several possibilities. First, the difference of measurement of ^{14}C and ^{35}S from biological samples (^{14}C was measured by dry combustion, while ^{35}S was measured by wet digestion) could have led to different recovery rates of the two radiotracers. Second, under the action of enzymes released by plant roots, part of added amino acids could have been degraded in nutrient solution to inorganic compounds ($^{14}CO_2$, NO_3^-, NH_4^+ and $^{35}SO_4^{2-}$; Fig. 8.1) prior to being taken up independently (Jones, Healey, *et al.*, 2005). This rapid enzymatic degradation of amino acids may contribute to a higher ^{35}S recovery in plant materials due to the fast uptake rates of inorganic sulphate by maize roots (Astolfi *et al.*, 2004). Some $^{14}CO_2$ may also have been lost in respiration during the washing and drying of the root and shoot material. In addition, rapid post-uptake metabolism of amino acids may also explain the anomalous relationships between ^{14}C and ^{35}S.

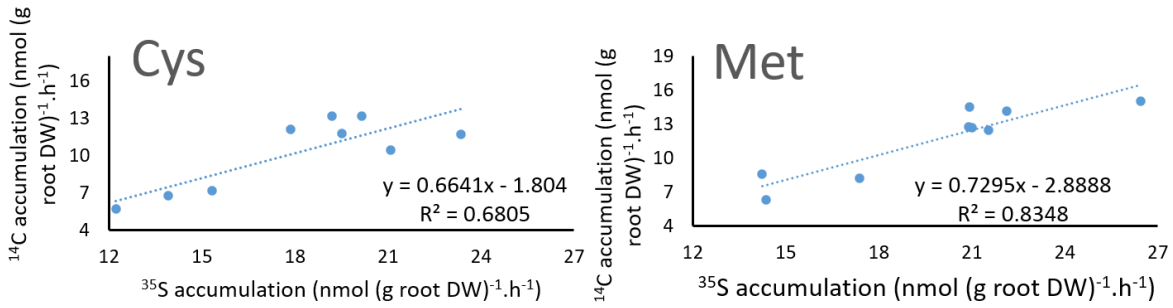

Fig. 8.9 Relationship between the total accumulation of ^{14}C and ^{35}S radiotracer (nmol g^{-1} root DW h^{-1}) in maize roots and shoots after 24 h of exposure to the labelled solution containing 100 µM cysteine or methionine (*n* = 9).

8.3.4. Efflux of cysteine, methionine and inorganic sulphate from maize roots

Previous studies on the loss of organic compounds from intact roots have focused on the accumulation of amino acids over long experiment periods, yet according to our results from experiment one and two, plant roots have the capacity to actively take up amino acids rapidly, which may in turn result in a lower detection of root efflux. Experiment three examined S efflux from maize roots. Rapid sampling was used to minimize the negative effect of root re-capture of the compounds lost from root efflux. It also helped to increase the concentration gradient between the root cytosol and the root bathing solution. The results showed that of the ^{35}S loaded into the plant, 37.1, 27.6, and 27.5% the Cys, Met, and sulphate-S was recovered in the root bathing medium, respectively. Efflux rates exceeded influx rates for all three compounds, which is in line with previous studies performed under axenic conditions (Lesuffleur and Cliquet, 2010). The results showed that Cys and Met efflux rates were in the same range, between 1.0 ± 0.1 and 4.2 ± 0.3 µmol g^{-1} root DW h^{-1} for Cys, and 0.7 ± 0.2 and 3.2 ± 0.1 µmol g^{-1} root DW h^{-1} for Met (Fig. 8.8), indicating rapid efflux of low molecular S compounds within the short monitoring period. The release of sulphate was similar to that of the amino acids, rates ranging from 1.2 ± 0.1 to 4.2 ± 0.3 µmol g^{-1} root DW h^{-1}. In this study, the efflux rate of Cys and Met from attached maize roots was about 3-13 fold greater that the

rate of uptake, implying a minor effect of amino acid uptake on the overall efflux pattern of amino acids over the short monitoring period. Application of $BaCl_2$ allowed the separation of organic and inorganic S in the root exudates. The results revealed that efflux of all three S compounds were in the form they were taken up, suggesting efflux of low molecular weight compounds in short period occurs via passive leakage. This is in line with previous studies, which suggested that amino acid efflux rates are small or insignificant, this efflux is generally regarded as not carrier-mediated but occurs by passive leakage (Jones and Darrah, 1993; Paynel, Murray and Bernard Cliquet, 2001), and could be recaptured by roots.

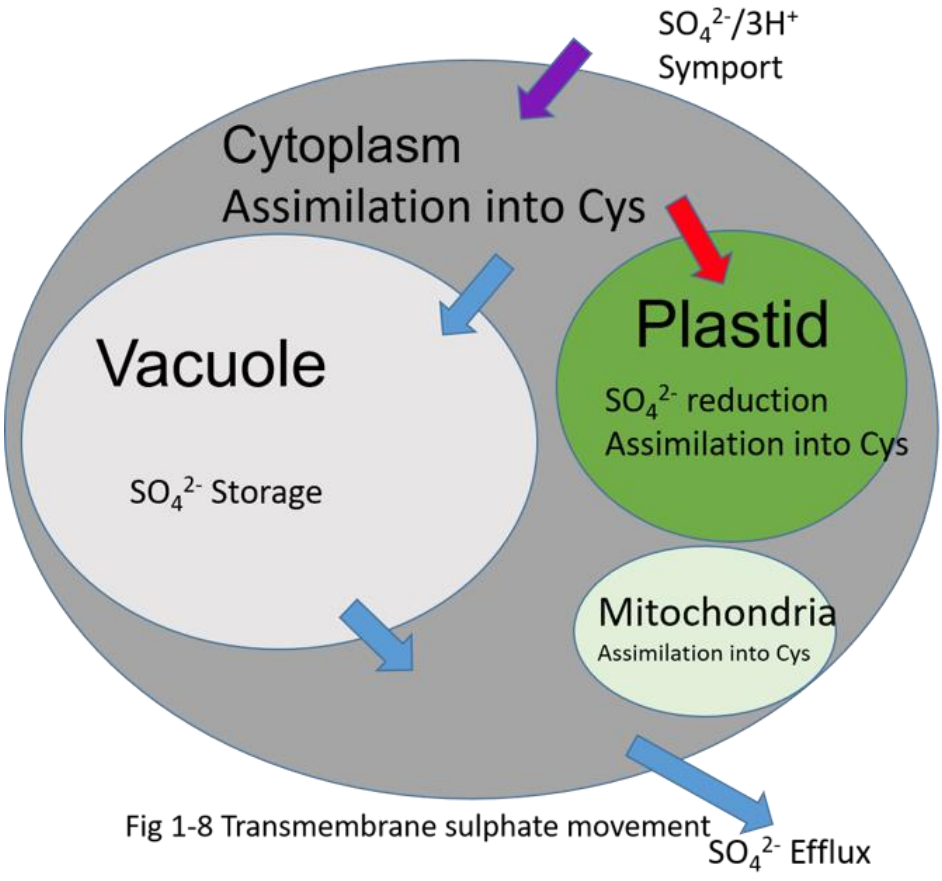

Fig 1-8 Transmembrane sulphate movement

Fig. 8.10 Transmembrane sulphate movement

Table 8.1 Parameters of amino acids (Cys and Met) and sulphate release from intact maize roots. Efflux data was fitted to Eqn. 2. The parameters *a* and *c* represent the ^{35}S held in the cytoplasm and vacuole respectively, while *b* and *d* are the efflux rate constants for these two pools, respectively. Values represent means ± SEM (*n* = 3).

Substrates	**Pool *a***	**Pool *c***	**$t_{½}$ (fast pool) hour**	**$t_{½}$ (slow pool) day**	A (μmol.(g root DW)$^{-1}$)	B (μmol.(g root DW)$^{-1}$)	**R^2**
Cys	0.56±0.07	1.09±0.01	2.98±0.62	1.78±0.26	1.03±0.18	0.66±0.02	0.99
Met	0.55±0.04	0.93±0.45	3.14±0.41	6.59±2.32	1.10±0.35	0.58±0.03	0.99
Na_2SO_4	0.58±0.04	1.27±0.02	3.72±0.33	1.39±0.24	1.14±0.05	0.71±0.06	0.99

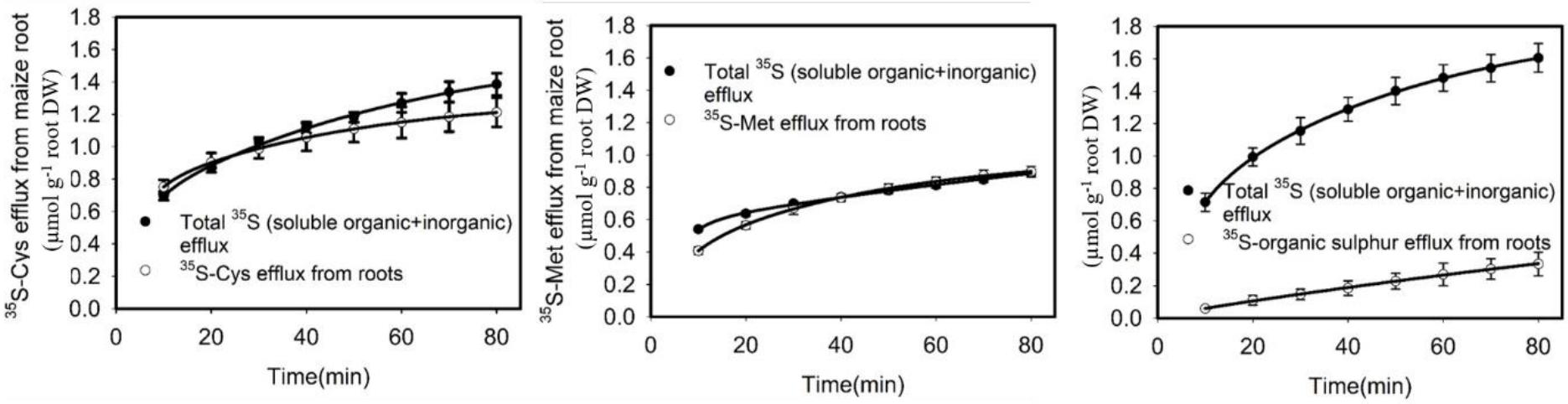

Fig. 8.11 Cumulative efflux of added ^{35}S-Cys, Met or sulphate per unit dry mass of maize roots. Prior to measuring efflux, plants were pre-treated with radioisotopes for 60 minutes. Efflux was determined by measuring the increase of radioisotope in the root bathing medium solutions. $BaCl_2$ was applied to separate organic and inorganic S in solution. Data represent means ± SEM (*n* = 3).

The release of ^{35}S into the bathing solution indicated two distinct compartments (Fig. 8.10), which may be interpreted as two pools: the cytoplasmic and vacuole compartments (Cooper and Clarkson, 1989; Paynel, Murray and Bernard Cliquet, 2001). The rate of ^{35}S release from the roots decreased sharply over the course of the efflux period. A double first order exponential decay equation fitted well to the efflux data ($R^2 > 0.99$; Fig. 8.11)). This predicted that the half-life for the slower exchanging compartment (vacuole) were 1.7, 6.6, and 1.4 d for Cys, Met, and sulphate, respectively (Table 8.1), while half-lives for the faster exchanging compartment (cytoplasm) were 2.9, 3.1 and 3.7 h for Cys, Met and sulphate, respectively. Based on calculation from Eqn. 4, the cytoplasmic S pool was estimated to range from 0.58 to 0.71 µmol g^{-1} root DW, which was smaller than concentration in the vacuole which ranged from 1.03 to 1.14 µmol g^{-1} root DW.

8.3.5 Cycling of sulphur compounds between maize shoot and root via split root systems

In experiment four, a split root system under sterile conditions was used to investigate the cycling of S compounds between shoot and root in young maize plants. Here, both the translocation of S compounds from 'donor root to shoot' and 'shoot to receiver roots' were monitored. Isotope labels (^{14}C and ^{35}S) were used to establish the quantitative significance of S cycling in S deficient plants. Overall, the results indicated that S compounds entered the plant via donor roots and were then cycled through the shoots and to the receiver-roots.

Calculation showed that > 50% of the ^{14}C tracer loaded from the donor root was cycled through the whole plant. The fraction of isotope tracer in each compartment (donor root, shoot, receiver root) is shown in Table 8.2. By the end of this incubation experiment, less than 10% of the ^{14}C-Cys from the donor root was transported and retained in the shoot within 24 h, while a much higher proportion (nearly 50%) was respired from the shoot, ^{14}C-Cys partitioning in the receiver root reached a similar level as the donor root (around 20%). Similarly, a high

proportion of ^{14}C derived from Met was cycled from donor root to the whole plant, although less ^{14}C was respired and a higher proportion was retained in the shoot relative to Cys.

The distribution pattern for ^{35}S differed from that of ^{14}C in that a higher proportion of S was retained in the donor root tissue. Nearly half of the ^{35}S taken up from the nutrient solution was retained in the donor root, ca. 40% was transported to the shoot, from where less than half was subsequently translocated to the receiver root. One possible explanation for the difference in ^{35}S and ^{14}C responses is that after being taken up by the root tissues, the amino acids are metabolized (deaminated, transaminated etc.) prior to transport to the shoots (Warren, 2012a). Overall, the actual amount of sulphate cycled from nutrient solution to the donor root was around three times higher than that of the two amino acids. By the end of the 24-h cycling period, however, negligible amounts of radioactivity were observed in the nutrient solution in the receiver root compartment. Therefore, it is likely that under S deficient conditions, maize plants are highly effective at maximising S use efficiency within the plant and minimise root exudation (or recapture any S lost to the external medium).

Table 8.2 Translocation and utilization of three sulphur compounds by maize plants over a 24-h period. Both root compartments received the same amount of nutrient solution. Three independent measurement from replicate plants were made for each treatment. Values represent means ± SEM (n = 3).

	^{14}C-Cys	^{35}S-Cys	^{14}C-Met	^{35}S-Met	^{35}S-Na_2SO_4
$^{14}CO_2$ partitioning (% ^{14}C taken up)	47.2 ± 9.3		32.4 ± 2.9		
Shoot tissue	6.0 ± 1.9	20.5 ± 5.2	11.4 ± 1.4	29.0 ± 2.5	21.1 ± 2.5
Donor root	22.9 ± 3.1	63.7 ± 2.7	32.0 ± 1.6	49.2 ± 3.2	62.9 ± 1.9
Receiver root	23.9 ± 5.0	15.8 ± 3.2	24.1 ± 1.8	21.8 ± 2.9	16.0 ± 3.7

8.4 Discussion

A complication about quantifying intact amino acid uptake is that theoretically the same correlation in isotope enrichment could be observed if the dual labelled amino acids were mineralized to inorganic compounds external to the root and then taken up (e.g. by carbon-sulphur lyases). In this short plant uptake experiment, considering the efforts to minimize bacteria growth in nutrient solution before the conduction of experiments, it is likely that the pre-mineralization of Cys and Met was negligible.

Previous studies have addressed the importance of carbon-sulphur-lyases in the degradation of amino acids to inorganic compounds, among which methionine gamma-lyase degrades Met to a-keto acids, ammonia and thiols (Rébeillé *et al.*, 2006; Goyer *et al.*, 2007; Huang, Joshi and Jander, 2014), while D-cysteine desulfhydrase degrades Cys to pyruvate, sulphide and ammonia (Schmidt, 1982; Ramírez and Whitaker, 1998; Riemenschneider *et al.*, 2005). Therefore, it is possible that enzymatic transformation of Cys and Met took place during our experiment, and the breakdown products of these metabolites were taken up separately by maize roots, however, to our knowledge these enzymes do not exist extracellularly.

Data from experiment two revealed that plants utilized inorganic sulphate preferentially over Cys and Met, and that organic S supply has a negative effect on sulphate uptake. This agrees with the widely accepted view that initial root uptake of inorganic sulphate is energy dependent through a proton/sulphate coupled co-transport in the plasma membrane of root cells and is well adjusted to the S status of the plant. When other sulphur sources (Cys or GSH) are provided to the plant, uptake is repressed in a negative feedback loop (Herschbach and Rennenberg, 1994; Bolchi *et al.*, 1999; Hawkesford *et al.*, 2003; Davidian and Kopriva, 2010; Noctor *et al.*, 2011), while during S starvation, uptake is enhanced by activating the expression of high affinity sulphate transporters (Maruyama-Nakashita *et al.*, 2004). However, the fact that maize plants could take up DOS does imply that it may have ecological significance under

some circumstances. The results clearly showed that a considerable proportion of the supplied amino acids was absorbed intact, this was illustrated by plots of excess ^{14}C against excess ^{35}S in roots (Fig. 8.3), with 66% and 73% of Cys and Met taken up intact separately. Observation that both Cys and Met inhibit uptake of each another supported our hypothesis that these amino acids enter root cells via the same transport system.

It is well documented that influx of amino acids involves proton-coupled amino acid transporters (Bush, 1993; Delrot, Atanassova and Maurousset, 2000), whereas passive efflux is driven primarily by large differences in concentrations between the inside (10 mM) and the outside (0.1 – 10 µM) of roots cells (Jones and Darrah, 1993; Moore *et al.*, 2003). However, the mechanisms behind S efflux from root tissue are still unclear. In this study, rapid efflux of intact S compounds was detected from maize roots in experiment three. Considering S influx across the plasma membrane is energy dependent process, it is therefore surprising that S appears to leak out again rapidly. We therefore assume that after uptake, each compound transitorily accumulates in the cellular compartments (cytoplasm, vacuole) that are sensitive to efflux. Once they enter a complex reductive metabolic pathway, they are less likely to leak out again. Even so, the total amino acid efflux in the present study may have been under-estimated since amino acids could be re-absorbed by plant roots and these re-absorbed amino acids would not have been detected in efflux. In addition, high exogenous amino acid concentrations applied may have stimulated influx and diminished efflux which is assumed to be concentration dependent.

8.5 Conclusions and outlook

We have presented direct experimental evidence to support our hypothesis that plants (e.g., *Zea mays* L.) can directly take up S-containing amino acids intact under hydroponic conditions. These results indicate that dissolved organic matter in the form of free amino acids

(e.g. Cys and Met) may represent a readily available source of N, S and C for plants. We also provided evidence for the rapid redistribution of S within the plant following root uptake. S efflux from roots indicated two distinct S compartments, with the vacuole being the slower releasing compartment and the cytoplasm being the smaller storage compartment.

However, many gaps in our understanding remain. First, the correlation between ^{14}C and ^{35}S in plant tissues could arise if the amino acid were mineralized in the nutrient solution and the products taken up independently. However, this would require enzymes such as L-cysteine desulfhydrase which are unlikely to exist as exoenzymes in sterile hydroponic culture. Therefore, stronger evidence using compound-specific ($^{13}C/^{34}S$) isotope ratio mass spectrometry (IRMS) could be used to examine this further.

Second, despite evidence that plants take up free amino acids from hydroponic solution, it is unclear to what extent Cys and Met are taken up intact by plants *in situ*, where plant roots and rhizosphere microorganisms compete intensely for organic N (A G Owen and Jones, 2001). It is also not clear whether plant roots are capable of taking up other dissolved organic S forms (e.g., peptides and proteins). These may also play an important role in the N and S dynamics where inorganic N and S are inadequate for plant growth.

It has previously been suggested that most amino acids are minor components of the xylem sap (e.g. glycine) and must be metabolized within the roots (e.g. to glutamine) before transport to the shoots, where post uptake metabolism takes place (Warren, 2012b). To our knowledge, we know little about the metabolic fate of Cys and Met following uptake (i.e. which are the dominant pathways involved in their transformation and in which root cells this takes place.

Also, to avoid disturbance in sampling solution due to microbial decomposition, the majority of studies on root efflux have been carried out under sterile conditions. However, as it is difficult to maintain sterile root systems over long periods, most efflux experiments only

last for a short time (up to several weeks). Sodium hydroxide trapping method normally involves placing plants in a closed system that is not at a steady state. This major drawback also makes it difficult to maintain steady atmosphere in the system for a longer experimental period. Therefore, there is a need to develop new methods for maintaining the root system under sterile conditions, so that exudates from various root types could be collected for longer experiment period.

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8.7 References

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Chapter 9

Summary, discussion and recommendations for future research

9.1 Introduction

In this section, the main findings of the experimental work (presented in Chapters 3-8) are summarised and discussed in relation to the overall initial objectives of the thesis and published literature. The broader implications of the findings are also addressed. Detailed discussions of the results from individual experiments are provided alongside an outline of the main strengths and potential limitations of the research. This is followed by an assessment of the challenges and uncertainties identified in the thesis that need to be addressed by future research.

9.2 Synthesis of findings

The overarching aim of this thesis was to address three fundamental issues relative to the behaviour of S-containing amino acids in the plant-soil system:

(i) To gain further insight into the size of the organic and inorganic S pools from a range of grassland sites across an altitudinal gradient, as well as the mineralization rates of S-containing amino acids (Cys and Met);

(ii) Determine the relative contribution of amino acid-S to plant S uptake compared to inorganic SO_4^{2-} under sterile hydroponic conditions;

(iii) Quantify the short-term competition for two S-containing amino acids between the rhizosphere microbial community and maize plant roots.

The results of each experimental chapter, in relation to the thesis objectives, are presented below:

The literature review highlighted that soil organic S is present as a heterogeneous mixture of S compounds, many of which likely remain uncharacterised. This pool can be separated into S immobilised within the soil microbial biomass and mesofaunal community, that present in soil organic matter and that present in living plant biomass (Michael A. Kertesz

and Mirleau, 2004a). It is also clear that most organic S is present in soil in an insoluble form and that a significant proportion may be chemically and/or physically protected, and therefore not susceptible to mineralization (Eriksen, Lefroy and Blair, 1995a). In contrast, there appears to be a small pool of organic S that can be readily mineralized (here termed labile organic S, LOS; Vermeiren et al., 2018). Examples of compounds in the LOS pool include S-containing proteins, peptides and amino acids. To better understand soil S dynamics, it is therefore essential to explore the size and turnover rate of this LOS pool. Carbon-bonded S (including S-containing amino acids, organic sulphonates) is a key component of this labile S pool and the breakdown product of many higher molecular weight organic-S moieties. To gain a better understanding of the S forms and their transformation in soils, radiolabelled and stable isotope tracers (^{14}C, ^{35}S, ^{32}S) were applied to track the turnover of Cys and Met in soils.

In chapter 3, P-containing extractants were used to extract total dissolved S (DOS plus SO_4^{2-}, TDS) from a range of different grassland soil types. Evidence showed that the concentration of DOS in all soils ranged from 11 to 309 mg kg^{-1} DW soil, constituting 24 to 94% of TDS in all selected grassland soils. Statistical analysis also showed that the DOS concentration correlated well with Al, B, Fe, K, Mn and NH_4^+ concentrations in soil extracts and MBC. A better understanding of the factors governing the seasonal and spatial variation in DOS in contrasting soils is therefore a prerequisite to make more accurate S budgets in soil.

Chapter 4 investigated the short-term, concentration-dependent microbial uptake and mineralization of ^{14}C-labelled Cys and Met in five grassland soils collected from an altitude-driven primary productivity gradient. The results revealed that ^{14}C-Cys-and ^{14}C-Met were directly and rapidly assimilated by soil microbes, with half times ranging from 0.34 – 2.14 min. This is an order of magnitude (or more) faster than mineralisation rates determined from measurement of $^{14}CO_2$ evolution and indicated that the DOS pool may be turning over many thousands of times in a year. This considerable delay between microbial ^{14}C-amino acid

removal from the soil solution (plant uptake) and subsequent $^{14}CO_2$ evolution (mineralisation) indicates that the degradation of ^{14}C-labelled Cys and Met in soil solution was mainly through microbial biological process rather than abiotic removal processes. This rapid turnover mirrors findings for other low molecular weight solutes in soil (e.g. sugars, organic acids; (Paul W. Hill, Farrar and Jones, 2008; Gunina *et al.*, 2017)) suggesting that the low molecular weight DOC, DOS, DOP and DON pools are all turning over very fast in concert with each other.

On the other hand, Cys and Met also constitute important sources of directly available S for both plant nutrition and microbial growth. Traditionally, gross S mineralization rates can be estimated by measuring sulphatase activity, or by periodically leaching the soils to prevent re-immobilization of newly formed sulphate. However, the legitimacy of both methods has been questioned by researchers; sulphatase enzymes only account for part of the enzymes responsible for S mineralization, while leaching sulphate changes soil conditions leading to overestimation of gross mineralization rates. In addition, enzymatic approaches for measuring transformation rates in soil have also been heavily criticised due to a lack of standardisation in methodological approaches (e.g. substrates, concentrations, pH, detection method etc; (Burns *et al.*, 2013)). In addition, difficulties have arisen due to problems in directly measuring S transformation processes and being able to design realistic laboratory studies which reflect the real world. Alternatively, the application of the S isotope dilution technique allows the determination of both gross and net S transformation rates. In Chapter 6, by quantifying 0.01 M $Ca(H_2PO_4)_2$ extractable $^{35}SO_4^{2-}$ and stable SO_4^{2-} concentrations every 7 d in a closed incubation experiment (for 70 d), the size of the labile organic S pool, and soil S turnover rates in grassland soils with contrasting soil properties were quantified via an established isotope-based method.

Chapter 7 investigated concurrent microbial mineralization and utilization of C, N and S derived from Cys and Met in a grassland soil over a 7-d incubation period, using ^{14}C and ^{35}S

dual-labelling. Results indicated that microbial communities retain the capacity to utilize Cys-C and Met-C in the presence of inorganic N (NH_4^+ and NO_3^-) and S (SO_4^{2-}) and that inorganic products derived from the amino acid are excreted into the soil, benefiting plants. In accordance with results from chapter 4, I confirmed that the decomposition of S-containing amino acids (Cys and Met) in my grassland soil mainly occurs as a result of microbial oxidation.

Results from Chapter 8 showed that a considerable proportion of Cys (66%) and methionine (73%) was also rapidly taken up intact by plant roots under sterile hydroponic conditions, even when sulphate was available, indicating that organic S may constitute a readily available source of S to plants, particularly in S-limiting ecosystems. This uptake pathway be important in providing an alternative source of S to plants and in recapturing amino acids previously lost in root exudates or when they are directly adjacent to decomposing organic matter. I also provided evidence of the rapid redistribution of S within the plant after been taken up by the root system.

Chapter 5 investigated the competition for S-containing amino acids between plant roots and microorganisms in the rhizosphere (^{14}C, ^{35}S). Results showed that the capture of free Cys and Met by maize plants was very low compared to soil microbes, with < 10% of the added amino acid captured by the plant, compared to the rhizosphere microbial community (around 80%). I conclude that both Cys and Met are sources of available C, N and S for maize plants, both pre- and post-mineralization. Therefore, studies relying solely on soil organic matter mineralization rates may have underestimated its N and S supply potential to plants.

In this thesis, I addressed a range of fundamental questions relating to the potential significance of amino acids to the overall plant S budget, with emphasis on S supply in different agroecological contexts. The results clearly showed that i) when studied in isolation, both soil microorganisms and maize roots have the capacity to take up free amino acid-S from soil solution, and ii) when allowed to compete, soil microorganisms outcompete maize roots for

amino acid-S in soil. Taken together, these findings have important implications for ecosystems S cycling and the more accurate design of ecosystem S cycling models.

9.3 Methodological strengths and limitations

Soil organic matter contains an important source of plant-available S and good methods are needed to estimate its potential contribution to total plant S supply. My work brings together a wide range of experimental techniques, leading to meaningful conclusions about the potential significance of Cys and Met to graminaceous plant nutrition.

In the experimental chapters where the aim was to capture the intrinsic variability in S cycling in different soils and keep conditions closer to natural conditions, soil samples were collected, transported immediately back to laboratory, and kept in the dark at ≤ 5 °C before conducting the experiments. All experiments conducted in this thesis commenced within ≤ 48 h of soil collection, to minimise artefacts which may arise from sample preparation and storage (Ross, 1992; Jones and Willett, 2006; Meyer, Welp and Amelung, 2019). However, it should also be considered whether the fast turnover measured in the laboratory was a result of C starvation in the microbial community following the removal of plant roots and rhizodeposition. On the other hand, the sometimes large concentrations of substrate added to the soil may not reflect steady state concentrations in the soil solution which are often at the nanomolar level (Jones, Shannon, *et al.*, 2005; Boddy *et al.*, 2007; R C I Broughton *et al.*, 2015). Therefore, there is a strong need for actual *in situ* field experiments to better estimate soil S turnover (Oburger and Jones, 2009).

A few previous studies indicate that DOS constitutes a significant part of the S flux from terrestrial to aquatic environments (Wang *et al.*, 2012b) where it can be turned over rapidly (Brailsford *et al.*, 2020). In chapter 3, spatial variation of two S fractions: DOS and inorganic sulphate were assessed based on S fractionation results from seven grassland soil

samples. Instrumental methods of S determination, such as ion chromatography (IC) and inductively coupled plasma atomic emission spectrometry (ICP-AES), gave rapid and precise estimation of SO_4^{2-}-S and organic S concentrations. However, despite previous studies showing that extractable organic S was directly related to mineralizable organic S (Watkinson, Perrott and Thorrold, 1991), it is advisable for future studies to develop a sound understanding of the labile organic S pool, which can become directly available to plants through mineralisation. Furthermore, future work should also include evaluations of the seasonal variation of DOS content in soil solution due to dilution or microbial activity caused by changes in moisture regime (i.e. rainfall, drought), seasonal and daily temperature patterns, management regime (e.g. tillage), crop growth stage, the presence of livestock etc.

High molecular weight C inputs (e,g. protein, hemicellulose, lignin, and cellulose) into soil are first broken down extracellularly to smaller units by the soil microbial community (e.g. fungi and bacteria). These low molecular weight breakdown products (such as peptides, amino acids etc) are then taken up into the cell, entering catabolic or anabolic pathways. Traditionally, the measurements of substrate turnover in soil typically has relied on the addition of isotopically labelled substrates (e.g. ^{13}C, ^{14}C) to soil in the laboratory and measurement of $^{14}CO_2$ evolution. An important advantage of using the ^{14}C tracer technique compared with traditional methods is that it is possible to calculate the distribution of C between different measured C pools accurately with high sensitivity, low cost, and ease of sample preparation in comparison to other non-isotopic approaches (e.g., LC-MS, NMR). However, while isotopic based measurements of $^{14}CO_2$ evolution greatly facilitate our understanding of the transformations involved in amino acid turnover in soil, it may underestimate their rates of cycling in the soil due to the delay between microbial uptake and mineralization (Paul W. Hill, Farrar and Jones, 2008). There are also issues about isotopic pool dilution and incomplete extraction of C pools that need to be considered (H. C. Glanville *et al.*, 2016). In Chapter 4,

therefore, I adopted the centrifugal drainage procedure to study substrate depletion from soil solution. This method allows small quantities of soil samples to be used so that soil solution can be recovered extremely rapidly; this method also allowed us to explore the temporal decoupling of Cys and Met-^{14}C uptake and subsequent mineralization.

Of all four S containing amino acids (Met, Cys, homocysteine, and taurine), only Cys and Met are biosynthetically incorporated into proteins and are therefore expected to represent the main forms of DOS entering soil. In chapter 7, with dual labelling (^{14}C, ^{35}S), I linked S-amino acid C and S dynamics to the size and activity of the microbial biomass in grassland soils. However, previous studies have indicated S mineralization seems to be driven by the form of S in the starting materials (Churka Blum *et al.*, 2013b), where the mineralization process of the ester S pool is mostly governed by the enzymatic hydrolysis, while mineralization of C-bonded S is governed by the need of C to provide energy to the microorganisms. Therefore, my study emphasizes the importance of a more detailed analysis of uptake and mineralization of ester S in soil solution.

Due to the continual degradation and synthesis of S fractions by soil organisms, it is not easy to examine the dynamics of S mineralization and immobilization within soil as these occur concurrently. The use of ^{35}S (Chapter 6) allowed the tracing of separate S pools and the determination of both gross and net transformation rates. The model I adopted in my study provided more realistic estimates of transformation rates as it takes the re-mineralisation of previously immobilized ^{35}S into account. However, as my study was carried out in a closed laboratory incubation system with no plants and rhizosphere microbial activity, future research and models should also take into account leaching, plant uptake, fertilization etc., performed under field conditions where possible at various points during the growing season. It would also be useful to investigate the diversity and function of sulfatases in soil and their rates of reaction.

Although it has been suggested that plants have the physiological capacity to take up a wide range of DOM in the form of amino acids from the environment prior to microbial degradation to inorganic nutrients, this has not been thoroughly studied for S-containing amino acids. In chapter 8, radiolabelled compounds were used to measure plant uptake. ^{14}C and ^{35}S were combined in parallel treatments to directly trace plant uptake of the carbon or S skeleton of added amino acids, but the results did not indicate whether the amino acid was taken up intact or cleaved prior to uptake of the molecule (Hill and Jones, 2019). It would be possible to better study plant uptake at a molecular level, if $^{15}N/^{34}S/^{13}C$ labelled S-containing amino acids were also utilised coupled with compound-specific isotope ratio mass spectrometry (Knowles *et al.*, 2010; Charteris, 2019). Future work should therefore build from the results in this thesis to increase our understanding of the molecular mechanisms behind DOS processing in soils and their subsequent incorporation in different plant species.

The availability of amino acids to plant roots may be constrained by differences between amino acids in their potential for mineralization, microbial assimilation, and sorption to soil solids, as well as the relative soil reserves of inorganic nutrients. Most studies to date have focused on the direct uptake of organic N solutes such as amino acids. However, the degree to which S-containing amino acids contribute to the overall S budget of plants, remains poorly understood. This is especially the case in the rhizosphere where microbial activity can be an order of magnitude greater than in the bulk soil, and competition for labile organic S between plant roots and soil microorganisms could be intense. The main aim of Chapter 5 was therefore to quantify the competition between rhizosphere microorganisms and plant roots for two S-containing amino acids namely, Cys and Met. In agreement with previous studies, only small amounts of S-containing amino acids were captured by maize plant roots. My study also indicates the clear need for *in situ* studies of plant acquisition of Cys and Met where amino acid concentrations are low, in order to learn more about the realistic contribution of low

molecular weight S containing compounds to plant N and S nutrition. In addition, the capacity of roots to take up alternative forms of organic S (e.g. oligopeptides, glutathione, S-lipids) remains to be evaluated.

Sulfurous air pollutants may act as both toxins and nutrients to plants (De Kok and Tausz, 2001). Atmospheric S gases can be taken up directly by the foliage, forming bisulfite (HSO_3^-) and sulfite (SO_3^{2-}) due to its high solubility in the apoplastic water of the mesophyll (Omasa *et al.*, 2012). Sulfite may directly enter the S reduction pathway and be reduced to sulphide, incorporated into Cys and subsequently into other S containing compounds (Romero *et al.*, 2014). Therefore, some plants may even benefit from elevated levels of atmospheric S gases, since they contribute to plant S nutrition, especially when inorganic sulphate is low in soil. In chapter 5, the total recovery rate of ^{35}S in the soil-plant system was < 80% for all five treatments. It is possible that volatile S compounds (VOS) were produced during the incubation, and this was not measured in my experiments. Future studies are needed to assess the magnitude of evolution of VOS and inorganic S compounds from both soil and plants. It is likely that VOS will be just as important in soils as it is in marine environments (Carpenter and Milyo, 2012; Tang, 2020).

9.4 Future research

The studies presented in this thesis have provided pivotal information about the significance of amino acid-S to plant roots and soil microbes. However, several research gaps have also been identified during the research. It is clear from the results presented in this study that a much deeper analysis of many areas covered in this subject are still required. Some of these are detailed below:

9.4.1 Determination the dynamics of inorganic and organic S compounds in soil solution

Determining S availability in soil based on extraction methods is very challenging since many forms of available S are founds in soils, including readily soluble inorganic sulphate, adsorbed sulphate and a portion of organic S (carbon bonded S or ester S). Most of the methods of soil S testing involving extraction of soil with a weak salt solution have been criticised for poor precision and lack of accuracy due to the effects of serious chemical interferences. In addition, these extraction methods fail to estimate the pool of labile organic S which becomes available to plants through mineralization. Some loss of organic S is also expected during the extraction procedure due to microbial mineralisation (Rousk and Jones, 2010). More recently, isotope dilution methods have provided a much deeper fundamental understanding of soil N cycle processes, modelling of gross N fluxes and understanding of individual microbial metabolic pathways (Sprent, Knowles and Blackburn, 1993). In my research, I also adopted this method to quantify the size of total labile S pool and gross mineralization/immobilization rates. Further experiments and modelling, which include a wider range of soils and organic S substrates are required to improve our understanding and prediction of the dynamics of inorganic and organic soil S. It would also be useful to see if the rates of S cycling are coupled to those of other nutrients such as P and K.

9.4.2 What specific members of microbial community play a dominant role in soil S cycling?

Soil microbial communities are strongly involved in the biological and biochemical mineralization of soil S. S oxidizing bacteria, such as *Thiobacillus*, *Thiothrix*, *Chlorobiaceae*, *Beggiatoa*, *Chromatiaceae*, and *Ectothiorhodospiraceae*, are able to oxidize organic S to inorganic sulphate. In addition, the biochemical S mineralization is driven mainly by arylsulfatases, which are partly produced by plant roots but mostly produced by soil microbes.

It seems likely that particular microbial species or genera in the rhizosphere may play a greater role in S cycling than others, but to date, assays for determining the overall rate of entire metabolic processes such as respiration, or specific enzyme activities do not allow any identification of the specific microbial species directly involved in each measured process. The development of modern molecular techniques which do not rely on cultivation (e.g. gene knockouts, mutants metagenomics, transcriptomics) now allow us to better explore the composition and function of soil microbial communities. Despite this, little is known about the specific microbial species or genera that play important roles in the soil organo-S cycle. For a better understanding of the microbial role in S cycling, therefore, future studies incorporating traditional approaches (e.g. phospholipid fatty acid analysis) with modern molecular techniques (e.g. proteomics, transcriptomics, metabolomics, stable isotope probing; Nannipieri et al., 2003) are needed. This may also be coupled to stable isotope imaging techniques (e.g. NanoSIMS) for imaging microbial S dynamics (Higuchi *et al.*, 2020; Stuart *et al.*, 2020). A better understanding of the mechanisms and processes regarding the effects of soil microbes on the availability of nutrients is particularly important for the design of new biofertilizers. The right combination of microbes in these products may enhance the solubility and plant availability of a wide range of different macro- and micro-nutrients (N, P, K and S etc.) making this an economically and environmentally friendly approach to improving plant nutrition (Miransari, 2013).

9.4.3 Predicting S availability from C/S ratio of plant residues

Where S inputs from fertilizer and atmospheric deposition are low, the release of S from organic forms becomes important for the supply of S to plants. Therefore, estimation of the potential contribution that the organic S pool makes to plant available S, especially following the addition of organic S materials such as composts, manures, biosolids, fresh plant residues

etc., is critical. S mineralization seems to be driven by the form of S added (Churka Blum *et al.*, 2013b), with the S mineralization process mostly governed by biological mineralization of C-bonded S-rich residues, with liberation of inorganic sulphate as a secondary product. In contrast, the application of highly oxidized S compounds is mainly governed by enzymatic hydrolysis of the ester S pool. Therefore, future studies providing valuable information for modelling of S transformations from different crop residues in more soil types are recommended.

9.4.4 Is organic S a significant S source for plants *in situ*?

Although my study did not take into account plants from widely differing habitats, results showing uptake of S-containing amino acids in maize provides an indication of the potential role of Cys and Met for plant S nutrition under certain conditions. However, it is not clear to what extent amino acid-S contributes to the total S budget of the plant when grown under field conditions, where concentrations of free amino acids and inorganic SO_4^{2-} may differ. The concentration of Cys and Met in soil solution *in situ* is typically in the 1-50 µM range, while the concentration of SO_4^{2-} in soils is typically one or more orders of magnitude greater. My research points to the need for longer term studies on the acquisition of S-containing amino acids by various plant species in the field to explore the actual importance of these low molecular weight organic S for plant S nutrition. In addition, to date, most studies on amino acid absorption by plant roots have mainly focused on gross influx rates, excluding the possibility of simultaneous root leakage, which could affect the calculation of net uptake rates. Clearly, in the future, more experimentation is needed to expand our mechanistic understanding of this root uptake processes, with the application of new approaches and techniques (e.g. cloning and *ex situ* expression of transporters to better understand their function, signal

transduction cascades which are triggered under S deficiency, internal S recycling in plants and microbes).

9.4.5 Seasonal and spatial responses in microbial biomass sulphur

Some of the more recent studies have shown a growing interest in soil microbial biomass S, as it not only contains a labile pool of nutrients but also drives the cycling of organic matter and nutrients in soil, thereby affecting S availability to plants (Banerjee and Chapman, 1996). Previous studies have compared seasonal responses of microbial biomass C and N to changes to environmental factors such as temperature (Van Gestel *et al.*, 2011), moisture (He *et al.*, 1997), plant growth (Franzluebbers, Hons and Zuberer, 1994) and fertilizer application (Ge *et al.*, 2010), to assess the importance of the microbial biomass in regulating the plant availability of C and N in soil. However, research for other major plant nutrients, such as S, are still limited.

Microbial biomass-S in soil is considered as intermediary between soil inorganic and organic S, and therefore the retention or release of this S pool is vital to support plant growth. Although only a relatively small fraction of the soil organic S resides in microbial biomass-S, this fraction is relatively labile and is thought to be the most active S pool for soil S turnover. Microbial biomass S can form a significant proportion of the organic S pool which is involved in cycling and potentially available to plants (Chapman, 1987), and its availability depends on its turnover rate. Therefore, additional studies are particularly needed to improve our understanding of the seasonal variations of microbial biomass S under more soil types with different plant species. In addition, the partitioning of microbial S between C-bonded S and C-O-S and how these two fractions turnover seasonally is also of particular interest.

9.5 Concluding remarks

In this thesis, I addressed fundamental questions related to amino acid-S such as their mineralization rates, contribution to plant nutrition that could have key implications for soil nutrient management purposes. This thesis provided the following essential information: 1) the mineralization of S containing amino acids is extremely rapid, representing an important short-term source of C, N and S in grassland soils; 2) maize plant roots can acquire S-containing amino acids directly under hydroponic conditions, even when inorganic sulphate was available, indicating low molecular organic S compounds like S-containing amino acids could constitute a significant proportion of S supply to plant growth under certain conditions; and 3) when in competition, the capture of free Cys and Met-C (or S) by the maize plants was low compared to soil microbes, with < 10% of the added amino acid captured by the plant roots, compared to the rhizosphere microbial community (around 80%). Despite these advances, there is still much more to explore about S cycling in both an agricultural and natural ecosystem settings. Additional research will be important to enhance our understanding of how to optimise the formulation and use of S fertilisers in agriculture and for understanding the impacts and legacy of anthropogenic S deposition on natural ecosystems.

9.6 References

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Appendix 1

Chapter 6 Estimation of sulphur transformation rates in grassland soils using ^{35}S isotope pool dilution (This chapter is not complete due to author being unable to read ^{32}S data for the final isotope dilution model)

Abstract

Due to reductions in fossil fuel-derived SO_2 emissions and atmospheric sulphur (S) deposition, many agroecosystems are now experiencing a greater incidence of S deficiency. This has led to a renewed interest in understanding the processes which regulate the supply of S to plants. In well drained grassland soils, most S is locked up in soil organic matter (SOM), however, a large proportion of this organic S is poorly bioavailable to soil microorganisms. The primary objective of this study was to quantify the size of labile organic S pool, and to characterize soil S turnover rates in grassland soils with contrasting soil properties. To achieve this, we used an isotope ($^{35}SO_4^{2-}$) pool dilution method in a closed incubation experiment over a 70-d period. After addition of ^{35}S-labelled SO_4^{2-} to each soil, we measured the progressive immobilization of this ^{35}S into the SOM pool on a weekly basis. The amount of $^{35}SO_4^{2-}$ remaining in the soil at each measurement time was estimated by extracting the soil with 0.01 M $Ca(H_2PO_4)_2$. Gross S mineralization rates and the size of total labile S pools were quantified using an established isotope pool dilution model. The modelling approach provided a more accurate estimations of gross S cycling processes, as it takes into account the immobilization of SO_4^{2-} into the labile organic S pool (LOS), and the re-mineralization of this newly formed LOS pool into account. In addition, it is clear that S cycling (mineralization, immobilization) in the soil is microbially mediated, and affected by nutrient conditions. For example, S mineralization in soil is attributed to either biological or biochemical processes, where biological processes are governed by the microbial search for energy from carbon-bonded S, and where the biochemical process is via enzymatic hydrolysis of sulphate esters controlled by S supply. On the other hand, S immobilization also depends on soil conditions, as carbon availability largely controls soil microbial activity, whereas SO_4^{2-} availability was found to retard ^{35}S immobilization. Therefore, a secondary objective was to evaluate effects of additions of various substrates (glucose, SO_4^{2-}-S or a combination of both) on the turnover of ^{35}S in soil.

Keywords: Isotope pool dilution method; Labile organic S; S turnover; Grassland soil.

6.1 Introduction

Sulphur (S) is an essential element for plant growth and development (Zhao *et al.*, 1997) as well as being involved in a range of processes involved in biotic and abiotic stress mitigation (Bloem, Haneklaus and Schnug, 2005). It is a key component of the amino acids, cysteine and methionine, as well as several co-enzymes. Historically, agricultural production in most European nations was not limited by S availability due to the emissions and subsequent atmospheric deposition of S from fossil-fuel burning. However, the almost complete removal of SO_2 from many power plant emissions and the adoption of low S containing fertilizers within Europe has led to an increase in S deficiency in many agricultural regions (Engardt *et al.*, 2017). The increased recognition of the importance of maintaining an adequate S supply for optimal crop growth has therefore led to a renewed interest in understanding the key factors that regulate S cycling in soil.

Typically, more than 95% of the S present in soil is bound within organic matter (SOM) (Ghani, McLaren and Swift, 1993), typically in the form of sulphate ester S and carbon-bonded S (Norman M Scott and Anderson, 1976; Lou and Warman, 1992). Although a proportion of this organic S pool is quasi-stable due to chemical and physical protection, the unprotected proportion is thought to be extremely dynamic, with S being continuously cycled between inorganic and organic forms (McLaren, Keer and Swift, 1985). This is likely to be particularly true in the rhizosphere where microbial activity is greatest and plant demand continually removes inorganic S from the soil solution. Specifically, the transformation of soil organic-S to inorganic SO_4^{2-} (i.e. S mineralization as well as enzymatic cleavage), and the reverse process, immobilization (the incorporation of SO_4^{2-} into soil organic-S), are two key processes governing S availability in soils (Scherer, 2001).

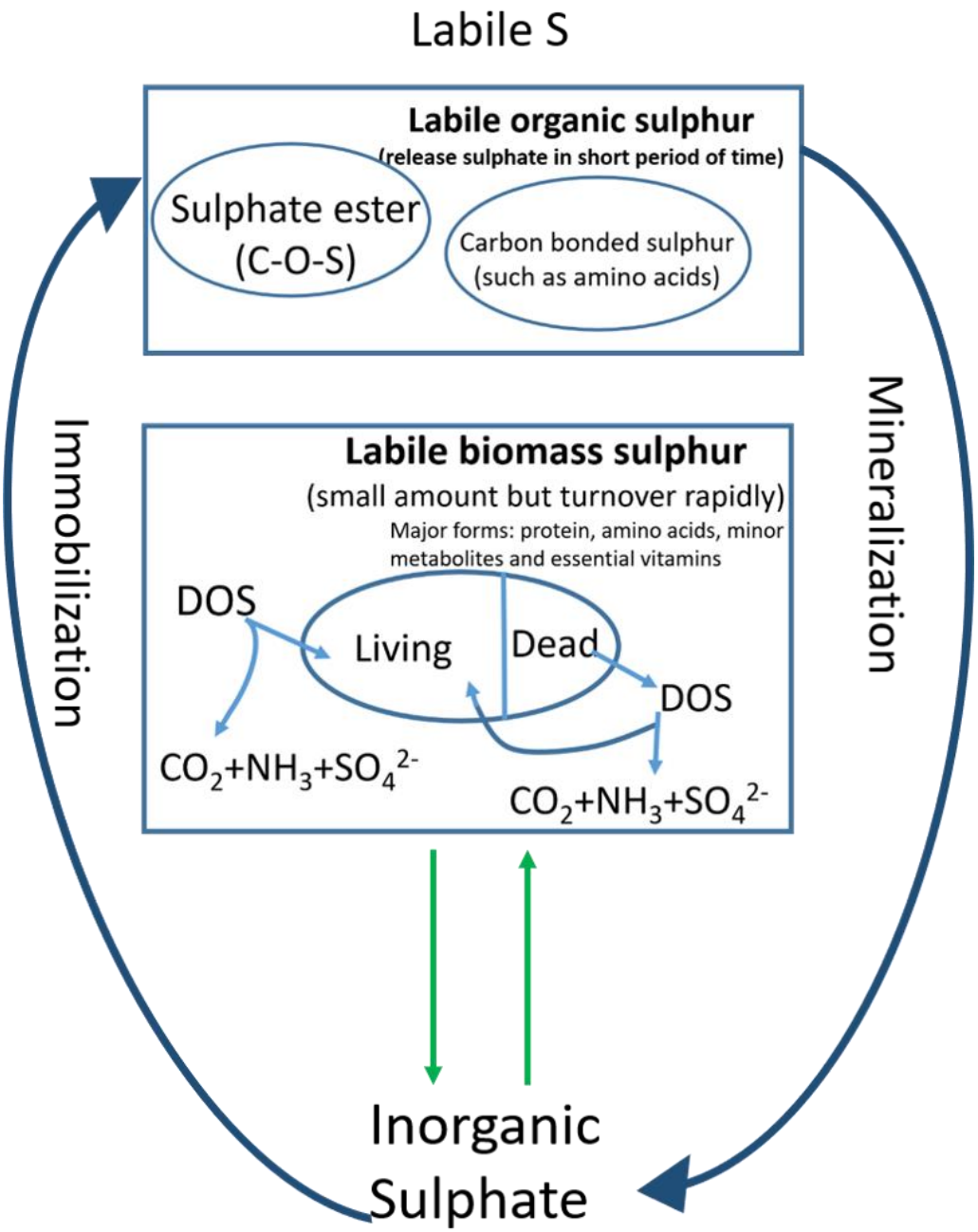

Fig. 6.1 Schematic representation of the two ongoing processes in our inorganic sulphate incubation experiment. On one hand, there is quick immobilization of ^{35}S in newly formed microbial biomass, which will decay and again be released as inorganic sulphate. On the other hand, inorganic sulphate is also slowly turned into labile organic sulphur, which could be mineralized to inorganic sulphate again, this process is mediated by microorganisms. In our study, however, we only took immobilization and mineralization between inorganic sulphate and labile organic sulphur into account.

In well drained surface soils, the amounts of inorganic SO_4^{2-} present is typically too small to satisfy plant demand unless it is regularly replenished. Consequently, mineralization plays a key role in resupplying this pool and maintaining plant S nutrition (Williams, 1967). Studies on the turnover of organic S in soils will, therefore, facilitate an improved prediction of S response to reduced S inputs (Haque and Walmsley, 1972; A N Ganeshamurthy and Nielsen, 1990). In grassland soils, a significant fraction of the soil organic S is either insoluble or physically protected on clay surfaces, and is therefore not directly available for microbial attack (Eriksen et al., 1995). This pool is defined as non-labile organic S. By contrast, labile soil organic S can be readily mineralized and the size of this pool can be used as an indicator of soil quality (Fig. 6.1; (Maynard, Stewart and Bettany, 1984)). To date, numerous soil extraction methods have been developed to assess the size of the labile organic S pool (e.g. by extracting with hot water or with alkaline solutions) (Dick, Kost and Chen, 2008); (Spencer and Freney, 1960; Fox, Olson and Rhoades, 1964). However, these methods are often time consuming, and strong extractants may alter the nature of organic S during the extraction procedure, while milder extractants may result in incomplete recovery from the soil (Freney, Melville and Williams, 1969). Therefore, it is difficult to gain a clear idea of the size and dynamics of the labile S pool(s) using a conventional chemical extraction approach.

Previously, low molecular weight (MW) organic-S compounds (e.g. methionine (Fitzgerald and Andrew, 1984), cysteine (Hale and Fitzgerald, 1990), sulphate esters (Houghton and Rose, 1976) and sulphoquinovose (Fitzgerald, 1983)) have been added to soil and the generation of inorganic S measured. However, most soil organic-S in soil is thought to be associated with higher MW substances (e.g., protein-humic complexes), so it may be inappropriate to extrapolate results from S mineralization studies that solely focus on simple organic compounds.

It is highly likely that the net rate of S mineralization in soil will be greatly influenced by the availability of other key nutrients (e.g., C, N and P), as well as the amount of inorganic S present. Their availability will also influence the final form of S incorporated into the microbial biomass. For example, low SO_4^{2-} concentrations in soil may stimulate the production of sulfohydrolases by plant roots or soil microorganisms (Maynard, Stewart and Bettany, 1984), leading to increased biochemical mineralization of ester sulphates. In contrast, a high soil SO_4^{2-} content may stimulate S immobilization into higher ester sulphates, since sulphate ester production is a mechanism for soil microorganisms, especially fungi, to store S when there is an adequate supply of SO_4^{2-} (Ghani, McLaren and Swift, 1992). In addition, amendment with a readily available C source (e.g. glucose) may stimulate rapid immobilization of ^{35}S into newly formed microbial biomass, leading to an increased incorporation of SO_4^{2-} into organic-S (S J Chapman, 1997; Shahsavani and Gholami, 2009).

Quantifying the dynamics of soil S is difficult as mineralization and immobilization processes take place simultaneously (Michael A Kertesz and Mirleau, 2004). The mineralization rate of soil S can be investigated using either “closed” or “open” incubations techniques in the laboratory. The closed incubation technique allows mineralized sulphate to accumulate (D G Maynard, Stewart and Bettany, 1983; Valeur and Nilsson, 1993), but this technique does not take account of the loss of newly formed SO_4^{2-} (e.g. via immobilization, plant uptake or leaching). ‘Open’ incubation techniques involve periodic leaching of SO_4^{2-} (D G Maynard, Stewart and Bettany, 1983; Ghani, McLaren and Swift, 1991), or quantifying plant S uptake plus increases in soil SO_4^{2-} at regular intervals (Eriksen *et al.*, 1995; Mclaren, 2001). The ‘open’ approach more closely replicates field conditions, where mineralized sulphate is either taken up by plant roots or lost by leaching. However, periodic leaching limits the potential for SO_4^{2-} immobilization (as it is removed from the system) and can therefore only be used to quantify gross mineralization. Further, repeated leaching may alter soil conditions (e.g.

removing NO_3^-, altering soil pH). Together all these factors may lead to overestimation of S mineralization with the 'open' approach.

An alternative approach is the use of stable or radio-isotopes (e.g. ^{32}S, ^{34}S, ^{35}S) to monitor the flow of S between different pools in soil. Previous studies using $Na_2{}^{35}SO_4$ have shown the added ^{35}S label can become readily incorporated into both hydriodic acid reducible S (sulphate esters) and C-bonded S (Freney, Melville and Williams, 1971), and the nature of the resulting organic S compounds largely determines the subsequent mineralization rate of S (Zhao, Wu and McGrath, 1996). The isotope pool dilution technique provides quantitative information on nutrient dynamics in the soil-plant system and when combined with mathematical modelling can be used to determine both gross and net S transformation rates in soil (Di, Cameron and McLaren, 2000; Eriksen, 2005; Vermeiren *et al.*, 2018b).

In the following study, the rates of ^{35}S released to and removed from the soil inorganic pool were estimated using the isotopic pool dilution method. The objectives of this study were to (1) quantify the size of the total labile S pool (sum of labile organic S and inorganic SO_4^{2-}) in grassland soils using ^{35}S as a tracer; (2) compare the modelled results of the labile S pool with those measured by conventional chemical extractant techniques; (3) evaluate gross S transformation rates with an established model; and (4) assess the effects of C availability (glucose) or SO_4^{2-} availability on S transformation rates.

6.2 Materials and methods

6.2.1 Field sites and soil sampling

Topsoil (0 - 10 cm) and subsoil (10 - 20 cm) was collected from seven sites along a natural grassland productivity gradient, located in Abergwyngregyn, Gwynedd, North Wales. Approximately 2 kg of soil was collected from each of three random points located 2 m apart from each other, placed in gas-permeable plastic bags and transferred immediately to the

laboratory. These samples represent the three replicates used for all experiments. After collection, all soils were sieved to < 2 mm and stored at 4 °C. Soil bulk density and moisture content of the original top- and subsoil was assessed by oven drying (80 °C, 24 h) intact 100 cm^3 cores collected from the field. Soil pH and electrical conductivity (EC) measurements were performed on 1:1.25 w/v soil-distilled water suspensions using standard electrodes. Selected properties of these soils are presented in Table 3.1 and 3.2.

6.2.2 Extraction of total labile S

Three established single-step extraction tests were performed to estimate total labile S on each untreated soil.

For the hot water extraction (Ghani, Dexter and Perrott, 2003; Vong *et al.*, 2010), 5 g of field-moist soil was weighed into 50 ml polypropylene centrifuge tubes, after which, deionized water (25 ml) was added, and the suspensions manually shaken. The mixture was then incubated for 18 h at 80 °C. The second extractant was 0.5 M $NaHCO_3$ (pH 8.5), mirroring the common Olsen-P method used to measure available P (Abdu, 2006). $NaHCO_3$ (20 ml) was added to 5 g of field-moist soil and the suspension placed on an end-over-end shaker for 1 h at 20 °C. The third extractant was 0.25 M KCl (Blair, Lefroy and Chinoim, 1993). This procedure involved the addition of 25 ml of 0.25 M KCl to 5 g of field-moist soil after which the suspension was manually shaken and then incubated at 40 ± 0.5 °C for 3 h.

For all three extractions, the samples were left to cool (20 °C) before being centrifuged (3500 rpm, 20 min), filtered (0.45 µm sterile PES syringe filter; Fisher Scientific), before S analysis using an inductively coupled plasma optical emission spectroscopy (ICP-OES; Varian 710ES, Agilent Technologies, USA).

6.2.3 Incubation experiment using ^{35}S isotope pool dilution

The experiment had 4 treatments to test how the availability of inorganic S and glucose-C affected ^{35}S cycling in soil. These were undertaken in a factorial design as follows:

1. -S -C (control)
2. -S +C (glucose addition only)
3. +S -C (sulphate addition only)
4. +S +C (glucose and sulphate addition)

The -S treatments just contained a very small amount of S (1 µg SO_4^{2-}-S kg^{-1}) to label the native SO_4^{2-} pool, but importantly without changing its intrinsic concentration. The +S additions were designed to greatly increase the native SO_4^{2-} pool and involved the addition of 30 mg SO_4^{2} (as Na_2SO_4) kg^{-1} soil (equivalent to 30 kg S ha^{-1}), while the C addition was 5 g C (as glucose) kg^{-1} soil, respectively. The +S treatment represents that typically used as a fertiliser dose in agricultural fields (Kulczycki, 2021).

To maintain the natural moisture content of the soil, the $^{35}SO_4^{2-}$ tracer (± glucose) was added to the soil using an inert quartz sand carrier. To prepare the carrier, a solution of $Na_2{}^{35}SO_4$ (American Radiolabelled Chemicals Inc., St Louis, MO) and/or glucose was mixed with pure sterile quartz sand and left to dry at room temperature. Glucose was chosen as a readily available C source, as it is highly abundant in root exudates (Rovira, 1969b). For the experiments, the ^{35}S-labelled quartz sand was mixed with the soil in a ratio of 1:10 (w/w; quartz sand: soil). Briefly, 25 g of field-moist soil were placed in individual polypropylene containers (5 cm internal diameter; 7.3 cm height; Fisher Scientific, Leicestershire, UK). Quartz sand (2.5 g) carrying the ^{35}S (± glucose) was then thoroughly mixed with the soil to give a final ^{35}S activity of 0.35 kBq g^{-1} soil.

The soil was then compressed to match the bulk density measured in the field. All containers were then incubated at 20 °C in the dark for 70 d. Containers were loosely covered

with plastic lids and opened regularly to ensure adequate aeration. Subsamples of soil (2 g) were taken from each container every 7 d for analysis of extractable $^{35}S\text{-}SO_4^{2-}$ and $^{32}S\text{-}SO_4^{2-}$. To measure the amount of inorganic $^{35}SO_4^{2-}$ remaining in the soil at each sampling point, the 2 g sub-sample was extracted with 10 ml of 0.01 M $Ca(H_2PO_4)_2$ on an end-over-end shaker (30 min, 200 rev min^{-1}), centrifuged (4000 rpm, 10 min) and filtered (0.45 µm syringe filter) prior to analysis. A 1 ml aliquot of the filtrate was used for analysis of $^{35}S\text{-}SO_4^{2-}$ by liquid scintillation counting using a Wallac 1404 scintillation counter with automated quench correction (Wallac EG&G, Milton Keynes, UK) and Optiphase HiSafe 3 scintillation fluid (PerkinElmer, Waltham, MA). The $^{32}S\text{-}SO_4^{2-}$ content of the filtrate was measured by ion chromatography using an ICS2100 ion chromatograph (Dionex Corp., Sunnyvale, CA; (Zhao and McGrath, 1994)). The ^{35}S present in our 0.01 M $Ca(H_2PO_4)_2$ extracts represented the non-immobilized ^{35}S, and the difference between ^{35}S initially applied and ^{35}S found in the $Ca(H_2PO_4)_2$ extracts was assumed to be ^{35}S that had been immobilized. Prior to data analysis, the ^{35}S measurements were all corrected for the natural decay of ^{35}S (half-life 87.1 d).

^{35}S has been used to successfully trace a range of S cycling processes in soil including microbial immobilisation, reduction, sorption, leaching and plant uptake (Di et al., 2000). The isotope pool dilution method is best used when the ^{35}S radiotracer is combined with measurements of its stable isotope counterpart (i.e. ^{32}S). The ratio of introduced label (^{35}S-labelled) with the native pool (^{32}S-labelled) can then be used to calculate flux rates (Table 6.1). For example, W_x can be calculated according to equation:

$$W_x = W_d \times A_d / A_r, \qquad \text{(Eqn. 1)}$$

Where W_d, A_d and A_r are known parameters.

Table 6.1 Summary of the main pools used in the isotope dilution analysis.

	Total radioactivity	Weight	Ratio
Native analyte	0	W_x	0
Adding radioactive analyte	A	W_d	A_d
Mixture	A	$W_x + W_d$	A_r

6.2.4 Compartment model

The fluxes of S in soil can be described as follows:

Labile organic S $\xrightarrow{M}$ SO_4^{2-} $\xrightarrow{I}$ S immobilization

Where *M* is defined as the mineralization rate and *I* is the immobilization rate, both in mg kg^{-1} soil day^{-1}. Some of the S immobilized during the incubation can be re-mineralized, however, this cannot be measured. Overall, this can be expressed by the following equation:

$$f = (1-f_{bio}) \times (f_s + (1-f_s) \times \exp^{k_{los} \times t} \quad \text{(Eqn. 2)}$$

Where f_{bio} represents the fraction of ^{35}S that is quickly incorporated into the microbial biomass, and f is the fraction of ^{35}S in the total added S, k_{los} is the rate constant for exchange between the sulphate and LOS pool, f_s is the fraction of ^{35}S in the total labile S pool (Total labile S = sulphate + LOS) present as SO_4^{2-} at steady state. By fitting data into this equation, the best fitted parameters for f_{bio}, k_{los} and f_s can be obtained.

Since previous studies have reported that glucose addition can result in rapid incorporation of ^{35}S into microbial biomass, with little change in total biomass S, this indicates that original non-labelled biomass-S was replaced by ^{35}S. Based on parameters (f_{bio}, k_{los} and f_s) derived from above, LBS, LOS and *M* can be obtained according to following equations separately:

$$[LBS] = [SO_4^{2-}\text{-S}] \times \ln^{(1 / (1- f_{bio})};$$

$$[LOS] = (1 / f_s - 1) \times [SO_4^{2-}\text{-S}];$$

$$M = I = k_{los} \times [SO_4^{2-}\text{-S}] \times [LOS] / ([SO_4^{2-}\text{-S}] + [LOS]).$$

Subsequently, the effect of glucose (5 g C kg^{-1} soil) and sulphate (10 mg S kg^{-1} soil) addition on sulphur transformation was also explored by repeating the same experiment as described above after substrate addition.

$$f = \exp([LBS] / [SO_4^{2-}\text{-S}] \times \exp(-k_{bio} - 1) \quad \text{(Eqn. 3)}$$

Where [LBS] is the concentration of labile biomass S (the SO_4-S immobilized in biomass when all glucose is decomposed), which can be derived by fitting data to equation (3), and where $[SO_4^{2-}\text{-S}]$ is considered to be a constant. These two parameters can be obtained by fitting equation (2) to the data with k_{bio} set as a constant.

Equation (2) and (3) were added to account for both LOS and LBS, and since no analytical solution can be derived from equation 3, numerical solutions were derived using time steps of 0.25 d.

$$d_f / d_t = (M / [LOS] \times (1- f) - I / [SO_4^{2-}\text{-S}] \times f) - (f \times [LBS] / [SO_4^{2-}\text{-S}] \times k_{bio} \times \exp(k_{bio} \times t)) \quad \text{(Eqn. 4)}$$

6.3 Results

6.3.1 Labile sulphur pool size

6.3.2 Total labile S in relation to S concentration extracted by different extractants

6.3.3 Gross S transformation rates estimated with an established model

6.3.4 Effects of glucose or SO_4^{2-} addition on S transformations

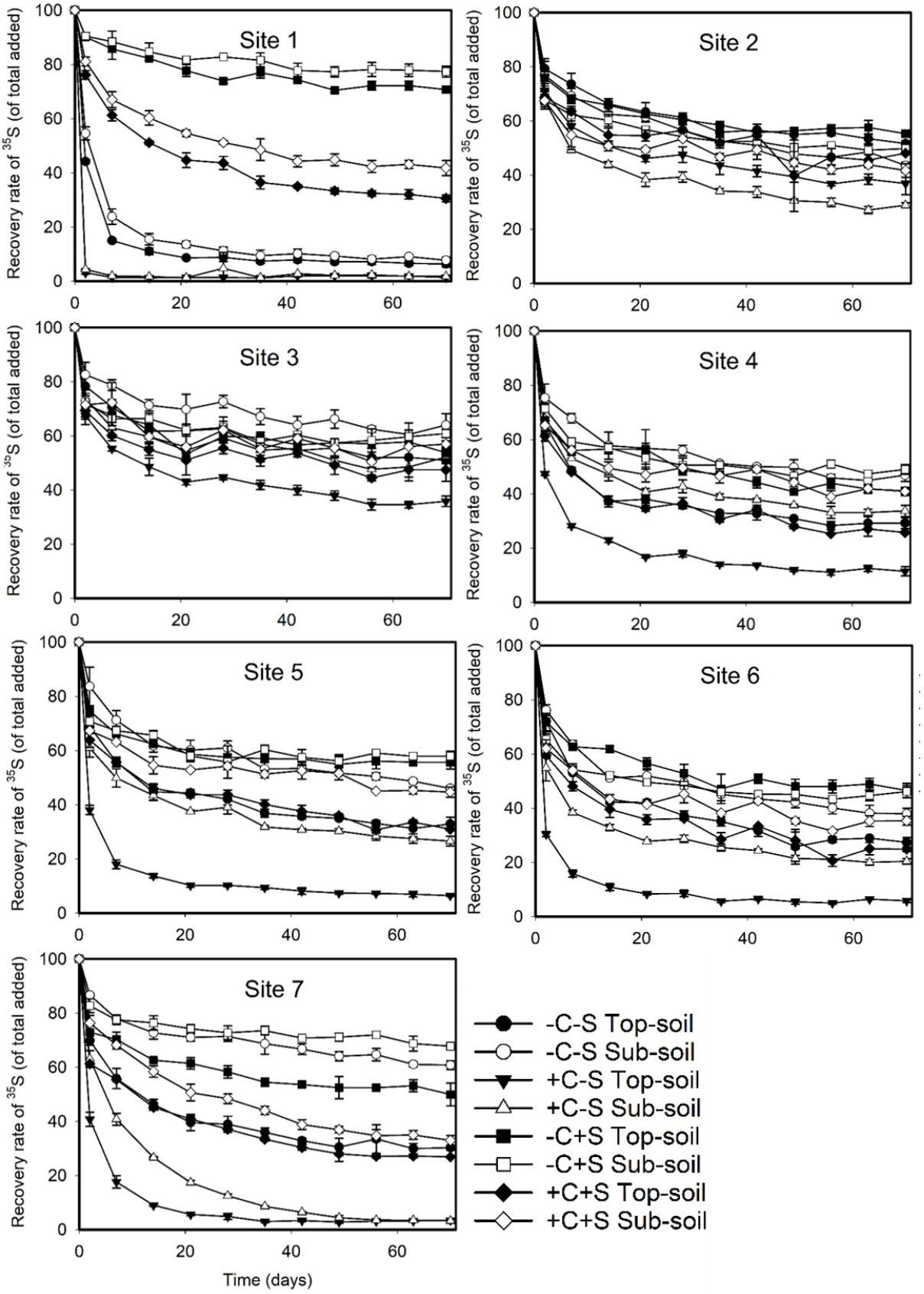

Fig. 6.2 Changes with time in the recovery rate of extractable specific activity of ^{35}S for soils 1, 5, 6 and 7 with or without substrate amendments. Values represent means ± SEM (n = 3).

6.4. Discussion and conclusions

Results of the recovery of ^{35}S in soils showed that, the –S-C treatment had the highest recovery rate, followed by +S-C, +S+C and –S+C treatments. Within a time period of 10 weeks, soil incorporated a large proportion of carrier free $Na_2{}^{35}SO_4$ into the non-extractable fraction, ranging from 27.0 ± 4.8 to 92.8 ± 1.6% (Fig. 6.2), depending on specific soils and substrate amendments. The addition of carrier sulphate was found to retard the incorporation of ^{35}S into the organo-sulphur pool, while the addition of glucose as a readily utilized carbon source was found to enhance this incorporation process due to an encouragement of rapid microbial growth. Our recovery test with quartz sand extracted by 0.01 M $Ca(H_2PO_4)_2$ showed a recovery rate ca. 100% along our 70-day-incubation experiment for all sampling times (Fig. 6.2).

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