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RESEARCH ARTICLE

Experimental strategies to measure the microbial uptake and mineralization kinetics of dissolved organic carbon in soil

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ABSTRACT

Soil organic matter turnover rates are typically estimated from mass loss of the material over time or from on rates of carbon dioxide production. In the study, we investigated a new way to characterize the concentration-dependent kinetics of amino acids used by measuring microbial uptake and mineralization of ^{14}C-alanine. We measured the depletion from soil solution after additions ^{14}C-alanine. The microbial uptake of ^{14}C-alanine from soil solution was concentration-dependent and kinetic analysis indicated the operation of at least three distinct alanine transport systems of differing affinities. Most of the ^{14}C-alanine depletion from the soil solution occurred rapidly within the first 10–30 min of the incubation after 10 μM to 1 mM substrate additions. At alanine concentrations less than 250 μM, the kinetic parameters for K_m and V_{max} of the higher-affinity transporter were 60.0 μM and 1.32 μmol g^{-1} DW soil h^{-1}, respectively. The mineralization of alanine was determined and the half-time values for the rapid mineralization process were 45 min to 1.5 h after the addition at alanine concentrations below 1 mM. The time delay after its uptake into microbial biomass suggested that alanine uptake and subsequent respiration were uncoupled pattern. The microbial N uptake rate was calculated by microbial mineralization, and an estimated K_m value of 1731.7±274.6 μM and V_{max} value of 486.0±38.5 μmol kg^{-1} DW soil h^{-1}. This study provides an alternative approach for measuring the rate of turnover of compounds that turnover very rapidly in soil.

1 Introduction

Soil organic matter (SOM) is a very important carbon (C) sink in terrestrial ecosystem, and research on SOM transformation has increased our understanding of the C cycle in soil biogeochemistry. Macromolecular SOM needs to be degraded into low molecular weight organic substances

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(LMWOS) by enzymes that act as sinks and source of nutrients; and transformation of LMWOS is a key process for making nutrients bioavailable to plant and microbial community (Dippold et al., 2014; Balland-Bolou-Bi et al., 2019), particularly when inorganic nutrient becomes limiting (Nordin et al., 2001; Jones and Kielland, 2002; Farrell et al., 2013). Within LMWOS, amino acids are the important compounds rich in C and nitrogen (N) that play an important role in soil C and N cycling (Jones, 1999; Jones and Hodge, 1999; Ge et al., 2009). In topsoil, 7%–50% of the total organic N exists as amino-N (Näsholm et al., 2009; Knicker, 2011). Current evidence suggests that uptake of amino acids by the microbial community not only provides bioavailable N, but also supplies C for energy production and growth (Jones and Murphy, 2007; Geisseler et al., 2010; Farrell et al., 2014a; Brailsford et al., 2019). In contrast, plants only utilize these compounds for the N contained within (Persson et al., 2003). Despite the differences in usage of the dissolved organic N (DON) from soil, strong competition exists between plant roots and the rhizosphere microbial community for this often-scarce resource (Kuzyakov and Xu, 2013). Evidence for this intense competition comes from the ubiquitously low concentrations of amino acids in soil solution and from the rapid removal and subsequent mineralization of isotopically-labeled DON compounds when added to soil (Kuzyakov and Xu, 2013; Farrell et al., 2014b; Gunina et al., 2017).

A good understanding of plant–microbial–soil N cycling is critical to predict ecosystem responses to anthropogenic disturbance (Hill and Jones, 2019). As such, it is important to characterize and mathematically describe the kinetics of N uptake by both roots and microorganisms. In the case of bacteria and fungi, this kinetic analysis is typically performed in monoxenic culture when the organisms are in exponential growth and under high rates of nutrient enrichment (Declerck et al., 2001; Chavarría-Hernández and Torre, 2001; Silvani et al., 2014). These conditions are not representative of soil and are likely to lead to significant overestimation of transport rates. Alternatively, many studies have modeled the kinetics of CO_2 evolution as a proxy to describe substrate uptake and use by the microbial community (Jones and Hodge, 1999). However, rather than providing a measure of uptake from soil solution, this approach largely provides an estimate of the speed of internal transamination/deamination reactions occurring post-uptake (Hill et al., 2008). Again, this will lead to large biases and an underestimation of transport rates. In addition, underestimation can also occur due to incomplete or slow recovery of evolved CO_2 as a result of the following: (i) $HCO_3^-{}_{(aq)}$ effluxed from the cell not rapidly forming $CO_{2(g)}$, (ii) HCO_3^- becoming fixed in inorganic carbonates in soil, (iii) the subsequent diffusion of CO_2 from the soil being slow relative to the rate of microbial assimilation, and (iv) some of the produced CO_2 being refixed by microbial autotrophs (Ström et al., 2003; Ge et al., 2016). In addition, when high substrate concentrations are used, metabolic overload can lead to the uptake of the solute from soil but a lack of mineralization (Jones, 1999).

Studies using isotopic tracers have found that at low exogenous concentrations, amino acids and peptides can be removed from soil within a matter of minutes (Ge et al., 2010; Hobbie and Hobbie, 2012; Hatté et al., 2017), which is 1–2 orders of magnitude faster than their subsequent mineralization (Fisk et al., 1998; Lipson et al., 2001; Vinolas et al., 2001). Accurately measuring substrate depletion kinetics over such short timescales therefore represents a major methodological challenge. Currently, our estimates of the concentration-dependent use of DON by soil microorganisms are largely based upon studies performed in pure culture or on respiration responses (CO_2 production) after substrate addition to soil. These are usually performed at artificially high DON addition rates (≥10 mM) relative to the intrinsic concentration of individual DON compounds in soil (≤ 5 μM). Secondly, the rate of microbial assimilation is frequently monitored over long time periods (e.g., 0–7 days) relative to the rate of depletion (0–7 min). This has led some to scientifically question the traditional isotopic CO_2 methodology (Jones et al., 2005; Hobbie and Hobbie, 2013). In the study, we explored an alternative approach for measuring the concentration-dependent uptake kinetics of highly labile DON compounds in soil; and alanine was chosen as the substrate because it is the one of the most abundant amino acid in soil (Dippold et al., 2014). In contrast to the traditional approach by extraction of soil solution, we hypothesized that adding a large volume of substrate to a small amount of soil would allow more accurate estimation of microbial uptake by (i) slowing the rate of microbial uptake, (ii) maintaining linear uptake rates for longer time periods, and (iii) maintaining exogenous substrate concentration at near steady-state.

2 Materials and methods

2.1 Soil sampling

The selected soil was a Eutric Cambisol soil collected from *Lolium perenne*-dominated agricultural grassland in the Bangor University, Henfaes Research Station Abergwyngregyn, Gwynedd, UK (53°14′N, 4°01′W). The mean annual rainfall and temperature for this region are 800 mm and 9.8°C, respectively. Soil samples were taken from the top 15 cm at three points (representing three replicates), placed in gas-permeable plastic bags, and brought to the laboratory directly after sampling, followed by sieving (2 mm) to remove earthworms, stones, and roots. Soil moisture content was measured by oven drying at 80°C for 24 h and averaged 27.6± 0.95%. Soil pH and electrical conductivity were assessed in a 1:1 (v/v) soil-distilled water extract, and were 6.15±0.22 and 78.6±6.2 (μS cm^{-1}), respectively.

2.2 Alanine depletion from soil

This study contains two experiments. The aim of the first

experiment was to assess alanine depletion with different amounts of soil and to determine a better proportion of soil amount to solution volume. The aim of the second experiment was to determine the dynamics of alanine removal from the soil solution depending on the substrate concentration. The two experiments were carried out at 20±1°C.

For the first experiment, 0.1, 0.25, 0.5, 1, 2, and 4 g of field-moist soil were placed in 50-mL centrifuge tubes respectively, followed by the addition of 40 mL of uniformly ^{14}C-labeled alanine (0.2 kBq mL^{-1}; American Radiolabeled Chemicals, St Louis, USA) at three concentrations (10, 100, and 1000 μM). Four replicates were analyzed for each combination of soil weight and substrate concentration. The tubes were immediately shaken at 350 r min^{-1} for 6 h, and 1-mL aliquot of soil solution was collected for each time interval at 5 min, 10 min, 30 min, 1 h, 2 h, 4 h, and 6 h and centrifuged at 14 000 r min^{-1} for 2 min. The ^{14}C activity was subsequently measured the clear supernatant with a Wallac 1404 liquid scintillation counter (Wallac EG & G Milton Keynes, UK) after mixing with HiSafe 3 scintillant (Fisher Scientific, Loughborough, UK).

For the second series of experiments, 0.5 g soil was incubated with 40 mL of 1, 10, 100, 250, 500, 750, 1000, 2500, 5000, and 10000 μM uniformly ^{14}C-labeled alanine (0.2 kBq mL^{-1}) on the shaker for 30 min. Four replicates were prepared for each substrate concentration. The ^{14}C activity of each sample was determined as described above.

2.3 Alanine mineralization

To assess alanine mineralization, 5 g of fresh soil was added to a 50 mL centrifuge tube, followed by the addition of 0.5 mL of uniformly ^{14}C-labeled alanine solution (0.2 kBq mL^{-1}). A total of 10 alanine concentrations (1, 10, 100, 250, 500, 750, 1000, 2500, 5000, and 10000 μM) were applied with four replicates, respectively. A scintillation vial containing 1 mL of 1 M NaOH was immediately placed into the centrifuge tube after adding the alanine solution and the tube was sealed with a cap and kept at 20±1°C. The vials with NaOH solution were replaced after 30 min, 60 min, 90 min, 2 h, 3 h, 4 h, 6 h, 8 h, and 24 h. After 24 h, all the soils were extracted with 25 mL of 1 M NaCl on a shaker at 350 r min^{-1} for 15 min. The ^{14}C activity of the CO_2 captured in the NaOH solution and that of the centrifuged solution were determined as described above.

2.4 Data analysis and statistics

For the substrate depletion experiments, a first-order double-exponential decay curve was fitted to the experimental data points according to the following Eq. (1). The Eq. (1) can be used to evaluate the microbial ^{14}C uptake rates, from which the maximum soil solution ^{14}C activity can be calculated (Ge et al., 2012; Wilkinson et al., 2014),

$$y = y_0 + a_1 \times \exp(-b_1 \times t) + a_2 \times \exp(-b_2 \times t) \quad (1)$$

where y is the ^{14}C remaining in the soil solution, y_0 is an asymptote, b_1 and b_2 are the exponential coefficients describing depletion by soil microorganisms, a_1 and a_2 describe the pool sizes of the fast and slow phase, and t is time.

The half-life ($t_{1/2}$) of the soil solution alanine (a_1) was defined by the following equation:

$$t_{1/2} = \ln(2)/b_1 \quad (2)$$

The concentration-dependence uptake of alanine was described using an Eadie–Hofstee diagram, and the Michaelis–Menten kinetic parameters K_m and V_{max} can be obtained according to the following equation:

$$V = V_{max} \times S/(K_m + S) \quad (3)$$

where V is the rate of alanine uptake, S is the added alanine concentration, V_{max} is the maximum uptake, and K_m is the alanine concentration at which half maximal uptake occurs.

The mineralization of amino acids and peptides is biphasic, and includes a primary rapid phase and a subsequent, slower second phase (Hill et al., 2011). The $^{14}CO_2$ evolution from the soil after the addition of ^{14}C-labeled alanine was fit to a first-order double exponential decay equation (Hill et al., 2011):

$$y = a_3 \times [1 - \exp(b_3 \times t)] + a_4 \times [1 - \exp(b_4 \times t)] \quad (4)$$

where b_3 and b_4 are the exponential coefficients describing the primary slower phase mineralization of the microbial biomass, and a_3 and a_4 describe the size of the pools. Therefore, the $t_{1/2}$ of alanine in soil can be calculated according to

$$t_{1/2} = \ln(2)/b_3 \quad (5)$$

According to Farrell et al. (2013), the uptake of alanine (Φ [μM N kg^{-1} soil d^{-1}]) for the soil solution concentration Q can also be calculated by the following equation:

$$\Phi = b_3 \times Q \quad (6)$$

where Q is the soil solution concentration of alanine (μmol N kg^{-1}).

All analyses were conducted in Sigmaplot 10.0 (SPSS Inc., Chicago, IL).

3 Results

3.1 Alanine depletion from soil

Depletion of ^{14}C-alanine increased with increasing soil weight (from 0.1 g to 4 g soil) and was accurately described by a double-exponential decay equation for all alanine concentrations ($r^2 > 0.99$ in most cases; Eq. (1), Fig. 1). However, as the concentration of alanine increased, the ratio of ^{14}C-alanine depletion decreased. After 1 h, 4.56%, 10.8%, 19.8%, 35.3%, 57.3%, and 72.8% of added ^{14}C-alanine from 10 μM solution was removed from 0.1 g to 4 g soil, respectively, representing removal of 2.7%–27.8% and 0.86%–8.28% of ^{14}C-alanine from 100 μM and 1000 μM solution, respectively (Fig. 1). The majority of ^{14}C-alanine depletion from the soil solution

occurred rapidly within the first 10–30 min of the incubation (half-lives for the rapid phase of the double exponential decay were mostly below 25.6 min in the 10 μM solution and below 11.2 min in the 100 μM and 1000 μM solution, excluding the measurements for the smallest masses). As the mass of soil increased, depletion of ^{14}C-alanine linearly increased by 19.8%–75.4% in 10 μM solution, and 4.4%–72.6% in 100 μM solution, and 1.6%–14.6% in 1 mM solution after 6 h.

The equilibrium alanine concentration decreased with increasing length of incubation times for the soil solutions (Fig. 2). Moreover, the equilibrium concentration was the lowest for the lowest alanine concentration (10 μM). However, the equilibrium concentration for the soil-solution samples 1:400, 1:160, and 1:80 was not significantly different ($p < 0.0001$) when compared to the initial concentration.

3.2 Uptake kinetics of alanine

The uptake of ^{14}C-labeled alanine from the soil solution after the addition of a 1 μM to 10 mM alanine was described by a double-exponential decay equation ($r^2 > 0.996$; Eq. (1), Fig. 3A). A total of 21.7±1% and 11.3±0.5% of the alanine was removed from the soil solution within 30 min for 1 and

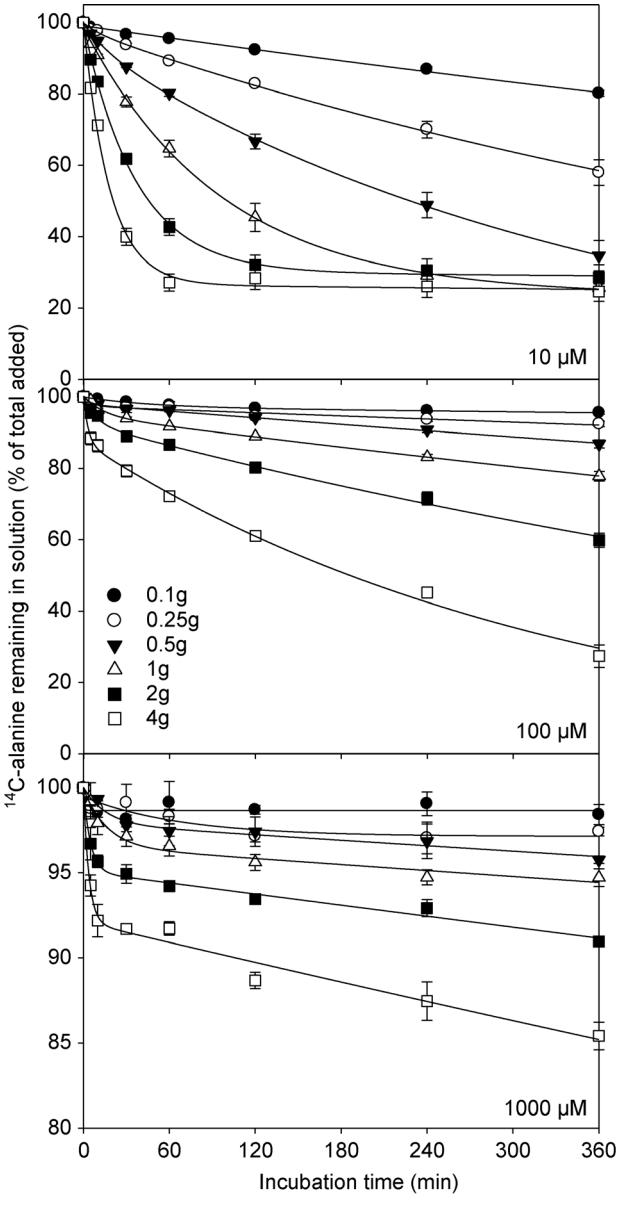

Fig. 1 Amount of ^{14}C-labeled alanine remaining in solution after the addition of ^{14}C-labeled alanine pulses (10 μM, 100 μM, and 1 mM) to grassland soils of different weights. Values represent means±standard error (n = 3). Lines represent fits of double first-order kinetic equations to the experimental data.

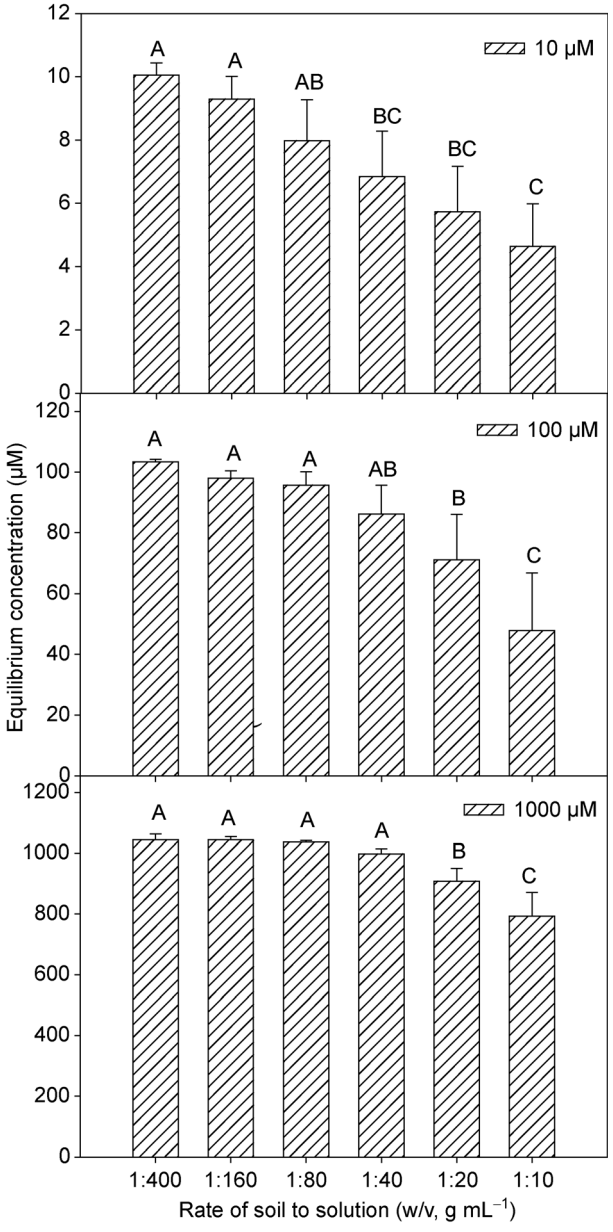

Fig. 2 The equilibrium alanine concentration of soil solutions after removal of 1 mL of solution seven times within 6 h for different soil to solution ratios.

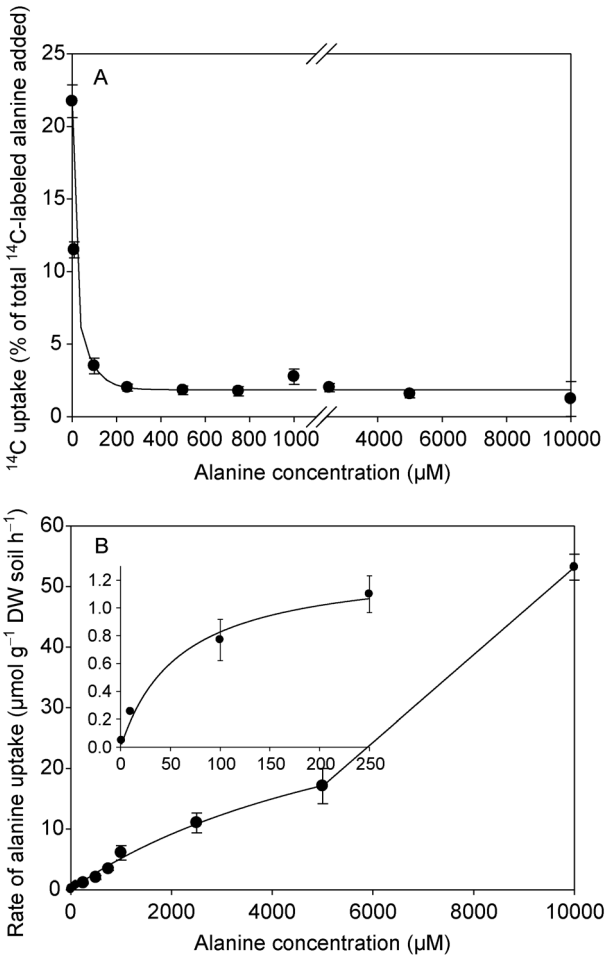

Fig. 3 Uptake of ^{14}C-labeled alanine by soil microbes. Values represent means±standard error (n = 4). (A) Amount of ^{14}C uptake over 30 min from the soil solution after the addition of a ^{14}C-labeled alanine (1 μM to 10 mM). Lines represent double-first-order kinetic Eq. fitted to the experimental data. (B) Concentration-dependent uptake kinetics of alanine measured after 30 min substrate incubation in soils. Lines represent the Michaelis–Menten kinetic equation fit to the experimental data. DW refers to dry weight.

10 M initial alanine concentrations, respectively. With increasing alanine concentration, from 100 μM to 10 mM, the proportion of ^{14}C-labeled alanine removed remained relatively constant and ranged from 3.5±0.5% to 1.2±1%. At concentrations less than 250 μM, the rate of alanine uptake from the soil solution was best described by Michaelis–Menten kinetics (r^2 > 0.987; Eq. (3), Fig. 3B). Values for K_m and V_{max} of the higher-affinity transporter were 60.0 μM and 1.32 μmol g^{-1} DW soil h^{-1}, respectively.

3.3 Substrate mineralization in soil

The $^{14}CO_2$ evolution following the addition of ^{14}C-labeled alanine was well-described by a double-exponential decay equation for all alanine concentrations (r^2 > 0.973 in all cases; Eq. (4), Fig. 4A), demonstrating a typical microbial growth curve with a rapid increase at the early stage of incubation and following slower phase. The exponential coefficient and half-time ($t_{1/2}$) values for the rapid mineralization process are shown in Table 1. The $t_{1/2}$ values increased with increasing alanine concentration. The proportions of respiration (a_3) by soil microbes in the "fast" phase ranged from 31.3±0.61% to 57.1±1.09% within 45 min to 1.5 h of addition, respectively, at alanine concentrations below 1 mM (Table 1). When the concentration of alanine increased from 1 mM to 10 mM, the $t_{1/2}$ values increased in magnitude by 1 to 4 times, but a_3 only increased by 7% to 12%. The $t_{1/2}$ values were 2.53, 3.89, and 6.08 h for 2.5, 5, and 10 mM alanine, respectively (Table 1). The exponential coefficients for ^{14}C depletion showed a concentration-dependent trend of microbial mineralization. Furthermore, there was a concentration-dependent trend for the mineralization rate of alanine, which was fitted to the

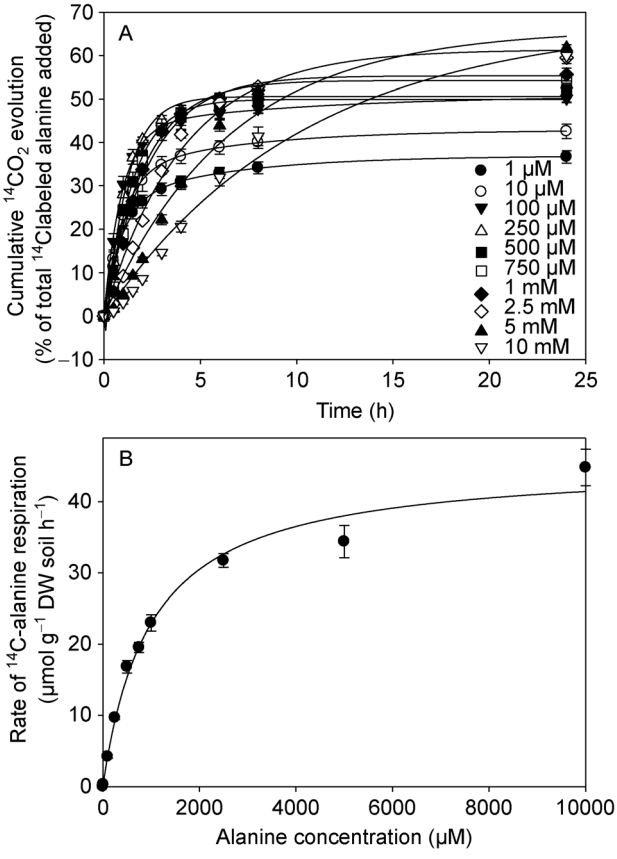

Fig. 4 Mineralization kinetics of ^{14}C-alanine to CO_2 by soil microbes over 24 h. Values represent means±standard error (n = 4). (A) The proportion of ^{14}C-labeled alanine (1 μM to 10 mM) added to a sample of soil that is transformed to $^{14}CO_2$ over time, and (B) the rate of ^{14}C-alanine respiration by soil microbes against the concentration of added alanine solution within a 60 min incubation. Lines represent the Michaelis–Menten kinetic equation fitted to the experimental data.

Table 1 Coefficients of double-first-order curve fits to the depletion of ^{14}C from soil by microbial mineralization over time.

L-alanine concentrations in solution (μM)	a_3	b_3	a_4	b_4	$a_3 t_{1/2}$(h)
1	31.3±0.61	0.895±0.03	68.6±0.59	0.0035±0.0001	0.78±0.03
10	37.5±0.76	0.898±0.05	62.6±0.69	0.0037±0.0003	0.78±0.04
100	45.7±0.54	1.041±0.05	54.7±0.50	0.0040±0.0001	0.67±0.03
250	50.7±0.73	0.828±0.02	50.7±0.69	0.0034±0.0001	0.84±0.02
500	50.3±1.93	0.658±0.02	51.1±1.68	0.0026±0.0002	1.06±0.04
750	55.4±0.76	0.533±0.01	47.2±0.68	0.0016±0.0003	1.30±0.02
1000	57.1±1.09	0.487±0.01	45.8±1.05	0.0014±0.0002	1.43±0.03
2500	64.3±0.73	0.274±0.002	39.5±0.64	$<10^{-10}$	2.53±0.01
5000	69.4±0.54	0.178±0.004	35.2±0.40	$<10^{-10}$	3.89±0.08
10000	68.9±0.44	0.11±0.005	34.6±0.59	$<10^{-10}$	6.08±0.26

Michaelis–Menten kinetic equation ($r^2 > 0.987$; Eq. (3), Fig. 4B). The K_m and V_{max} values of mineralization were 987.6 μM and 45.6 μmol kg^{-1} DW soil h^{-1}, respectively.

We used Eq. (6) to estimate microbial N uptake rate (Fig. 5). The microbial N uptake rate was a concentration-dependent trend best described by the Michaelis–Menten kinetic equation ($r^2 > 0.9767$; Eq. (4)). The K_m and V_{max} values of microbial N uptake rate were 1731.7±274.6 μM and 486.0±38.5 μmol kg^{-1} DW soil h^{-1}, respectively.

4 Discussion

4.1 Influence of the proportion of soil weight and substrate mass

Traditional methods for measuring the LMWOS removal from soil solutions by soil microbial uptake usually rely on the determination of the rate of $^{14}CO_2$ evolution to determine the substrate uptake rate (Mcfarland et al., 2010; Fischer et al., 2010; Gunina et al., 2017). However, there will be a time gap between uptake and mineralization to CO_2. In this study, to screen a suitable proportion of soil-solution in different alanine concentrations, we added 0.1 to 4 g of soil to 40 mL ^{14}C-alanine solution. The results indicated that the depletion of ^{14}C-alanine can be accurately predicted by a double-exponential decay equation, and was concentration-dependent and linearly increased with increasing soil mass. Moreover, we found the substrate-to-soil ratio of 1:80 maintained a near-constant external alanine concentration. Therefore, the ratio of 1:80 best reflected the soil microbial uptake of N with a relatively stable equilibrium concentration.

4.2 Dynamics of amino acid uptake from soil solution

By directly determining radiolabeled alanine removal from the soil solution, our results showed that ^{14}C-alanine uptake was concentration-dependent and a multi-stage process. This results indicated that there were at least three transport systems for alanine uptake by the soil microbial community: two saturable carrier systems (at concentrations 0–250μM and 250–5000μM) and one non-saturable carrier system (>5000μM). Previous studies indirectly calculated uptake rates based on the evolution of CO_2 from the radiolabeled substrate (Wilkinson et al., 2014) or by using Eq. (6) to indirectly estimate microbial N uptake rate (Farrell et al., 2013). In these studies, it was difficult to distinguish different affinity systems, and (as noted in Wilkinson et al., 2014) this method may overestimate the values of V_{max} and K_m (Farrell et al., 2013). In this study, we obtained the K_m (60.0 μM) and V_{max} (1.32 μmol g^{-1} DW soil h^{-1}) values for the high-affinity system by measuring removal from the soil solution. However, the calculated values obtained from Eq. (6) for the K_m and V_{max} of microbial N uptake were 1731.7±274.6 μM and 486.0 ±38.5 μmol kg^{-1} DW soil h^{-1}, respectively. Therefore, a better reflection of the rate of alanine uptake may be obtained by directly determining substrate removal from the soil solution to estimate ^{14}C-alanine uptake.

4.3 Dynamics of amino acid mineralization by soil microbial community

A biphasic $^{14}CO_2$ evolution was observed for all experimental

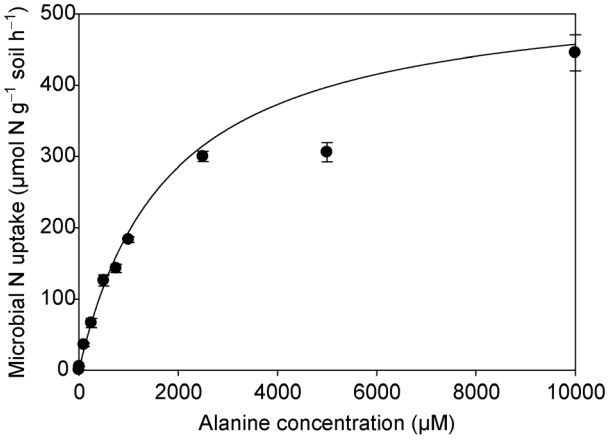

Fig. 5 Microbial N uptake kinetics estimated after a 24 h mineralization period for different alanine concentrations in the soil solution. Symbols represent calculated data point (mean±SE), while lines represent curves fitted to a single Michaelis–Menten equation.

alanine concentrations, and is supported by earlier findings (Boddy et al., 2007, 2008; Wilkinson et al., 2014). The amount of alanine-C was rapidly mineralized (a_3), and this a_3 pool accounted for 31%–50% of the added ^{14}C-labeled alanine below 1 mM. The half-lives of the a_1 pool ranged from 40 min to approximately 1 h. At higher concentrations of ^{14}C-labeled alanine (1–10 mM), the proportion of $^{14}CO_2$ produced following rapid substrate mineralization (a_3) increased by 7% to 12%. Meanwhile, the $t_{1/2}$ of a_3 pool ranged from 1.5 to 6 h. Overall, the $t_{1/2}$ of the rapid mineralization phase is much slower than previously reported values for alanine (Wilkinson et al., 2014). For example, Wilkinson et al. (2014) reported that the half-life for ^{14}C-labeled alanine ranged from 1 to 9 min in the initial rapid mineralization pool (below 10%) for four different soils. The half-life ranged with an increase in substrate concentration (from 0.5 to 10 mM), and more than 55% of added ^{14}C-alanine was allocated to the a_1 pool (Wilkinson et al., 2014). The main difference for the $t_{1/2}$ values was because the concentration of ^{14}C-alanine used in this study was greater than that in previous studies during the rapid mineralization phase (Boddy et al., 2007; Wilkinson et al., 2014). The $t_{1/2}$ values for the slower secondary pool (a_4) were not accumulated by the secondary mineralization of the microbial biomass (b_4), because it could be similar to glucose mineralization to uncertainty over the connectivity between respiratory substrate pools (Boddy et al., 2007; Hill et al., 2008).

5 Conclusion

The ^{14}C-alanine uptake by soil microorganisms was directly determined based on removal from soil solution (instead of determining the production of CO_2 from the radiolabeled substrate), in order to reduce or remove the time lag in measurements relating to C decomposition to CO_2. The ratio of soil weight to solution volume in 1:80 best reflected the soil microbial uptake of ^{14}C-alanine with a relatively stable equilibrium concentration. Direct monitoring of the removal of ^{14}C-labeled alanine from the soil solution made it possible to obtain multiple transporter system kinetics for alanine uptake by the soil microbial community. Moreover, we found that mineralization of alanine by microorganisms showed a significant delay after its uptake into microbial biomass suggesting that the uncoupled pattern of alanine uptake and subsequent respiration was possibly due to a dilution of alanine in labile metabolite pools. Therefore, our results may provide a useful method for directly determining uptake kinetics of the fast turnover rates of LMWOS by soil microbial uptake of ^{14}C-labeled alanine.

Acknowledgments

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