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REGULAR ARTICLE

Biogeochemical transformations of amino acids in soil assessed by position-specific labelling

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Abstract
Background and aims Amino acid turnover in soil is an important element of terrestrial carbon and nitrogen cycles. This study accounts for their driver - the microbial metabolism - by tracing them via the unique isotopic approach of position-specific labeling.
Methods Three ^{14}C isotopomers of alanine at five concentration levels combined with selective sterilization were used to distinguish sorption mechanisms, exoenzymatic and microbial utilization of amino acids in soil.
Results Sorption and microbial uptake occurred immediately. Unspecific microbial uptake followed a linear kinetic, whereas energy-dependent uptake followed Michaelis-Menten. Less than 6 % of the initially added alanine was sorbed to soil, but after microbial transformation products were bound to the soil matrix at higher proportions (5–25 %). The carboxyl group (C-1) was rapidly oxidized by microorganisms, whereas C-2 and C-3 positions were preferentially incorporated into microbial biomass. Dependency of C metabolization on amino acid concentration reflected individual alanine transformation pathways for starvation, maintenance and growth conditions.
Conclusions This study demonstrates that position-specific labeling determines the mechanisms and rates of C cycling from individual functional groups. This approach reflected underlying metabolic pathways and revealed the formation of new organic matter. We therefore conclude that position-specific labeling is a unique tool for detailed insights into submolecular transformation pathways and their regulation factors.

Keywords Position-specific tracers · Amino acids stabilization · Sorption · Exoenzyme and uptake kinetics · Metabolic tracing · Soil organic matter formation · Sterilization and inhibition methods · Biochemical pathways

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Introduction

Studies on transformation of organic substances in soils are important for understanding of C and N

cycles in terrestrial ecosystems. Plant residues and rhizodeposits are the main sources of organic matter in soils (Rasse et al. 2005). Therefore, many studies have focused on decomposition, microbial utilization and stabilization of C from these sources (von Luetzow et al. 2006).

During decomposition of litter, macromolecular compounds are depolymerized by enzymes into low molecular weight organic substances (LMWOS) (Cadisch and Giller 1996). LMWOS are the lightest (<250 Da) components of DOC (Boddy et al. 2007) from substance classes such as organic acids, amino acids, mono- and disaccarides, amino sugars, phenols and many more (van Hees et al. 2005). In addition to litter, rhizodeposition is a source of LMWOS in soil. Microorganisms determine the fate of LMWOS in soil because they either produce them, decompose them to CO_2 and NH_4^+ (catabolism) or incorporate them in cellular compounds (anabolism). The importance of LMWOS is not connected with their pool size (Fischer et al. 2007), but with the huge fluxes that pass through this pool. Therefore, the transformation pathways of LMWOS represent a crucial step of soil C and N fluxes, and a molecular-level knowledge of these processes is needed (van Hees et al. 2005).

Within the LMWOS, amino acids play an important role because they are the quantitatively most important compounds coupling the C and N cycle. In topsoil, amino bound N constitutes 7–50 % of the total organic N (Stevenson 1982; Gardenas et al. 2011). Thus, many recent studies focused on the fate of N-containing LMWOS (Kuzyakov 1996; Lipson et al. 2001; Hobbie and Hobbie 2012; Jones et al. 2004; Knowles et al. 2010; Vinolas et al. 2001a) and investigated the three major pathways of amino acid utilization in soil: 1) sorption (Jones 1999), 2) extracellular transformation, and 3) intracellular metabolization (Vinolas et al. 2001a, b) which can be separated by selective inhibition of biotic processes.

Sorption strongly depends on the functional group of the amino acid (Jones and Hodge 1999): it can occur by ion exchange of positively charged amino groups, by ligand exchange of carboxyl groups and by hydrophobic interactions with alkyl groups. To date, nearly all studies assumed sorption of the entire molecule by soil sorbents. Only a few studies on glycine sorption indicated abiotic degradation of the sorbed amino acid (Wang and Huang 2003, 2005).

Amino acids can be transformed extracellularly, mainly by exoenzymes attached to cell surfaces (Geisseler et al. 2010). Deamininating (Killham and Rashid 1986) and oxidizing (Bohmer et al. 1989; Braun et al. 1992) extracellular systems are described in the literature, but neither their relevance nor the differences between extra- and intracellular pathways have been investigated (Burns 1982).

Intracellular amino acid metabolization follows the uptake by transport systems (Hediger 1994; Anraku 1980; Hosie and Poole 2001). Uptake kinetics of some amino acids has already been elucidated (Vinolas et al. 2001a, b). Barraclough (1997) showed that the majority of N mineralization of amino acids occurred inside the cells. Knowles et al. (2010) described for the first time the decoupling of N and C metabolization in soil, discovering a preferential retention of amino acid N with respect to C. Nonetheless, as they used uniformly labeled tracers, they could not determine the fate of the C skeleton. We hypothesize that the fate of amino acid C and N in soil is mainly determined by the dominating intracellular metabolization pathways. Therefore, identification of microbial metabolization is a crucial step for understanding and predicting C and N fluxes.

In addition to abiotic factors such as temperature (Dijkstra et al. 2011c; Vinolas et al. 2001b) or soil properties (Kemmitt et al. 2008; Gonod et al. 2006), the concentration of a substrate is a key driver of the intracellular metabolization (Fischer and Kuzyakov 2010; Dijkstra et al. 2011a; Schneckenberger et al. 2008). Soil amino acid concentrations range from 0.5 μM in root-free bulk soil to 5 mM directly next to bursting cells (Fischer et al. 2007; Jones and Hodge 1999). We expect cellular uptake and metabolism always dominate the amino acid removal from soil solution and that sorption only plays a relevant role at low substrate concentrations. For our study, we chose alanine as a representative amino acid for the neutral amino acids as it is one of the most dominant amino acids in soil solution (Fischer et al. 2007). In addition, alanine was chosen because it is very close to the basic C metabolism of the cell: by oxidative deamination alanine can be transferred to pyruvate, which is a suitable substrate for metabolic tracing experiments in plants and soils (Tcherkez et al. 2005; Dijkstra et al. 2011a).

To elucidate intra- and extracellular alanine transformation pathways, we used the approach of position-specific labeling. This tool is commonly used

in biochemistry to investigate metabolization pathways, but has rarely been applied in soil science (Haider and Martin 1975; Fischer and Kuzyakov 2010; Fokin et al. 1993, 1994; Dijkstra et al. 2011a, b, c; Kuzyakov 1997; Nasholm et al. 2001). It overcomes the limitations of uniform labeling because it allows differentiating between incorporation of fragments vs. incorporation of entire molecules.

Coupling of position-specific labeling with soil sterilization enables us to separate abiotic splitting of alanine from extracellular and from cellular metabolism. We assume that extra– and intracellular transformation differ from each other as they are based on different enzymes. By comparison of the kinetics of alanine removal from soil solution in the non-inhibited and respiration-inhibited treatments, the relevance of extra- versus intracellular transformations of alanine was compared. We hypothesize that under soil conditions microbial uptake systems and intracellular metabolization dominate the fate of alanine in soil. Comparing our results with known microbial metabolization pathways enables the identification of metabolic changes depending on substrate concentration.

Materials and methods

Soil

Topsoil (0–10 cm) from the Ap horizon of a silt loam haplic Luvisol (WRB 2006) was collected from a field in Bavaria with a crop sequencing of barley, wheat and triticale (49.907 N, 11.152 E, 501 m asl, mean annual temperature 6–7 °C, mean annual precipitation 874 mm). The soil had a pH_{KCl} of 4.88 and pH_{H2O} of 6.49, total organic C and total N content were 1.77 % and 0.19 %, respectively, and potential CEC was 13.6 $cmol_c\ kg^{-1}$. Soil was sieved to 2 mm, and all roots were removed with tweezers. Soil was stored at field moisture at 5 °C not longer than 1 week until the experiment started.

Chemicals and radiochemicals

Stock solutions with 1, 10, 100, 1,000, and 10,000 μM alanine and an equal activity of 10^4 DPM ml^{-1} (Disintegrations Per Minute and ml) were prepared from U-^{14}C-labelled alanine and the position-specifically labeled isotopomers 1-^{14}C-, 2-^{14}C- and 3-^{14}C-labeled alanine (American Radiolabeled Chemicals Inc, St. Louis, USA) as well as non-labeled alanine (Sigma-Aldrich, Taufkirchen, Germany).

Sterilization solutions were produced with 1 mM NaN_3 to inactivate aerobic microbial respiration or with 1 mM NaN_3 and 1 mM $HgCl_2$ to denaturate all proteins and reach full inhibition of biotic processes. Effectiveness of the chosen azide inhibitor was evaluated by a qualitative 2,3,5-triphenyltetrazoliumchloride incubation (TCC, Sigma-Aldrich, Taufkirchen, Germany). Therefore 0.63 μg of the yellow dye TCC were added to the 1 ml of soil suspension in this preexperiment.

Experimental setup

The effects of two factors on alanine transformations in soil were investigated: 1) the concentration of alanine, and 2) the extra- and intracellular as well as abiotic processes of alanine removal from soil solution, separated by sterilization. Therefore, three sterilization treatments were used (Fig. 1): 1) treatments without any inhibition, where three groups of processes occured: intracellular metabolism, extracellular transformation and physicochemical sorption, 2) treatments with inhibition of aerobic respiratory chains by azides (Burns 1982), where only extracellular processes are active and sorption could occur, and 3) treatments with full inhibition, where microbial metabolism as well as exoenzymes were inhibited by $HgCl_2$ (Wolf et al. 1989; Stevenson and Verburg 2006) and only sorption could remove alanine from the soil solution (Fig. 1). We define here as extracellular transformations all processes (decomposition, decarboxylation, condensation, etc.) localized in the soil solution or periplasm (Glenn 1976) which don't depend on intracellular energy metabolism (i.e. proton gradient or ATP) and can not be inhibited by NaN_3. Biotic transformations sums up extra- as well as intracellular processes.

The experiment consisted of two parts (Fig. 1): In the first part – the incubation experiment- the processes removing alanine from the supernatant were investigated. The incubation was performed in 24-deep-well plates (6 ml volume per well) on a rotational shaker at 200 rpm with 200 mg field fresh soil per replication. Before adding the alanine, the soil was pre-incubated for 1 h with 0.5 ml of 1 mM sterilization solutions or distilled water, respectively. Pre-incubation was performed under intensive shaking to

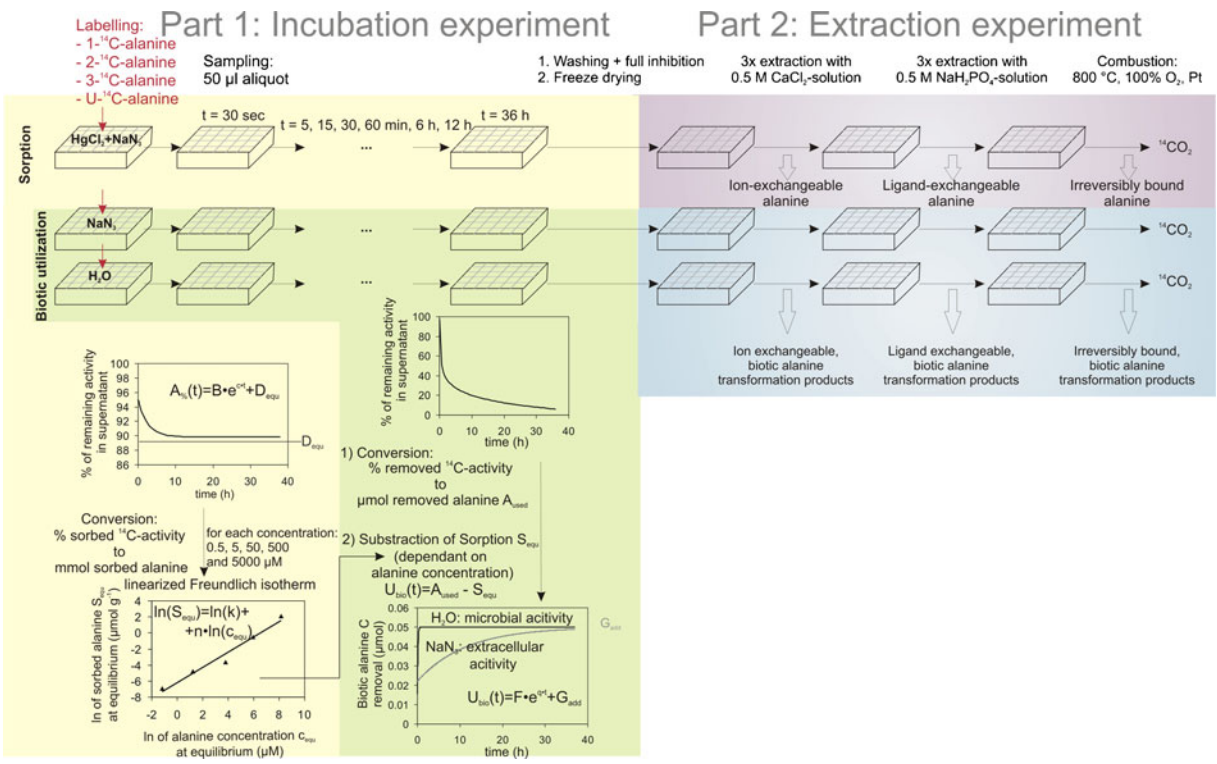

Fig. 1 Scheme of the experimental design for one of the five concentrations: in part 1 on the left side (incubation experiment) yellow-shaded plates shows fully-inhibited treatment to investigate sorption whereas green-shaded plates reflect biotic utilization (*upper line* with only extracellular activity and *lower line* with extra- and intracellular activity). *Yellow-shaded graphs* demonstrate the calculation of the sorbed proportion of alanine by the sorption isotherm, which is derived from the fully inhibited treatment. *Green-shaded graphs* reveal the calculation of the biotic utilization by substracting the sorption from the percentage of alanine removal from supernatant. In part 2 on the right side (extraction experiment) *purple-shaded plates* reflect the fully-inhibited treatment and thus extraction of untransformed alanine by the sequential procedure. *Blue-shaded plates* show desorption of biotic alanine transformation products (*upper plate* with only extracellular activity and *lower plate* with extra- and intracellular activity)

enable a homogenous sterilization of the entire soil volume under high oxygen supply. Thus, during pre-incubation anaerobic processes were prevented, the stored energy could be consumed and no new energy reserves were produced.

In the treatment with extracellular processes, the intracellular respiration was inhibited with 0.5 ml 1 mM NaN_3. Although chosen NaN_3-concentrations are far above those described for respiratory chain inhibition (Kita et al. 1984) some activity may remain in the soil suspension. This was evaluated by a triphenyl-tetrazolium chloride assay. This dye is intracellularly reduced by various dehydrogenases (Kvasnikov et al. 1974; Mohammadzadeh et al. 2006). An active intracellular metabolism leads to the formation of insoluble red formazan crystals within living cells. In the treatment with full inhibition, denaturation of proteins was achieved by adding 0.5 ml of 1 mM $HgCl_2$ and 1 mM NaN_3.

After pre-incubation, 0.5 ml of the alanine-solution was added. All experiments were performed with uniformly labeled alanine and the three isotopomers. The soil suspension was shaken for 30 s, centrifuged at 2,000 rpm and an aliquot of 50 µl was removed for ^{14}C measurement. After remixing, incubation was continued, and further 50 µl were sampled 5, 15, 30 and 60 min and 6 h, 12 h and 36 h after addition of ^{14}C labeled alanine. After incubation, the remaining supernatant was removed and soil was washed three times – first with distilled water, then with full inhibition solution and finally with distilled water.

In the second part of the study - the desorption experiment - we evaluated the binding mechanisms of alanine C in soil (Fig. 1). In treatments with full

inhibition, the extracted C reflects alanine C itself, as no biotic transformation occurred. In treatments with biotic activity, the microbial or extracellular transformation products were extracted. The washing step with $HgCl_2$ led to denaturation of membrane proteins and thus a loss of membrane integrity. This allowed the joint extraction of water soluble cytoplasm compounds and extracellular transformation products. Macromolecular compounds like proteins, polysaccharides or peptidoglycan as well as hydrophobic compounds like the membrane lipids could not be extracted by a salt solution.

For the desorption experiment, 0.5 ml of 0.5 M $CaCl_2$ solution was added to the soil and shaken for 2 h. The solution was centrifuged, and supernatant was removed and stored for ^{14}C analysis. Desorption was repeated three times, and the supernatants were combined to one solution, in which ^{14}C was analyzed. This desorption treatment with $CaCl_2$ enabled evaluating the amount of alanine being weakly bound, mainly by ion exchange. After extraction with $CaCl_2$ the same procedure was done three times with 0.5 M NaH_2PO_4 solution to extract the alanine bound by ligand-exchange. To estimate irreversibly bound alanine C, the soil was freeze-dried and combusted at 600 °C for 10 min under a constant O_2 stream with a HT 1300 solid combustion module of the multi N/C 2100 analyzer (Analytik Jena, Jena Germany). $^{14}CO_2$ released by combustion was trapped in 10 ml of 1 M NaOH. The irreversibly bound pool contains untransformed, irreversibly bound alanine C as well as macromolecular, hydrophobic or irreversibly bound microbial transformation products.

Radiochemical analyses

^{14}C activity of the supernatants was determined using a scintillation counter (Wallac 1450, MicroBeta® TriLux, PerkinElmer, Walham MA; USA) by adding 50 µl of the supernatant directly to 0.6 ml scintillation cocktail (EcoPlus, Roth Company, Germany) in transparent 24-well plates. Remaining supernatant, washing solution and desorption solution were measured in glass scintillation vials with the LS 6500 scintillation counter (LS 6500, Beckman-Coulter, Krefeld, Germany) with a 1:2 ratio of solution to scintillation cocktail and a 1:8 ratio for the $CaCl_2$ and NaH_2PO_4 solutions. ^{14}C activity in the NaOH solution was measured with a 1:2 ratio of sample to scintillation cocktail after 24 h of dark storage after disappearance of chemoluminescence. All measurements with the LS 6500 were also performed with blanks of the respective solutions ($CaCl_2$, NaH_2PO_4 or NaOH) and background corrected by subtracting this value from each measurement result.

Calculation of the kinetics of alanine utilization

To calculate the biotic utilization the amount of sorbed alanine C has to be subtracted from the total removal from soil suspension. Therefore, the decrease in ^{14}C activity in the supernatant of the fully inhibited treatment ($A_{\%}(t)$ in percent of added activity) was fitted to an exponential equation (Fig. 1) where B (% of added activity) and c (1/h) are the fitted parameters and D_{equ} is the remaining percentage of activity in the supernatant at equilibrium. The remaining activity D_{equ} was converted into the amounts of sorbed alanine per g soil (S_{equ} in µmol g^{-1}) and the dissolved alanine concentration c_{equ} (µM) were calculated and all five concentration treatments were fitted by a linearized Freundlich sorption isotherm with the sorption affinity constants k and n (Fig. 1). Based on Fischer and Kuzyakov (2010), the fitted sorption isotherm was used to calculate the sorbed amount of alanine (S_{equ} in µmol g^{-1}) at different concentration (c_{equ}) in the supernatant .

The biotic alanine C utilization $U_{bio}(t)$ (µmol) per well was calculated by subtracting the sorbed amount of alanine S_{equ} (µmol) from the amount of alanine $A_{used}(t)$ (µmol) removed from the supernatant (Fig. 1). For those concentration treatments in which nearly all of the added alanine was biologically used after 36 h (0.5 µM to 500 µM), biotic utilization $U_{bio}(t)$ was fitted by an exponential model (Fig. 1) which is approaching the total amount of added alanine G_{add} (µmol) after 36 h. The fitted parameters F (µmol) and q (h^{-1}) are the amount of initial utilization (µmol) and the rate of biotic utilization (h^{-1}), respectively. This equation was used to linearize the measured data for statistical tests.

For the whole concentration range, curves for the reaction kinetics v at a distinct substrate concentration (S_0+S_{add}: alanine concentration derived from soil solution+alanine concentration from the addition of labeling solution) were fitted to the initial rate of biotic utilization q (e.g. the slope of the function $U_{bio}(t)$ at an initial time point). The extracellular processes showed saturation kinetics according to Michaelis-Menten.

Thus, the Michaelis-Menten constant K_m, the maxima rate V_{max} and the alanine concentration S_0 (μM) of the soil was calculated from the results of curve fitting (Eq. 1).

$$v = \frac{V_{max} \cdot (S_{add} + S_0)}{K_m + (S_{add} + S_0)} \quad (1)$$

The curves for cellular uptake showed no saturation. Thus, their Eq. (2) contains an additional linear component expressed by the linear utilization rate constant L, as observed by Jones and Hodge (1999). Equations were linearized as proposed by Hobbie and Hobbie (2012).

$$v = \frac{V_{max} \cdot (S_{add} + S_0)}{K_m + (S_{add} + S_0)} + L \cdot (S_{add} + S_0) \quad (2)$$

Calculation of the distribution of alanine-C in transformation products

For the second part of the experiment, the desorption experiment, the distribution of the remaining alanine C in the soil suspension after 36 h was determined. Alanine C in the various bound fractions ($CaCl_2$-extractable, NaH_2PO_4-extractable, irreversibly bound) and dissolved as well as decomposed alanine C was calculated as relative percentage of the added ^{14}C activity.

For a presentation of transformation specifics of C from individual molecule positions, the Divergence Index DI_i was defined:

$$DI_i = \frac{n \cdot [C_i]}{\sum_{i=1}^{i=n} [C_i]} = \frac{3 \cdot [Ala_i]}{\sum_{1}^{3} [Ala_i]} \quad (3)$$

This index shows the fate of individual C atoms from the position i within a transformation process relative to the mean transformation of the n total number of C atoms in the substance. Thus, a DI_i of 1 means that the transformation of this C atom, e.g. Ala_1 positon, in the investigated pool corresponds to that of uniformly labeled substance (average of all C atoms e.g. $Ala_1 + Ala_2 + Ala_3$). The DI_i ranges from 0 to n, and values between 0 and 1 reflect reduced incorporation of the C into the investigated pool, whereas values between 1 and n show increased incorporation of the C atom into this pool as compared to the average. This index is not dependent on absolute amounts or proportions of the substance used in individual processes. Therefore, it enables comparing the distribution of individual alanine C atoms over the whole range of investigated concentrations.

Statistics

All experiments were done with six replications, and the values on figures and in tables present a mean±standard error of mean (± SEM). SEM of divergence index was calculated by Gaussian error propagation (Gottwald 2000). Measured variables were screened for outliers using the Nalimov test (Gottwald 2000) and tested for normal distribution using the Kolmogorov Smirnoff test. Less than 1 % of the values were excluded as outliers. Nested ANOVA, with the factor C position nested within the factor inhibition treatment, and nonlinear curve fitting were done using Statistica (version 7.0, Statsoft GmbH, Hamburg, Germany). ANOVA of the divergence index was calculated by a procedure proposed by Cohen (2002) from means and standard deviations. Nonlinear curve fitting of the Michaelis Menten equations was done minimizing least-squares with the nonlinear estimation tool of Statistica based on a Levenberg-Marquardt algorithm. Tests of regressions for linearity and significant differences of linear regression parameters were performed by GraphPad Prism (version 4.01, GraphPad Software Inc, San Diego CA, USA). Linear regressions were tested for deviation from linearity by Steven's Runs Test and significant differences between regression lines were identified by covariance analyses (ANCOVA) according to Zar (1984).

Results

Evaluation of results quality

We evaluated four quality aspects of the experimental data. The first approach tested for ^{14}C losses by calculating the tracer budget of the full inhibition treatments. For the five concentrations the sum of irreversibly bound, extractable and dissolved ^{14}C activity was between 90 and 97 % of the added ^{14}C activity. This high tracer recovery enables the calculation of respired alanine C in the treatments with biotic activity based on the difference between the added and recovered ^{14}C activity.

The second approach checked for sterilization efficiency of the $HgCl_2$+NaN_3 – solution based on the sorption kinetics in the soil with full inhibition: Sorption occurred completely within the first hour. Within the further 35 h the amount of alanine C in the soil suspension remained constant without any position-specific differences (Supplementary, Fig. A1 shows the example of 50 μM treatment). Thus, no respiration of alanine occurred in the fully inhibited soil.

In a third approach sterilization efficiency of the respiration-inhibited treatment with NaN_3 was tested by the dehydrogenase substrate tetrazoliumchlorid (Kvasnikov et al. 1974; Mohammadzadeh et al. 2006): after 36 h under identical incubation conditions only the non-inhibited treatment showed a clear red precipitation at the bottom of the well. Much longer incubation time was needed (> 3–5 days) until first precipitates could be observed in the respiration-inhibited soil.

The fourth approach evaluated the quality of position-specific data and was based on comparing the mean of the ^{14}C activity in three positions with the ^{14}C activity in uniformly labelled alanine. For the fitted curves of sorption and of biotic utilization (Fig. 2), no significant differences between uniformly labelled alanine and the mean of the three isotopomers could be detected.

Sorption of alanine to the soil matrix

For the treatment with full inhibition the amount of bound alanine at each concentration was calculated and an exponential curve was fitted to these data. Equilibrium of sorption (95 % of fitted line of sorption at equilibrium) was reached within 1 h. The linearized Freundlich Isotherms (Supplementary, Fig A2) showed no significant deviation from linearity based on Stevens Runs Test. ANCOVA of the fitted regression parameters of the linearized Freundlich data

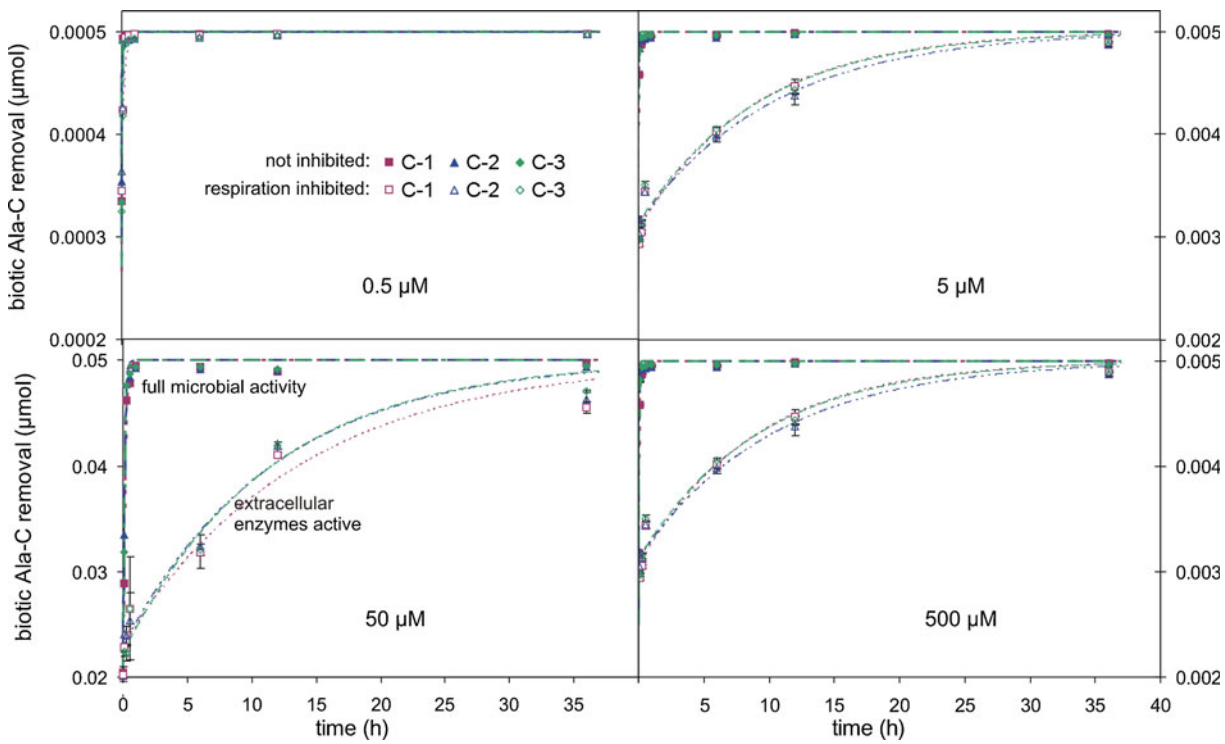

Fig. 2 Removal of alanine from soil solution by extra- and intracellular processes without inhibition (*filled symbols*) and by extracellular transformation in respiration-inhibited treatments (*open symbols*) depending on alanine concentration. Experimental points (means ± SEM, N=6) and fitted curves based on the exponential utilization model (see Fig. 1) are presented. Removal of alanine from soil solution is identical for treatments with low concentrations (lines for C-1, C-2 and C-3 overlap) and starts to differentiate for concentrations higher 50 μM

showed no differences between the individual C positions and U-labelled alanine for the slopes (p=0.9991) and the intercepts (p=0.9997). Thus, data of isotopomers and U-alanine were pooled and the affinity constants were determined by curve fitting on the entire dataset: k=0.002 and n=0.965.

The second part of the experiment revealed the extractability of the bound alanine. The sorbed portion ranged from 3 to 6 % of the added alanine (Fig. 3). Less than 7.3 % of the totally sorbed alanine was adsorbed irreversibly to the soil matrix (<0.4 % of the added alanine), and only a portion of <11.70 % could be extracted with NaH_2PO_4 (<0.7 % of the totally sorbed alanine). 83–90 % of the totally sorbed alanine were extracted with $CaCl_2$ (2.5 to 5.4 % of the added alanine) (Fig. 3).

Kinetics of biotic alanine utilization

An exponential curve was fitted to the data of biotic alanine utilization (Fig. 2) for the concentration range from 0.5 to 500 μM. Curve fitting was impossible for the 5 mM treatment because the equilibrium was not reached after 36 h. The rate of biotic utilization p (equation see Fig. 1) ranged from low to high concentrations from −11.3 to −0.05 s^{-1} for the exoenzymatic and from −41.7 to −0.13 s^{-1} for the non-inhibited treatment. For lowest alanine concentration extracellular and biotic removal of alanine from the soil suspension were similiarly fast: more than 95 % of the added alanine was used within the first 5 min. In contrast, biotic alanine utilization is significantly faster than extracellular removal (p<0.001) at medium and high alanine concentrations (5–500 μM). In the 5 and 50 μM treatments, microbial uptake removed 95 % of the added alanine in less than 15 and 30 min, respectively, whereas extracellular systems needed about 1 day.

The effect of C position on alanine removal from the soil solution was tested using the linearized function of $U_{bio}(t)$. No significant difference in the removal of alanine C from the three positions by extracellular processes or total biotic utilization was detected over the 36 h of experiment duration.

The extracellular alanine transformation rate (Fig. 4) followed Michaelis-Menten kinetics for all alanine C positions (p<0.001, R^2>0.95) (Table 1). Significant differences in the kinetics of extracellular transformations of C from individual positions were identified (F=44.4, p<0.0001). The initial alanine concentration S_0 in the soil was 0.39 μM and thus in the range of the lowest amino acid concentration added.

The rate of alanine removal from soil suspension in the non-inhibited treatment followed a mixed linear and Michaelis-Menten model (p<0.001, R^2>0.99) i.e. had no saturation level within the range of amino acids concentration investigated (Fig. 4). The linear uptake rate constant L did not differ significantly between the positions (slope: F=2.45, p>0.05; intercept: F=2.76, p>0.05). In contrast at low alanine concentrations (<50 μM) the K_m values were always lowest for the carboxyl group (K_m=16.4 μM) and highest for methyl groups (384 μM), and V_{max} behaved opposite (p<0.001) (Table 1). ANCOVA revealed significantly different uptake behaviour of the individual alanine C positions (F=314; p<0.0001).

Biotic transformation products of alanine

Sequential desorption by $CaCl_2$ and NaH_2PO_4 gives first information about physico-chemical properties of alanine transformation products (Fig. 1). The extractability of alanine C significantly changed due to biotic transformation. Comparing full-inhibited treatments with those with extracellular activity revealed that - with the exception of the highest alanine concentration - extracellular transformation always caused an increase in the irreversibly bound and a decrease in the $CaCl_2$-extractable alanine C. In treatments with intracellular metabolization of alanine the increase of NaH_2PO_4-extractable alanine C is even higher. In summary, the portion of C associated with the soil matrix (in the irreversibly bound or NaH_2PO_4-extractable fraction) strongly increased after biotic transformations (Fig. 3). Note, however, that the irreversibly bound pool would contain many cellular components being pelleted out during centrifugation.

At very low concentrations (0.5–5 μM), nearly no alanine C remained in the soil solution; at medium and high concentrations, however, exoenzyms were saturated and were unable to use the whole added alanine C within 36 h (Fig. 2). Thus, significantly more alanine C remained in the supernatant if only extracellular processes used the alanine. Incomplete removal of the added alanine (>50 μM) always caused significant position-specific differences in

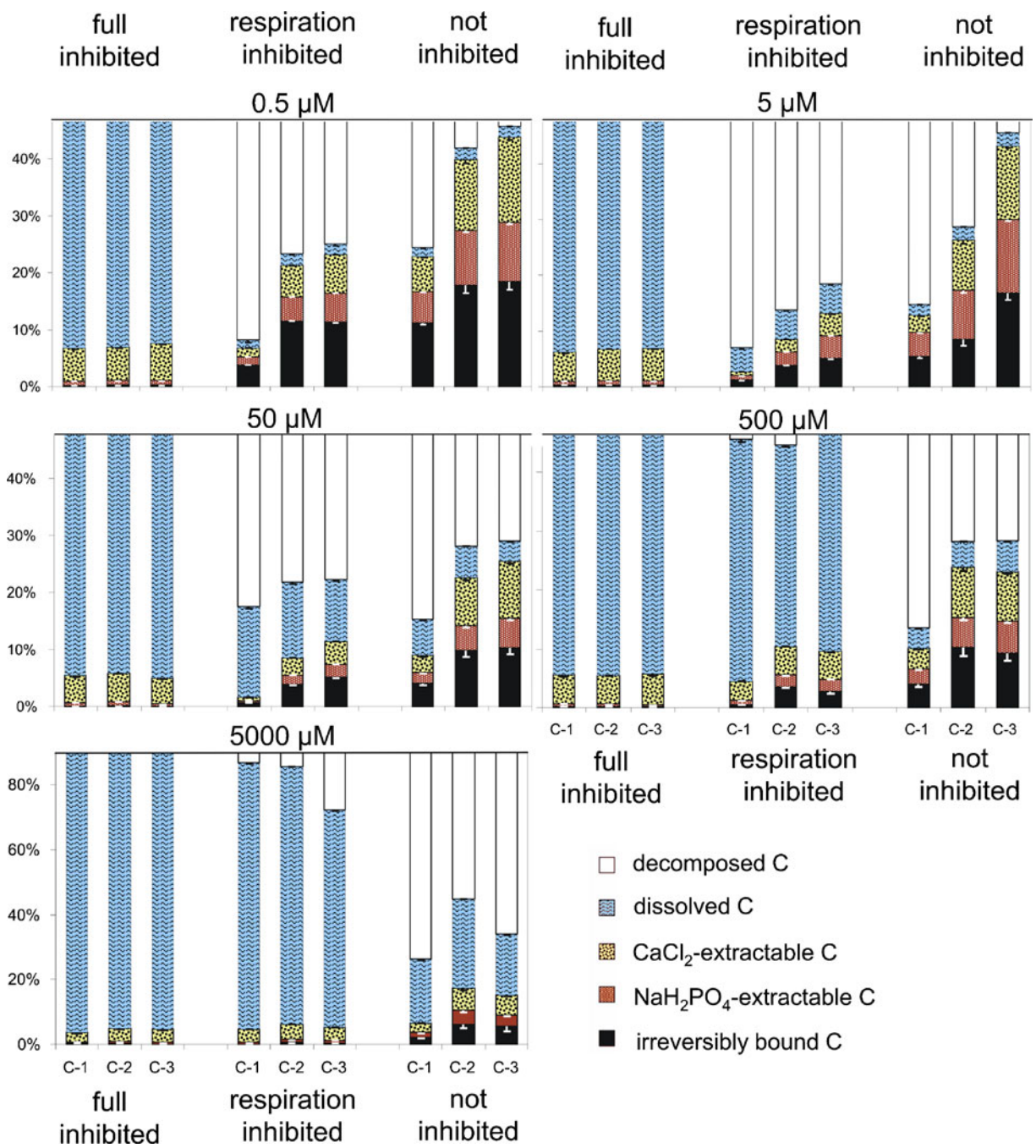

Fig. 3 Amounts of alanine C in differently bound, dissolved or degraded forms in treatments with full inhibition (*left*), treatments with respiration inhibition (*middle*) and treatments without inhibition (*right*). Values show means ± SEM (N=6) depending on alanine concentration

the dissolved alanine C (Supplementary Table A1), irrespective of the utilization mechanism. If only trace amounts of alanine remained in the soil solution (<50 µM), no significant position-specific differences were observed (Supplementary Table A1).

Position-specific differences of the alanine transformation pathways

Biotic transformations discriminated between the three C positions (Fig. 3). At all concentrations in treatments

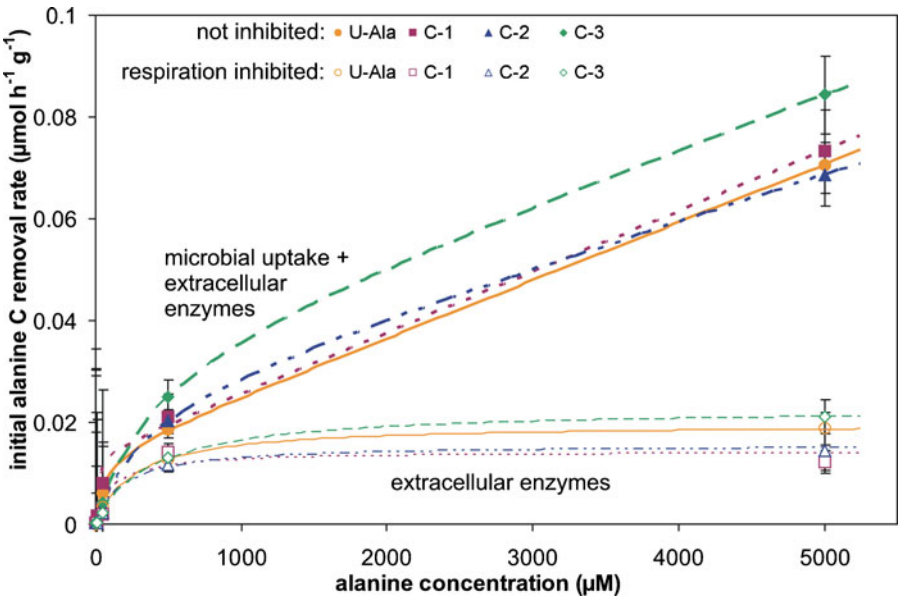

Fig. 4 Initial rate of alanine removal from soil suspension in treatment without inhibition (*filled symbols*) and treatments with respiration-inhibition (*open symbols*); Experimental points (means±SEM calculated by Gaussian error propagation, $N=6$) and fitted kinetic curves (parameters see Tab. 2) are presented

with biotic activity, the carboxyl group was preferentially decomposed to CO_2 ($p<0.001$), whereas the C-2 and C-3 positions of alanine were incorporated into various transformation products after 36 h (Fig. 3). Only in the absence of microbial uptake and metabolization the NaH_2PO_4-extractable products at 5 µM and the irreversibly bound forms at 50 µM exhibited a significantly higher portion of the C-3 versus C-2 alanine C. The methyl group was preferentially incorporated in transformation products at the 5 µM concentration in the $CaCl_2$-extractable products at 0.5 µM and in the NaH_2PO_4-extractable pool at medium alanine concentrations (50–500 µM) (Supplementary Table A1). Comparing absolute values of alanine C incorporation depending on concentration is hardly possible, because the very broad concentration range leads to a different utilization of alanine C (e.g. alanine as a growth substrate is incorporated in different absolut and relative amounts than under maintenance conditions). Thus, completely different portions of C are incorporated in the various pools (see Fig. 3: from 0.5 µM to 5 mM the dissolved alanine C pool changed from 1.2 to 8.0 %) and direct comparison of C positions is complicated. Less pronounced differences in the position-specific behavior will be lost, if percentages of alanine C allocation are compared. Instead a relative index ignoring

Table 1 Parameters of the Michaelis-Menten kinetics for treatments with inhibition of respiration (eq. 1) and treatments without inhibition (eq. 2). R^2 is the coefficient of determination and stars show significance of the respective non-linear fitting result (respectives curves are plotted in Fig. 4)

Position	Extracellular transformation			Microbial uptake and intracellular metabolization			
	K_M (µM)±SE	V_{max} (nmol h^{-1} g^{-1})±SE	R^2	K_M (µM)±SE	V_{max} (nmol h^{-1} g^{-1})±SE	L (nmol h^{-1} g^{-1})±SE	R^2
U-Ala	266.4±16.0**	19.70±0.26 **	0.9998	62.1±49.2***	14.29±3.45***	0.011±0.000***	0.9976
C-1	124.0±122.4**	14.21±2.75 *	0.9179	16.4±18.1***	13.89±3.53***	0.012±0.000***	0.9948
C-2	199.1±56.9**	15.51±0.91 **	0.9930	323.6±407.1***	25.87±16.05***	0.009±0.000***	0.9978
C-3	385.6±24.7**	22.69±0.35***	0.9996	383.9±512.3***	34.88±24.98***	0.010±0.000***	0.9981

* $p<0,05$; ** $p<0,01$; *** $p<0,001$

these absolute differences is needed to enable comparison of transformation over the investigated concentration range. The Divergence Index DI_i was calculated for degraded alanine C, $CaCl_2$-extractable, NaH_2PO_4-extractable and irreversibly bound transformation products (Fig. 5). The DI_i shows that C-1 was preferentially degraded by intracellular metabolism at all concentrations, whereas the C-2 and C-3 positions were preferentially incorporated into cellular compounds. A significant effect of concentration was observed for intracellular alanine C transformation to NaH_2PO_4-extractable and irreversibly bound products (Supplementary, Table A2): with increasing alanine concentration less splitting between the C-2 and C-3 position occurred, and with decreasing concentration the preferential C-1 decarboxylation by intracellular metabolism tended to be reduced.

In contrast, extracellular degradation showed a lower preferential decarboxylation. At the highest concentration the C-3 oxidation even exceeded the C-1 oxidation (Fig. 5). With increasing concentration a highly significant decrease in the discrimination between alanine positions for the extractable transformation products was observed. In addition a change in the preferential incorporation from C-2 to C-3 occurred with increasing concentrations (Fig. 5).

Discussion

Sorption of alanine occurs as a whole molecule

The goodness of fit of the Freundlich isotherms shows that the sorption capacity of a loamy soil for alanine has no limit within naturally occurring concentrations. Sorption of the dipolar ion alanine can occur by ion exchange via the amino group, ligand exchange via the carboxyl group and hydrophobic interactions via the methyl group and may explain the observed non-saturable Freundlich isotherm. Sequential desorption is an attempt to differentiate these mechanisms. As $CaCl_2$ is a very potent cation exchange reagent, it is likely that the majority of the alanine molecules were bound by ion exchange (83–90 %), i.e. via the positively charged amino group.

Wang and Huang (2003) observed abiotic degradation of amino acids by the interaction with mineral phases. In their experiment, the abiotic oxidations of

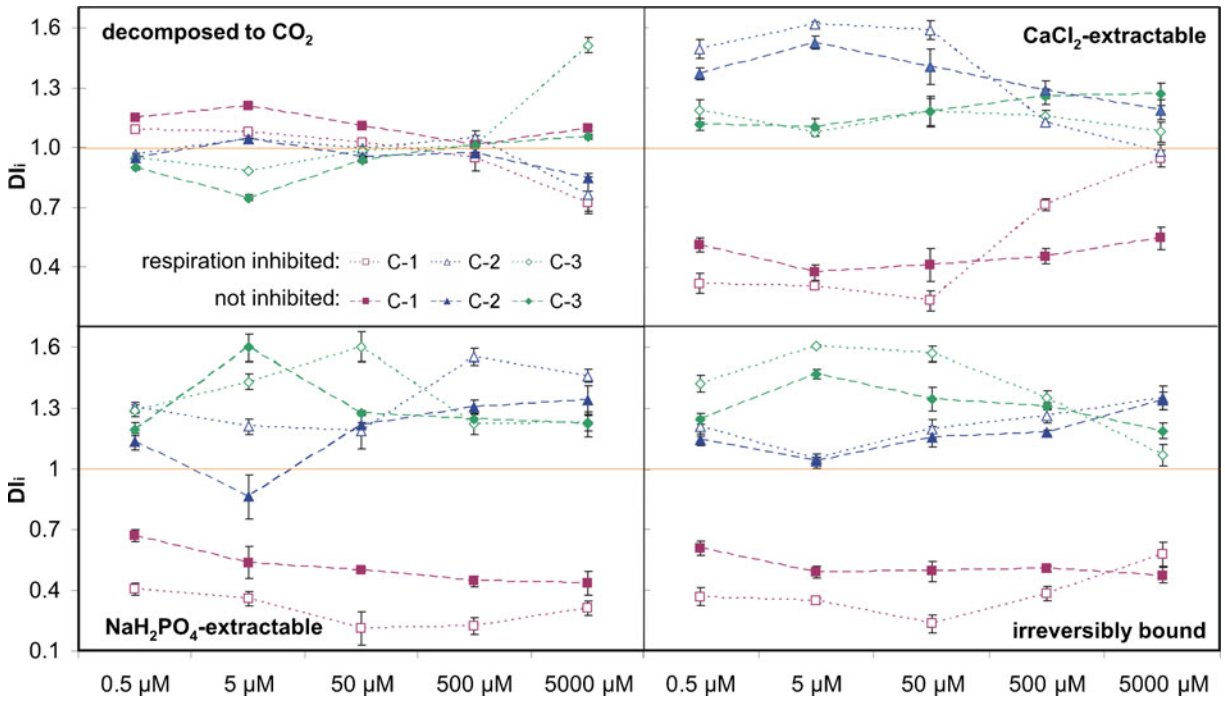

Fig. 5 Concentration-dependent position-specific transformation index DI_i (N=6, ± SEM) of alanine C positions for treatments without inhibition (*filled symbols*) and treatments with respiration inhibition (*open symbols*) for four pools. The SEMs were calculated by Gaussian error propagation

glycine showed highly significant position-specific differences with a preferential decarboxylation of the carboxyl group (Wang and Huang 2003, 2005). We did not observe this in our treatments with full inhibition, neither for the sorption isotherms nor for the desorbed transformation products. Thus, we conclude that sorption of the intact alanine C skeleton occurred. Theoretically, the used ^{14}C labeling did not enable to observe deamination of amino acids. However, deamination is very unlikely as the majority of the sorbed alanine is $CaCl_2$-extractable and thus, is mainly bound by cation exchange via the amino group. Thus, we conclude that abiotic cleavage of amino acids is of minor relevance. We explain this lack of abiotic molecule splitting by the short incubation time of our experiment and the physico-chemical differences of the investigated soils compared to the subsoils used by Wang and Huang (2003).

Kinetics of extracellular transformation and microbial uptake

In general, differentiation between extra- and intracellular metabolization in soil can either be performed by using selective inhibition of the exoenzymes (Martens and Frankenberger 1993) or by using selective sterilization that inhibits intracellular metabolism (Gibson and Burns 1977; Hope and Burns 1987). As a broad set of enzymes is available to transform alanine (a very common substrate), we used the inhibition of the active microbial cells. We tried to reduce artifacts of the inhibition (e.g. remaining activity in dead cells or continuing fermentation processes) by a pre-incubation with double concentrated NaN_3 and high oxygen supply (Burns 1982). The lack of visible red crystals after 36 h confirms that the inhibition of intracellular processes was successful. Thus, we conclude that extracellular enzymes which were stabilized for more than the 1 h of preincubation in the soil dominated the alanine removal from soil suspension. Extracellular kinetics as well as transformation products differed significantly from those formed by intracellular metabolism (Fig. 3). This confirms that remaining intracellular activity is of minor importance after NaN_3 inhibition. The observed Michaelis-Menten constants are in a similar range than values observed for other exoenzymes (Braun et al. 1992).

Under medium and high concentrations, microbial uptake systems were much faster than extracellular enzymes (Figs. 2 and 4). Rate of alanine utilization resembled removal of an amino acid mixture observed by Rousk and Jones (2010): they observed a loss of 90 % of ^{14}C activity within the first hour of incubation. At low concentrations this microbial alanine uptake also follows a Michaelis-Menten kinetics (Vinolas et al. 2001a). The linear kinetic term of microbial uptake at higher concentrations is similar to the kinetics observed by Vinolas et al. (2001a). These combined kinetics reflect different types of microbial uptake and enzyme systems involved in alanine utilization (Piperno and Oxender 1968; Anraku 1980): 1) high-affinity, energy-dependent active uptake systems at low concentrations and 2) additional uptake mechanisms with linear non-saturable kinetics at high substrate concentrations. The non-saturable kinetics showed identical behavior of all C positions. This experimentally confirmed the assumption of Jones and Hodge (1999) that the non-saturable kinetics is based on uptake by permeases or ion channels which do not split the molecule. In contrast, the processes that follow Michaelis-Menten kinetics revealed individual position-specific kinetics (Fig. 4). For extracellular enzymes this is a result of alanine splitting processes like decarboxylation. However, alanine uptake into cells also revealed molecule splitting. This can either be uptake of fragments or the uptaken alanine was split and fragments were secreted. Both possible processes can not be distinguished by our approach.

To summarize, C-1 decarboxylation by extracellular enzymes as well as C-1 removal by cellular uptake are the fastest biotic processes under low alanine concentrations. At medium and high alanine concentration microbial cells take up alanine without splitting by unsaturated enzyme systems. Thus, the position-specific transformations revealed that multiphase kinetics of LMWOS uptake by microbial cells reflect a change of the underlying biochemical processes and thus the fate of LMWOS depending on their concentration in soil.

Exoenzymatic transformation products

We know little about the exoenzymatic transformation of amino acids because the tacit assumption is that LMWOS are completely taken up into microbial cells. It was shown that some organisms such as *Cellulomonas cellulans*, *Corynebacterium* or *Proteus rettgeri* produce extracellular amino acid oxidase with broad substrate specificity (Braun et al. 1992). These enzymes catalyze the oxidative deamination of alanine, forming pyruvate, which could be decarboxylated in further steps. This

might e.g. be done by unspecific decarboxylation by manganese peroxidase (Hofrichter et al. 1998). Exact mechanisms cannot be identified without analysis of the enzymatic products, but it can be shown that the discrimination between C-2 and C-3 of the exchangeable products is higher compared to microbial metabolites (Fig. 5). This might indicate a stepwise oxidation reaction from C-1 to C-3, at least at low alanine concentrations.

At the two highest concentrations, alanine was not completely removed from the solution (Fig. 2) and thus, discrimination between C positions decreased due to an increasing portion of untransformed alanine (Fig. 5). This saturation of exoenzymes at high concentrations supports the hypothesis that mainly unspecific oxidizing enzymes are involved. The desorption experiment showed that a significant part of the not-extractable products are formed after exoenzymatic transformation. This is probably a first step of extracellular amino acid C stabilization in soil. Further metabolite tracing of the transformation products is needed to understand LMWOS stabilization mechanisms in soil.

Extracellular pathways were less important because active microorganisms take up LMWOS much faster (Fig. 4 and Table 1). Nonetheless, these pathways may explain transformation in aggregate cores or micropores, where microbial cells are size-excluded (von Luetzow et al. 2006). Thus, these transformation pathways are expected to take place parallel to microbial uptake and metabolization. However, if a potential overestimation of extracellular activity due to insufficient microbial inhibition by NaN_3 is considered, the extracellular transformation of alanine is of minor importance for microbially active soil.

Metabolic pathways and their intracellular transformation products

Intracellular metabolism products showed a strong increase of irreversibly bound and ligand exchangeable substances (Fig. 3). Non-extractable products were expected to be either macromolecular or hydrophobic e.g. proteins, peptidoglycan or lipids: they are not extractable in polar reagents with low salt molarity. Ligand-exchangeable transformation products are characterized by either hydroxyl or more probably carboxyl groups (Gu et al. 1994) like alcohols and mono- or poly-carboxylic acids.

Direct decarboxylation of alanine, producing amine, has been described only for cucumber and tea plants (Takeo 1978), and no evidence for this reaction within microorganisms is available. In contrast, the oxidation of the C-1 group was most likely caused by the fundamental alanine degradation pathway of prokaryotes under aerobic conditions: the oxidative deamination to pyruvate by alanine dehydrogenase (Caspi et al. 2008; Keseler et al. 2009; Gottschalk 1986). This reaction, shown in Fig. 6 (first arrow down from alanine), decouples the C and N fate in the microbial metabolism (Knowles et al. 2010). Additional pathways of alanine utilization like transamination or oxidoreduction also lead to the formation of pyruvate (Caspi et al. 2008; Keseler et al. 2009; Gottschalk 1986). Pyruvate as the main alanine transformation product allows a qualitative metabolic tracing approach (Dijkstra et al. 2011a). Citric acid cycle leading to the oxidation of the C-1 position by pyruvate dehydrogenase can be distinguished from alternative C utilization pathways like protein biosynthesis or gluconeogenesis (Fig. 6, arrows branching left from the main pathway), which commonly use the entire C skeleton of alanine (Keseler et al. 2009; Caspi et al. 2008).

In general for all concentrations, only a minor part of the C-1 group is incorporated into newly formed cellular compounds (Figs. 3 and 5). This reveals citric acid cycle as the major pathway of alanine i.e. pyruvate C metabolization (Fig. 6). Further cycling of the C skeleton through the citric acid cycle led to a partial oxidation of the C-2 position, which is maximized under medium alanine concentrations (Fig. 5).

At lowest alanine concentration the incorporation of C-1 slightly increased in the extractable transformation products (Fig. 5) and significantly increased in irreversibly bound microbial products (Supplementary, Table A2). This might reflect the slight increase of anapleurotic carboxylation pathways, protein biosynthesis or gluconeogenesis with increasing C deficiency (Dijkstra et al. 2011a). For these pathways the C skeleton of alanine needn't be split (Fig. 6, left). Which of these pathways is driven by microbial anabolism can be clarified only if transformation products (e.g. amino sugars or acids) are investigated compound-specifically.

At high alanine concentrations we experimentally simulated growth conditions like representative for soil hot-spots: the microbial community was not energy deficient because oxidative deamination of alanine along with decarboxylation of pyruvate to Acetyl-CoA are energy-supplying reactions producing ATP (Caspi

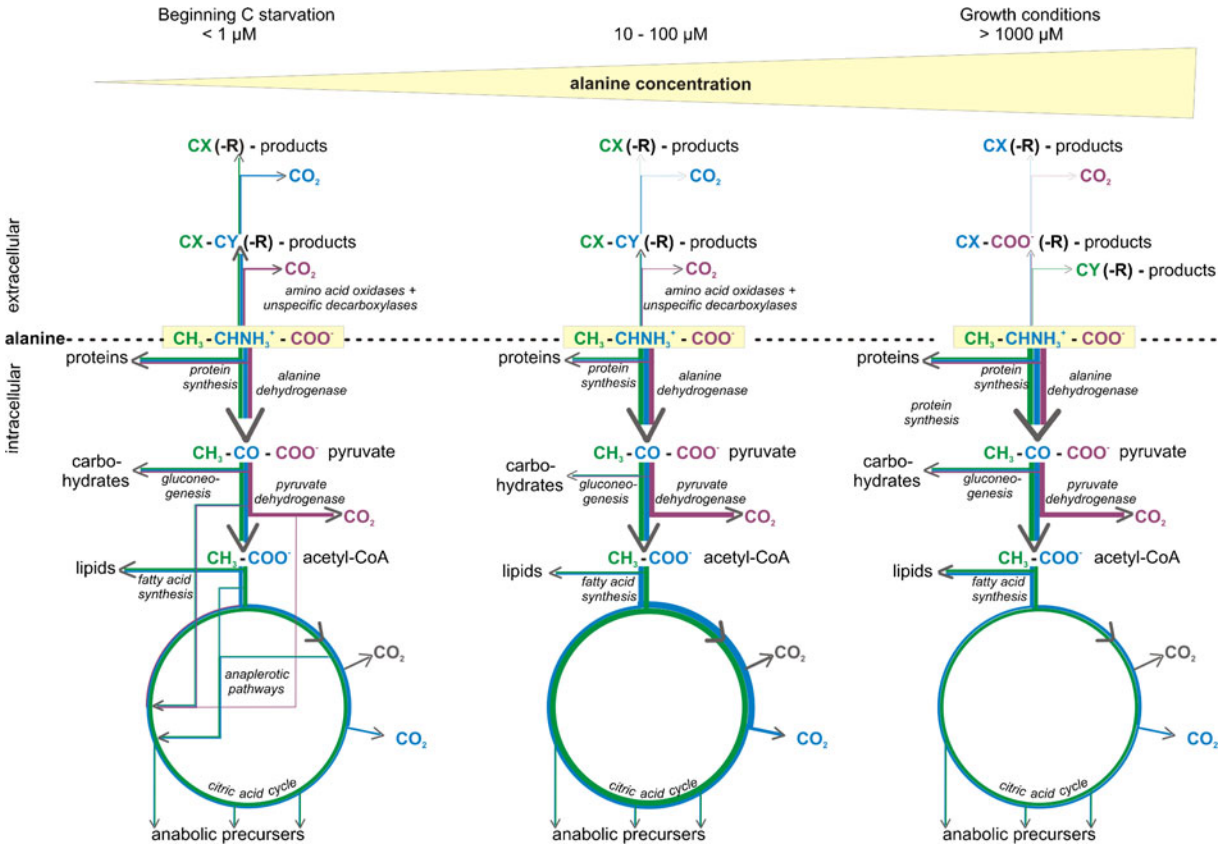

Fig. 6 General biochemical pathways of extra- and intracellular amino acids transformation in soil in dependence on alanine availability. *Line width* represents the qualitatively estimated relative shifts of alanine C between certain pathways dependent on the alanine concentration

et al. 2008; Keseler et al. 2009). Thus, C and energy were available in excess and allowed microbial growth, at least for a part of the microbial community, presumably bacterial groups (Rousk and Baath 2011). A high portion of C-2 and C-3 positions was found in irreversibly bound, cellular products under these conditions (Fig. 5). Biosyntheses pathways common for microbial growth like fatty acid synthesis start with Acetyl-CoA (Fig. 6, arrow to left) and do not split this C-2-C-3-fragment (Keseler et al. 2009; Caspi et al. 2008). Thus, such growth pathways produce not-extractable, macromolecular or hydrophobic compounds containing the C-2 and C-3 positions (Fig. 6, right). Hence, an increased incorporation by biosynthesis would explain the changes of alanine transformation under high C supply (Fig. 6).

To summarize, position-specific labeling enables to trace changes in microbial substrate metabolization due to changes in C availability: from starvation over maintenance and growth concentrations a shift in the microbial pathways (Fig. 6) leading to formation of different cellular compounds from alanine C could be observed in this study.

Conclusion and outlook

Next to multiple isotope labeling (Knowles et al. 2010) the position-specific labeling is a preeminent tool to identify and trace the major pathways of LMWOS transformation in soil (Dijkstra et al. 2011a, b). This study demonstrates that position-specific labeling provides a new insight into amino acid transformation on a submolecular level. Cellular uptake always outcompetes sorption and extracellular transformations, which are quantitatively relevant only at very low alanine concentrations or in specific microhabitats and occurs as a stepwise extracellular oxidation. In general, however,

cellular uptake and metabolization dominate the fate of alanine C in soil. Two mechanisms underlying the microbial uptake kinetic were identified: an unsaturable unspecific uptake of intact alanine at hot-spot concentrations and specific uptake mechanisms at low alanine concentrations. In addition, this tool also enabled us to detect minor changes of the intracellular alanine metabolization, which were a result of the switch from anabolic pathways characteristic for C deficiency to those common for growing cells. However, without a quantitative detection of the metabolic products this assay remains qualitative. Coupling this sensitive, submolecularly operating technique with compound-specific isotope analysis of the transformation products is the next step to shed light on the black box of C transformations in soil. As opposed to closer examination of transformation pathways, generalization (by further compounds, compound classes and environmental conditions) and upscaling are the future demands: Once, general principles controlling LMWOS metabolization and the effects of environmental conditions are identified, the fate of C entering the soil can be determined based on its chemical structure. A detailed understanding of the general principles of LMWOS transformation, the used pathways and the regulating factors is crucial to understand and predict the SOC dynamics under changing environmental conditions.

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