**Respiration and carbon use efficiency characteristics of soluble protein-derived carbon by soil microorganisms: A case study at afforested sites**

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**Abstract**

Soluble protein makes an important contribution to the release of carbon dioxide (CO2) in soils, however, knowledge on the respiration and C use efficiency (CUE) characteristics of soluble protein-derived C by soil microorganisms remains limited. To address this issue, we sampled surface soils (0−10 cm) from seven tree monocultures and investigated the temporal dynamics of turnover of 14C-labelled soluble protein-derived C by soil microorganisms. Two double first-order exponential kinetic decay models were applied to analyze the mineralization data (i.e., with and without abiotic protein-surface interactions). The model incorporating the immobilization of protein on the non-living solid phase exhibited the best fit to the experimental data (R2 > 99.6%). Our results suggest that 66.1−73.9% of the soluble protein-derived C was immobilized by the non-living solid phase in soils. After uptake by the soil microbial community, 8.0−13.8% of the C was rapidly respired as CO2, while 15.0−20.8% was used in anabolic processes, resulting in a CUE of 55.1−70.2%. However, there was little effect of forest type on protein turnover rate in the soil. The C:N ratio of soil microbial biomass (C/Nmic) was positively related to the CUE of protein and showed less variation within a forest type. Compared with soil microbial biomass C and N, C/Nmic could serve as a better indicator of the CUE of protein by soil microorganisms. This study sheds light on the respiration and CUE characteristics of soluble protein-derived C by soil microorganisms at afforested sites and enhances our understanding of the trade-off between the metabolism of protein-derived C by soil microorganisms and its immobilization by the non-living solid phase in soils.

**Keywords**: Soluble protein; soil microorganisms; C use efficiency; 14C tracer

**Introduction**

Protein represents an important source of organic nitrogen (N) input into soils in forest ecosystems, playing a crucial role in the cycling of both carbon (C) and N (Jan et al., 2009; Hill et al., 2011). This protein originates from plants, animals and microorganisms, including a wide diversity in terms of their size, charge and solubility (Warren, 2014). A single plant cell may contain several thousand different proteins and 25 billion individual protein molecules (Ckurshumova et al., 2011; Heinemann et al., 2021). Generally, soil proteins can be tightly bound to humus, physically trapped within soil aggregates and dead organisms, electrostatically held on mineral surfaces or free in soil solution (Rillig et al., 2007). Soil soluble protein is highly susceptible to exoenzymatic attack, and it is expected to play a critical role in the release of CO2 and the production of low molecular weight forms of N for plant and microbial uptake (Hill et al., 2011). However, knowledge on the release characteristics of CO2 of protein-derived C respired by soil microorganisms is still limited.

The breakdown of soluble proteins produces oligopeptides and amino acids, both of which can be directly taken up by soil microorganisms (Gao et al., 2023). Furthermore, the metabolism of amino acids or oligopeptides by soil microorganisms can be divided into two major processes: 1) catabolism, whereby microorganisms utilize a portion of the amino acids or peptides C for energy production, resulting in the rapid release of CO2. This process is typically associated with deamination of the amino acids and utilization of the resultant keto acids for energy production alongside the excretion of excess NH4+ and SO42-; 2) anabolism, whereby microorganisms utilize the amino acids/oligopeptides C to undertake cell maintenance or synthesize new biomass (Farrell et al., 2011; Glanville et al., 2016; Ma et al., 2021). The respiration characteristics of the amino acids or oligopeptides by soil microorganisms is similar, with an initial rapid phase followed by a secondary slower phase of CO2 evolution. The relative proportion between the amount of C allocated to these two processes is defined as the C use efficiency (CUE) of the amino acids or oligopeptides by soil microorganisms. An increasing amount of research discovers the importance of soil microorganisms to the contribution of soil organic matter (Ma et al., 2018), and its CUE serves as a key regulator of C storage in soils (Wang et al., 2021; Tao et al., 2023). However, the issue of the CUE of protein-derived C by soil microorganisms still warrants further research.

To date, most research has focused on the metabolism of amino acids and oligopeptides by microorganisms, and we lack corresponding knowledge on soluble proteins in soils (Jones et al., 2009; Farrell et al., 2011). Forest ecosystems are one of the most important C sinks in the biosphere (Pan et al., 2011). Afforestation is regarded as an important pathway of mitigating the impacts of global warming, and the selection of tree species is especially fundamental (IPCC, 2014; Bastin et al., 2019). Afforestation has been widely implemented since the 1990 s and has increased the area of planted forests globally by about 1.05 × 108 ha (Hong et al., 2020). In this context, an improved understanding how different tree species affect the respiration and CUE characteristics of protein-derived C by soil microorganisms is important. The structure and abundance of soil microorganisms are strongly influenced by tree species (Gunina et al., 2017a), and the C:N ratio of microbial biomass is expected to influence the allocation of protein-derived C between respiration and growth (Wang et al., 2023). In light of this, we investigated the influence of tree species on the breakdown of soluble protein-derived C by the soil microbial community, and evaluated microbial respiration and CUE characteristics for soluble protein. Specifically, we hypothesized that the temporary dynamics of the respiration of soluble protein-derived C by soil microorganisms would be similar to that of amino acids and oligopeptides, with an initial rapid phase followed by a secondary slower one.

**Materials and methods**

The study was conducted at the BangorDIVERSE experimental site, situated at the Henfaes Research Centre, Abergwyngregyn, North Wales, UK (53°14′16″N, 04°01′01″W). The experimental subject consisted of seven tree monocultures, namely black alder (*Alnus glutinosa* L.), silver birch (*Betula pendula* Roth), sycamore (*Acer pseudoplatanus* L.), European ash (*Fraxinus excelsior* L.), sweet chestnut (*Castanea sativa* Mill.), pedunculate oak (*Quercus robur* L.) and European beech (*Fagus sylvatica* L.). Each monoculture has four independent blocks of trees (*n* = 4), with each block being 15 m × 15 m in size. The selection of these seven tree species was based on their contrasting shade tolerance and successional stages, representing a range of taxonomic, physiological, and ecological types. The trees were planted on the agricultural grassland in 2004. The soils are classified as Fluventic Dystrochrept in the USDA system (Smith et al., 2013), and the climate is classified as Hyperoceanic, with a mean annual temperature of 10.6 °C and annual rainfall of 950 mm (Gunina et al., 2017b). Surface soil samples (0−10 cm) were collected from the plots of the monocultures of seven tree species. Four samples were randomly taken within each plot with a trowel and were combined into a composite sample. Composite soil samples were placed in plastic bags and immediately transported back to laboratory. The fresh samples were then sieved to pass 2 mm and stored at 4 °C for further analysis. Basic soil properties were measured based on our previous experiments conducted at the study site (Gao et al., 2023), including moisture content, pH, electrical conductivity, total C and N, dissolved organic C and total soluble N, nitrate, ammonium, amino acids, microbial biomass C and N.

The temporal dynamics of the respiration of protein-derived 14C were determined at 20 °C over 14 d, following the method described in Jones et al. (2009). The experiment used 14C-labelled soluble proteins (0.064 mg C l−1; 0.0063 mg N l−1) isolated from *Nicotiana tabacum* L. leaves (St Louis, MA, USA). The protein mixture undergoes secondary purification by ultrafiltration in an Amicon® stirred cell using a 3 kDa Ultracel® cutoff membrane (Millipore UK Ltd., Watford, UK) to remove any oligopeptides before being used (Greenfield et al., 2020). Briefly, 5 g (fresh weight) of sieved soil was placed into 50 ml sterile polypropylene centrifuge tubes. 0.5 ml of 14C-labelled protein solution (> 3 kDa; 3.32 kbq ml−1) was then added to the soil surface in each tube. To trap the evolved 14CO2, a vial containing 1 ml of 1 M NaOH was then placed inside the tube, and the centrifuge tube was immediately capped. The NaOH trap was positioned above the soil to enable free diffusion of CO2 from the soil surface. To quantify the rates of evolution of respired 14CO2, the NaOH traps were removed at 0.5, 1, 2, 4, 8, 24, 48, 96, 144, 192, 240, 288 and 336 h after the addition of 14C-labelled protein solution. The 14C activity in the collected solution was determined using a Wallac 1404 scintillation counter with automated quench correction (Perkin−Elmer Life Sciences, Boston, MA) after mixing with Scintisafe3 scintillation cocktail (Fisher Scientific, Loughborough, UK). Two double first-order exponential kinetic decay models were applied to fit the inverse of the mineralization data, and the goodness of fit was used to compare the advantages of the models:

$y=\left(Y\_{r}×e^{\left(-k\_{1}×t\right)}\right)+\left(Y\_{b}×e^{\left(-k\_{2}×t\right)}\right)$ (1)

$y=\left(Y\_{r}×e^{\left(-k\_{1}×t\right)}\right)+\left(Y\_{b}×e^{\left(-k\_{2}×t\right)}\right)+Y\_{0}$ (2)

where, $y$ (%) is the amount of 14C remaining in the soils, $t$ (h) is time, $Y\_{r}$ and $Y\_{b}$ are the amount of protein-derived 14C partitioned into microbial respiration and biomass production, respectively, $k\_{1}$(h−1) and $k\_{2}$(h−1) are the exponential coefficients for these two components (Jones et al., 2009; Gao et al., 2023), respectively, and $Y\_{0}$ is the constant of the model. The C use efficiency ($CUE$) by soil microorganisms was defined as:

$CUE=[Y\_{b}/(Y\_{b}+Y\_{r})]×100\%$ (3)

All these calculations were conducted on a dry weight soil basis. Correlation analysis of the parameters of Model 2 and soil fundamental properties was calculated by using Spearman’s rank correlation coefficient and visualized as a network by Cytoscape (v.3.9.1). The coefficients of variation for soil microbial biomass C, N and the C:N ratio of microbial biomass within a forest type were calculated to compare the degree of variation, and their differences were tested through one-way analysis of variance using LSD post hoc tests (SPSS 22.0; SPSS Inc., Chicago, IL, USA).

**Results and discussions**

Although the proteins isolated from *Nicotiana tabacum* L. leaves may not fully represent the diversity of proteins in the forest soils, they are still suitable for this research based on the following reasons: 1) no single plant species simultaneously exists in all the seven monocultures, which makes it difficult to choose a representative plant for protein extraction; 2) *Nicotiana tabacum* L. is herbaceous and the understory of these forests includes herbaceous vegetation; 3) the proteins used in this experiment exhibited rapid turnover rates in the forest soils (Fig. 1), which indicates that these forest soils could possess corresponding proteases, similar proteins and turnover processes. The temporal dynamics of 14CO2 evolution resulting from the microbial respiration of the soluble protein-derived C showed a biphasic mineralization pattern, characterized by a rapid initial phase of 14CO2 evolution and followed by a slower phase, irrespective of forest type (Fig. 1a). This pattern is similar to the results observed for amino acids or oligopeptides in soils from diverse ecosystems, including forests and grasslands (Jones et al., 2009; Gao et al., 2023). While both double first-order exponential kinetic decay models fitted the experimental data reasonably well, Model 2 demonstrated significantly higher goodness of fit than Model 1 (R2 > 99.6%; Fig. 1b). This indicates a strong immobilization of protein-derived C by the non-living solid phase in the soil (Rillig et al., 2007). By using Model 2, we found that the portion of protein-derived C used for microbial respiration was 8.0−13.8%, and the portion used for biomass synthesis was 15.0−20.8%. The CUE was 55.1−70.2%, and the portion of protein-derived C immobilized by the non-living solid phase in the soil was 66.1−73.9% (Fig. 1c-h). The reported values for CUE align with those previously documented for amino acids in soils from various ecosystems (Jones et al., 2018).

Based on the average values fitted by the seven tree monocultures (Model 2), the temporal dynamics of soluble protein-derived C in soil solution can be expressed as: y = (11.84 × e(-0.47 × t)) + (16.82 × e(-0.0075 × t)) + 70.83. The first part of the model represents catabolism, where soil microorganisms respired and released CO2 following the uptake of protein-derived C, and the process was primarily for energy production. The middle part represents anabolism, where soil microorganisms utilized protein-derived C for biomass synthesis, and the process rate was significantly lower than that of respiration ($k\_{2}$ < $k\_{1}$, *P* < 0.001; Fig. 2). The constant of the last part represents the immobilization effect of protein-derived C by the non-living solid phase surfaces in the soils (Fig. 2). This is different from the turnover of soluble amino acids or oligopeptides C in soils, which are primarily taken up and metabolized by soil microbial community and Model 1 generally provides a better representation (Jones et al., 2009; Gao et al., 2023). This could originate from the difference in the transformation rate between protein and amino acids/oligopeptides in soils. Compared with the mineralization rate of low molecular weight organic N (LMWON) in soils, e.g. amino acids and oligopeptides, the depolymerization rate of protein to LMWON is slower (Jan et al., 2009; Simpson et al., 2017). Therefore, certain proteins in the soil solution are likely to be electrostatically bound to mineral surfaces or became complexed with organic matter, hindering their cleavage by exoenzymes (Rillig et al., 2007). In contrast, amino acids and oligopeptides often possess a net neutral charge, and can be transformed more rapidly and exhibit weak binding to the solid phase (Jan et al., 2009; Simpson et al., 2017).

Soil properties significantly influenced the rate of catabolism ($k\_{1}$) and the CUE of protein by soil microorganisms (Fig. 3a; Table S1). The rate of catabolism was positively related to NH4+ and negatively related to CUE (Fig. 3a). This indicates that the N mineralization of protein could be closely coupled with the C catabolism of protein. The CUE of protein was positively related to the C:N ratio of soil microbial biomass, soluble organic C and N, and was negatively related to soil microbial biomass C and N and pH (Fig. 3a). Based on the following reasons, the C:N ratio of soil microbial biomass could serve as a better indicator of the CUE of protein by soil microorganisms: 1) soil protein simultaneously provides C and N for soil microorganisms; 2) this index concurrently integrates soil microbial biomass C and N and is more simplified; 3) the coefficient of variation for the index was significantly lower than that for soil microbial biomass C and N within a forest type and was more stable (Fig. 3b). Therefore, a higher C:N ratio of microbial biomass may indicate a lower allocation of C to respiration following the uptake of protein-derived C and a higher allocation to biomass synthesis, and *vice versa*. Soil pH may indirectly influence the CUE of protein by affecting the C:N ratio of soil microbial biomass (Fig. 3a).

**Conclusions**

In summary, our study reveals that the temporary dynamics of the respiration of soluble protein-derived C by soil microorganisms are similar to those of amino acids or oligopeptides. Nevertheless, the majority of protein-derived C in the soil solution becomes immobilized by the non-living solid phase. Only a small fraction of the protein-derived C is taken up by soil microorganisms, some of which is rapidly released as CO2 by respiration, while the remaining C is primarily utilized for biomass synthesis. Furthermore, our results highlight the potential importance of the C:N ratio of soil microbial biomass as a crucial indicator of the CUE of soluble protein by soil microorganisms. Understanding the respiration and CUE characteristics of protein-derived C by soil microorganisms and its interaction with soil properties provides valuable insights into the intricate C cycling processes in various environmental settings.

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**Fig. 1** The temporal dynamics of the respiration of soluble protein-derived 14C (a), the goodness of fit (R2) of Model 1 and 2 (b), the constant of model 2 ($Y\_{0}$; c), the amount of protein-derived 14C partitioned into microbial respiration ($Y\_{r}$; d) and biomass production ($Y\_{b}$; f), the exponential coefficients for these two components ($k\_{1}$, e; $k\_{2}$, g), and the C use efficiency (CUE; h) by soil microorganisms in seven tree monocultures. $Y\_{r}$, $Y\_{b}$, $k\_{1}$, $k\_{2}$, and CUE were based on Model 2. \*\*\* indicates statistically significant difference at the *P* < 0.001 level.



**Fig. 2** The temporal dynamics of soluble protein-derived C in soils (fitted by Model 2).



**Fig. 3** Pearson correlation network of the turnover parameters of protein-derived C in soils (Model 2; orange colour) and soil factors (green colour; a). The width of solid and dotted lines indicates the strength of correlation. The constant of model 2 ($Y\_{0}$), the amount of protein-derived 14C partitioned into microbial respiration ($Y\_{r}$) and biomass production ($Y\_{b}$), the exponential coefficients for these two components ($k\_{1}$ and $k\_{2}$), and the C use efficiency (CUE) by soil microorganisms in seven tree monocultures. Microbial biomass C (Cmic) and N (Nmic); the C:N ratio of microbial biomass (C/Nmic); soil total C (TC); soluble organic C (SOC) and N (SON); the C:N ratio of soil (C/N). The autocorrelation pathways are deleted, including CUE and $Y\_{r}$ ($Y\_{b}$), TC and C/N, C/Nmic and Cmic (Nmic). Coefficient of variation for soil Cmic, Nmic and C/Nmic within a forest type (b). The boundaries of the box indicate the 25% and 75% percentiles, and the whiskers indicate the 1% and 99% percentiles, respectively. The solid line in the box marks the median, and the dotted line marks the mean. Different lowercase letters indicate significant difference at the *P* < 0.05 level.