1 Title: Dissipation kinetics of asparagine in soil measured by compound-specific analysis with metabolite tracking.

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8
9 Abstract: Estimating the potential for direct plant acquisition of organic N , in particular amino acids, requires assessment of
10 their turnover times in soil. It is well known from ¹⁴C-studies that mineralization of amino acids occurs within hours, but
11 mineralization to ¹⁴CO₂ does not indicate the rate of disappearance of the intact amino acid or the possible formation of
12 metabolites during amino acid dissipation. We here used compound-specific isotope analysis with metabolite tracking to
13 investigate the dissipation rate of universally labeled intact ¹³C¹⁵N-asparagine at two concentrations and the subsequent
14 formation of compounds closely related to ¹³C¹⁵N-asparagine metabolism over three hours in agricultural soil. ¹³C¹⁵N-
15 asparagine was rapidly dissipated with half-lives of 55 and 231 minutes for the low and high ¹³C¹⁵N-asparagine
16 concentrations used. Universally labeled aspartic acid and interestingly also universally labeled glutamic acid were detected
17 in soil.. This highlights the fast turn-over of amino acid in soil and that the estimation of concentration of the formed
18 compounds is important when evaluating plant available organic N Efficiency of the compound-specific analysis
19 showed to be a powerful technique in following the pathway of amino acids in soil.

20 Keywords

21 Amino acid, Dissipation kinetics, Asparagine metabolites, Stable isotope application

Plants can absorb a wide array of organic N compounds in soil, ranging from amino acids to proteins (Ohlund and Nasholm 2004; Paungfoo-Lonhienne et al. 2008; Warren and Adams 2007). Among all organic N compounds amino acids are the most studied. In soil solution, amino acid concentrations range from 0.1 to 500 μM (van Hees et al. 2005) with the actual availability affected by production rates, adsorption to soil particles and uptake by microorganisms and plants. Microorganisms largely determine the fate of amino acids due to rapid mineralization to CO_2 or assimilation into cellular compounds. These transformations occur within minutes (Hill et al. 2012) to hours and to assess what fraction of amino acid is plant available, knowledge on kinetics of intact amino acid loss from soil is required.

Choosing an appropriate methodology to measure dissipation rate is important. Often degradation kinetics are estimated from CO_2 evolution after addition of ^{14}C -labeled compounds (Farrell et al. 2014a; Jones and Hodge 1999; Jones et al. 2009; Kielland et al. 2007), but mineralization of a ^{14}C -labeled compound into $^{14}\text{CO}_2$ underestimates the actual disappearance rate of the compound (Jones et al. 2005). Likewise, retention of CO_2 as bicarbonate (HCO_3^-) in soils with pH above 6.5 is another source of underestimation in ^{14}C -mineralization studies (Pepper et al. 1995). Glanville et al. (2016) also pointed out that $^{14}\text{CO}_2$ evolution is a poor estimate of substrate disappearance partly due to ^{14}C from the added compound is used for both anabolic and catabolic processes. Thus, the direct detection of molecules e.g. by gas- or liquid chromatography mass spectrometry (GC- or LC-MS) provides clear evidence for intact substrate dissipation from soil and simultaneously enables onward tracking of its transformation products. During transformations (deamination, transamination, decarboxylation) of some amino acids (e.g. asparagine), other compounds may be formed (e.g. aspartic acid). Therefore, integrated information on the disappearance rate of intact amino acid and formation of its transformation products in soil helps to obtain realistic estimates of what is available to the plant at certain time. Yet, very few studies traced intact amino acids in soil using compound-specific analysis, and even fewer tracked their transformation products (Geisseler and Horwath 2014). In the present study we investigated the dissipation rate of intact asparagine (Asn), a major transport molecule of N in plants (Lea et al. 2007) and the dominant amino acid in clover roots (Lesuffleur et al. 2007), using labeled $^{13}\text{C}_4^{15}\text{N}_2$ -Asn at two concentrations, 100 and 1000 μM , in agricultural soil over three hours. We also measured the formation of labeled Asn transformation products, aspartic acid (Asp), glutamine (Gln), glutamic acid (Glu), and ammonium (NH_4^+). The 100 μM treatment, although exceeded the concentration reported for the bulk soil (Warren 2014), was in the range of concentration found in the rhizosphere (van Hees et al. 2005). The high 1000 μM treatment was included to obtain higher sensitivity in detecting transformation products. Soil used in the study (sandy loam) was

collected in April, 2014. Soil was taken from eight different blocks from the A horizon (5-15 cm) at the CENTS (explain) long-term field experiment located in Foulum, Denmark (56°30' N, 9°35' E), and stored at 4°C. The properties of the soil were the following: water holding capacity 34.4%, pH 6.1, bulk density 1.25 g cm⁻³, total C 18 g kg⁻¹, total N 1.6 g kg⁻¹, organic matter 3.5%. (Chatskikh and Olesen 2007; Hansen et al. 2011). The full methodological details are provided in Online Resource 1. Briefly, 4.2 µl (690.3 µg ml⁻¹) and 5.5 µl (5225 µg ml⁻¹) of ¹³C₄¹⁵N₂-Asn water solution was added dropwise per 1 g of dry soil and mixed to obtain final concentration of 100 µM (20.8 µmol kg⁻¹ soil DW) and 1000 µM (208 µmol kg⁻¹ soil DW), respectively. Then, soil was incubated at room temperature (22°C) for 0, 10, 20, 30, 40, 50, 60, 90, 110, 140 and 180 minutes. At each concentration treatment, there were five replicates (n=5) per time point. Soil was sampled for immediate extraction following the procedure of Inselsbacher (2014). To each tube with soil 30 ml of 1 M KCl was added, containing norvaline (Sigma Aldrich, Denmark) as an internal standard (0.125 pmol µl⁻¹). Tubes were shaken for 60 min at 4°C on shaker (Intelli Mixer, In Vitro, Denmark) at 99 rpm. Then the tubes were centrifuged (Heraeus, Axeb, Denmark) at 4 600 g for 10 min at 4°C. From each of the soil extracts, 1000 µl was filtered using PTFE syringe filters (0.22 µm) (Mikrolab Aarhus A/S, Denmark) and transferred to LC-MS vials. The extracts were then derivatized with the AccQ•Tag Ultra Derivatization Kit (Waters Corp.) according to the manufacturer's protocol. The derivatized samples were analyzed for the presence of ¹³C₄¹⁵N₂-Asn, ¹³C₄¹⁵N-Asp, ¹⁵N-Gln, ¹³C₅¹⁵N-Glu and ¹⁵NH₄⁺ by HPLC (Agilent) coupled to 4500 QTRAP (ABSciex) using electrospray ionization (ESI) in positive mode with a voltage of 4500 eV. The acquisition method was based on Czaban et al. (2016) developed on basis of Armenta et al. (2010) and Johansson et al. (unpublished). The derivatized amino acids were separated on a Waters Xbridge C18 column (2.1 mm x 150 mm). The following separation gradients were used: 0–0.54 min (99.9% A), 5.50 min (90.9% A), 7.70 min (78.8% A), 8.50–9.00 min (40.4% A), 9.50–10.00 min (20.0% A), and 10.50–15.00 min (99.9% A). Eluent A consisted of Milli-Q water and 0.1% formic acid, eluent B consisted of acetonitrile and 0.1% formic acid, and the column flow rate was 0.5 ml min⁻¹. The column temperature was set to 55°C, and the sample injection volume was 1 µl. Mass spectrometric detection was performed in multiple reaction mode (MRM). Retention times and MRM transitions (Q1/Q3) are listed in Online Resource 1. The compounds of interest were analyzed using Sciex Analyst 1.6.2 software. The presence of Asn, Asp, Gln, Glu, ¹³C₄¹⁵N₂-Asn, ¹³C₄¹⁵N-Asp, ¹⁵N-Gln, ¹³C₅¹⁵N-Glu and ¹⁵NH₄ in soil samples was confirmed by comparison of retention times and MRM transitions with reference standard compounds: Asn (99%), Asp (98%), Gln (99%), Glu (99%), ¹³C₄¹⁵N₂-Asn (95%), ¹³C₄¹⁵N-Asp (95%), ¹⁵N-Gln (95%), ¹³C₅¹⁵N-Glu (95%), NH₄Cl (99%) (Sigma-Aldrich, Denmark). The calibration curves of the unlabeled

standards – Asn, Asp, Gln, Glu, and NH_4 – were prepared from authentic standard compounds in the range of 0.001 – 5 pmol μl^{-1} (≈ 0.07 – 70 $\mu\text{mol kg}^{-1}$ soil DW). The peak area of each standard was divided by the peak area of the internal standard (norvaline). The normalized peak area of the standards was plotted against the standard concentration that was entered in the column. A linear function was applied to the calibration curves. To determine the concentrations of the amino acids and ammonium in the samples, the peak area was normalized using the peak area of norvaline and calculated using the regression equations from the calibration of the standards. The minimum level at which each of the amino acids could be reliably quantified was determined by the limit of detection (LOD) (Online Resource 1). Based on the background noise level, the LOD was defined as the concentration of the analyte that generated a signal equal to two times the background noise. Recovery experiments were performed to evaluate the efficiency and the reproducibility of the analytical method. . For the recovery of Asn, Asp, Gln, Glu, 4.4 g of soil was adjusted to 50% of WHC and spiked with low and high concentration of the respective amino acid standard. For the low concentration 5.125 μl of each of the Asn, Asp, Gln, Glu water stock solution (100 ppm) was spiked per 1 g of soil DW to obtain final concentration of 0.05 pmol μl^{-1} (≈ 3.5 $\mu\text{mol kg}^{-1}$ soil DW). For the high concentration 125 μl of each of the Asn, Asp, Gln, Glu water stock solution (100 ppm) was spiked per 1 g of soil DW to obtain final concentration of 1 pmol μl^{-1} (≈ 70 $\mu\text{mol kg}^{-1}$ soil DW). For the recovery of $^{15}\text{NH}_4$, 4.4 g of soil was adjusted to 50% of WHC and 26.2 μl of the NH_4Cl water stock solution (1000 ppm) was spiked on soil to obtain concentration of 1.8 pmol μl^{-1} (≈ 126 $\mu\text{mol kg}^{-1}$ soil DW). The concentration of each analyte used for spiking the soil was calculated previously and depends on the approximate concentration known to occur naturally in the soil samples. Recovery experiments were prepared in five replicates. Spiked soil samples were extracted and analyzed as described before. The analyte recovered was calculated by subtracting the analyte concentration in a blank soil (water spiked soil) from the analyte concentration in a spiked soil and then the analyte recovered was calculated as percentage of analyte added (Online Resource 1). No $^{13}\text{C}_4^{15}\text{N}_2$ -Asn, $^{13}\text{C}_4^{15}\text{N}$ -Asp, ^{15}N -Glu, $^{13}\text{C}_5^{15}\text{N}$ -G and $^{15}\text{NH}_4$ were detected in the control soil, therefore, no corrections for the naturally abundant labeled compounds were made when calculating the dissipation kinetics of $^{13}\text{C}_4^{15}\text{N}_2$ -Asn and the resulting concentrations of transformation products. Three different kinetic models (Double First Order in Parallel, DFOP; First Order Multi Compartment, FOMC and Single First Order, SFO, Online Resource 1) (Etzerodt et al. 2008) were tested to determine the $^{13}\text{C}_4^{15}\text{N}_2$ -Asn dissipation (RStudio software version 0.99.46) and DT_{50} calculated from the rate constant, k , from the SFO model .

104 The concentration of $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ in soil decreased with time both at the low (100 μM) and high (1000 μM) concentration
 105 treatments (Fig. 1a and c). No lag-phase was observed, which indicates that $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ was continuously degraded; with
 106 the concurrent increasing concentration of $^{15}\text{NH}_4^+$ (Fig. 1b and d). Goodness of fit of the kinetic models was evaluated by
 107 inspection of the curve, residuals, degrees of freedom (DF) adjusted R^2 , and confidence intervals (Fig. 1 and Table 2).
 108 Among three tested models, the SFO model resulted in the best fit, but the two concentration treatments gave markedly
 109 different rate constants (k values). The k value was four times greater for $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ applied to soil at 100 μM than when
 110 supplied at 1000 μM (Table 1), corresponding to $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ half-lives of 55 minutes for the low substrate concentration
 111 and 231 minutes for the high (Table 1). At the low concentration, we suppose that the enzymatic capacity of the
 112 microorganisms was not exceeded and the kinetics was a true first order process. At the high concentration, saturation
 113 probably occurred and the rate of degradation depended on the number of organisms able to degrade the substrate and on its
 114 concentration (Okpokwasili and Nweke 2006). It is concluded that $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ was rapidly degraded in soil when supplied
 115 at rhizosphere-relevant concentrations (100 μM). Moreover, the concentration of unlabeled Asn remained constant (Online
 116 Resource 2), indicating that there must have been a balance between formation and disappearance of Asn in soil.

117 Three ($^{13}\text{C}_4^{15}\text{N-Asp}$, $^{15}\text{NH}_4$, $^{13}\text{C}_5^{15}\text{N-Glu}$) out of four analyzed $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ transformation compounds were
 118 detected (Fig. 1 and 2). None of them were found in the control soil, and the analysis of the $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ stock solution
 119 showed only $^{13}\text{C}_4^{15}\text{N-Asp}$ to be present at 1.6 % of the $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ stock content (data not shown). Therefore, detected
 120 compounds must have originated from the decomposition of added $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$, either via abiotic or biotic pathways
 121 (Jones and Hodge 1999). The presence of $^{13}\text{C}_4^{15}\text{N-Asp}$ and $^{15}\text{NH}_4$ could be explained by chemical $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ hydrolysis
 122 during extraction or enzymatic deamidation by microorganisms. In the 100 μM treatment enzymatic processes could have
 123 greater influence, since a decrease in $^{13}\text{C}_4^{15}\text{N-Asp}$ concentration was observed after 90 min (Fig. 2). It has been proposed
 124 that the rapid utilization of amino acids by soil microorganisms is caused by microbial C limitation, rather than N limitation
 125 (Farrell et al. 2014b). Perhaps, $^{13}\text{C}_4^{15}\text{N-Asp}$ disappearance was in consequence of fast $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ depletion ($t_{1/2}=55$ min),
 126 where the reduction in availability of one nutrient led to consumption of the other one. By contrast, increase in $^{13}\text{C}_4^{15}\text{N-Asp}$
 127 concentration in the 1000 μM treatment was likely the reason of microbial saturation with $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ (Fig. 2).
 128 Interestingly, $^{13}\text{C}_5^{15}\text{N-Glu}$ was also detected in the soil. In the 100 μM treatment it was in trace amounts below the limit of
 129 detection (Fig. 2b), but in the 1000 μM treatment its accumulation after 90 min was confirmed (Fig. 2e). It is difficult to
 130 unambiguously explain its formation in soil and link to $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ metabolization. One likely pathway could be the

¹³C₄¹⁵N₂-Asn incorporation by microorganism and utilization in citric acid cycle for subsequent ¹³C₅¹⁵N-Glu production and release to soil solution. The release of ¹³C₅¹⁵N-Glu could also result from disruption of microbial cells during soil extraction. Nevertheless, it demonstrates that ¹³C₄¹⁵N₂-Asn in soil can be transformed rapidly and used for production of even greater molecules. Furthermore, compound-specific analysis facilitates tracing the molecules that result from those transformations. Such knowledge is important to know to understand amino acid pool available for plant uptake.

The conclusion from these results is that the ¹³C₄¹⁵N₂-Asn dissipates rapidly in soil with half-life of the intact amino acid equal to $t_{1/2} = 55$ min, at the 100 μ M treatment. The direct analysis of intact molecule kinetics gives assessment of how much compound is actually present in soil at a time, which has to be considered when determining plant ability to absorb intact amino acids from soil. Furthermore, the detection of ¹³C₄¹⁵N-Asp and ¹³C₅¹⁵N-Glu provides novel and complementary information on the fate of ¹³C₄¹⁵N₂-Asn in soil proving the strength of the direct method and the importance of metabolite tracking when estimating amino acid availability for plant uptake.

142

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146 Conflict of Interest

147 The authors declare that they have no conflicts of interest.

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207 Figure captions

208 **Fig. 1** Dissipation of $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ concentration in soil over time. Soil was spiked with $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ at either low (100
209 μM , a and b) or high (1000 μM , c and d) concentration and analyzed for the presence of $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ and $^{15}\text{NH}_4$ at different
210 time points. At each concentration treatment there were five replicates per each time point ($n=5$). Symbols represent
211 experimentally derived data points ($n=5$), while lines represent fits to Single First-Order kinetics

212 **Fig. 2** Concentration of $^{13}\text{C}_4^{15}\text{N-Asp}$, $^{13}\text{C}_5^{15}\text{N-Glu}$ and $^{15}\text{N-Gln}$ in soil over 180 min at different $^{13}\text{C}_4^{15}\text{N}_2\text{-Asn}$ concentration
213 treatments: 100 μM (a, b, c) or 1000 μM (d, e, f). At each concentration treatment there were five replicates per each time
214 point ($n=5$)

Table 1 Single First-Order kinetic parameters for $^{13}\text{C}_4^{15}\text{N}_2$ -Asn dissipation in soil at different $^{13}\text{C}_4^{15}\text{N}_2$ -Asn starting concentrations, 100 μM or 1000 μM . k is the rate constant for the degradation process, C_0 is the percent of amino acid at time 0, DT_{50} is an estimated time needed for 50 % dissipation of an active substance under field conditions. 95% Confidence Intervals are presented in parenthesis.

	C_0 (%)	k	DF adjusted R^2	DT_{50} (min)
Low concentration (100 μM)	90.1 (87.2-92.9)	0.0125 (0.0117-0.0134)	0.995	55.3
High concentration (1000 μM)	104.4 (100.1-108.3)	0.003 (0.0025-0.0036)	0.955	231

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Electronic Supplementary Material 1

Soil collection and preparation

Soil used in the study (sandy loam) was collected in April, 2014. Soil was taken from eight different blocks from the A horizon (5-15 cm) at the CENTS long-term field experiment located in Foulum, Denmark (56°30' N, 9°35' E), and stored at 4°C. The general properties of the soil are given in Table 1 (Chatskikh and Olesen, 2007; Hansen et al., 2011). Prior to analysis, soil was sieved (3 mm) and the water content was determined after drying at 100°C for 24 hours.

Table 1 Selected properties of the soil used in the dissipation study

		Water			Bulk			
		Water	Holding		density	Total C	Total N	Organic
Texture		content (%)	Capacity (%)	pH	(g cm ⁻³)	(g kg ⁻¹)	(g kg ⁻¹)	matter (%)
soil	sandy loam	9.14	34.4	6.1	1.25	18	1.6	3.5

Amino acid dissipation analysis

The rate of Asn dissipation in soil was measured during 180 min at room temperature using universally labeled L-asparagine-¹³C₄, ¹⁵N₂ (¹³C₄¹⁵N₂-Asn) (95%, Sigma-Aldrich, Denmark). Briefly, 4.4 g fresh soil was weighed in a 50 mL falcon tube (VWR, Denmark) and water content adjusted to 50% of Water Holding Capacity (WHC) using Milli-Q water (Merck Millipore, Denmark). 4.2 µl (690.3 µg ml⁻¹) and 5.5 µl (5225 µg ml⁻¹) of ¹³C₄¹⁵N₂-Asn water solution was added dropwise per 1 g of dry soil and mixed to obtain final concentration of 100 µM (20.8 µmol kg⁻¹ soil DW) and 1000 µM (208 µmol kg⁻¹ soil DW), respectively. The low concentration treatment 100 µM, although exceeded the concentration reported for the bulk soil (Warren 2014), was in the range of concentration found in the rhizosphere (van Hees et al. 2005). The high concentration treatment (1000 µM) was included to obtain higher sensitivity in detecting amino acids closely related to ¹³C₄¹⁵N₂-Asn metabolism. After 0, 10, 20, 30, 40, 50, 60, 90, 110, 140 and 180 min of incubation (22°C) tubes with soil were taken for immediate extraction following the procedure of Inselsbacher (2014). Soils used as a control were spiked

41 with Milli-Q water in order to determine the natural abundance of labeled amino acids. At each concentration treatment,
42 there were five replicates (n=5) per each time point and control.

43 Extraction and derivatization of amino acids in the soil samples

44 The extraction method was based on Inselsbacher (2014). To each tube with soil we added 30 ml of 1 M KCl, containing
45 norvaline (Sigma Aldrich, Denmark) as an internal standard (0.125 pmol μl^{-1}). Tubes were shaken for 60 min at 4°C on
46 shaker (Intelli Mixer, In Vitro, Denmark) at 99 rpm. Then the tubes were centrifuged (Heraeus, Axeb, Denmark) at 4 600 g
47 for 10 min at 4°C. From each of the soil extracts, 1000 μl was filtered using PTFE syringe filters (0.22 μm) (Mikrolab
48 Aarhus A/S, Denmark) and transferred to LC-MS vials. The extracts were then derivatized with the AccQ•Tag Ultra
49 Derivatization Kit (Waters Corp.) according to the manufacturer’s protocol.

50 Amino acid analysis in the derivatized samples

51 The derivatized samples were analyzed with an HPLC (Agilent) coupled to 4500 QTRAP (ABSciex) using electrospray
52 ionization (ESI) in positive mode with a voltage of 4500 eV. The acquisition method was based on Armenta et al. (2010)
53 and Johansson et al. (unpublished). The derivatized amino acids were separated on a Waters Xbridge C18 column (2.1 mm
54 x 150 mm). The following separation gradients were used: 0–0.54 min (99.9% A), 5.50 min (90.9% A), 7.70 min (78.8%
55 A), 8.50–9.00 min (40.4% A), 9.50–10.00 min (20.0% A), and 10.50-15.00 (99.9% A). Eluent A consisted of Milli-Q water
56 and 0.1% formic acid, eluent B consisted of acetonitrile and 0.1% formic acid, and the column flow rate was 0.5 ml min^{-1} .
57 The column temperature was set to 55°C, and the sample injection volume was 1 μl . Mass spectrometric detection was
58 performed in multiple reaction mode (MRM). Retention times and MRM transitions (Q1/Q3) are listed in Table 2. The
59 compounds of interest were analyzed using AB Sciex Analyst 1.6.2 software.

60 **Table 2** Mass-to-charge ratio (m/z) used in selected ion monitoring and retention times.

Standard						
	available/ not available	Compound name	Short name	Retention time (min)	Q1 (m/z)	Q3 (m/z)
Unlabeled compounds	available	asparagine	Asn	4.25	303.1	171

	available	aspartic acid	Asp	5.56	304	171
	available	glutamine	Gln	4.98	317.1	171
	available	glutamic acid	Glu	5.93	318.1	171
	available	ammonium	NH ₄	2.61	188	171
	available	norvaline	Nor	9.51	288.1	171
Labeled compounds	available	¹³ C ₄ ¹⁵ N ₂ -asparagine	¹³ C ₄ ¹⁵ N ₂ -Asn	4.25	309.1	171
	available	¹³ C ₄ ¹⁵ N-aspartic acid	¹³ C ₄ ¹⁵ N-Asp	5.56	309.1	171
	available	¹³ C ₅ ¹⁵ N ₂ -glutamine	¹³ C ₅ ¹⁵ N ₂ -Gln	4.98	324.1	171
	available	¹⁵ N-glutamic acid	¹⁵ N-Glu	5.93	318.2	171
	not available	¹⁵ N-ammonium*	¹⁵ NH ₄	2.61	189.1	171

* ¹⁵N-ammonium represents analyzed compound, for which the analyzed mass was the monoisotopic mass with an addition of the mass of the same number of neutrons (1.008 u)

Amino acid quantification and method validation

The presence of Asn, Asp, Gln, Glu, ¹³C₄¹⁵N₂-Asn, ¹³C₄¹⁵N-Asp, ¹⁵N-Gln, ¹³C₅¹⁵N-Glu and ¹⁵NH₄ in soil samples was confirmed by comparison of retention times and MRM transitions with reference standard compounds: Asn (99%), Asp (98%), Gln (99%), Glu (99%), ¹³C₄¹⁵N₂-Asn (95%), ¹³C₄¹⁵N-Asp (95%), ¹⁵N-Gln (95%), ¹³C₅¹⁵N-Glu (95%), NH₄Cl (99%) (Sigma-Aldrich, Denmark). The calibration curves of the unlabeled standards – Asn, Asp, Gln, Glu, and NH₄ (Sigma-Aldrich, Denmark) – were prepared from authentic standard compounds in the range of 0.001 – 1 pmol µl⁻¹ (≈ 0.07 – 70 µmol kg⁻¹ soil DW). The peak area of each standard was divided by the peak area of the internal standard (norvaline). The normalized peak area of the standards was plotted against the standard concentration that was entered in the column. A linear function was applied to the calibration curves. To determine the concentrations of the amino acids and ammonium in the samples, the peak area was normalized using the peak area of norvaline and calculated using the regression equations from the calibration of the standards. The minimum level at which each of the amino acids could be reliably quantified was determined by the limit of detection (LOD) (Table 3). Based on the background noise level, the LOD was defined as the concentration of the analyte that generated a signal equal to two times the background noise. Recovery experiments were

performed to evaluate the efficiency and the reproducibility of the analytical method. For the recovery of Asn, Asp, Gln, Gln, 4.4 g of soil was adjusted to 50% of WHC and spiked with low and high concentration of the respective amino acid standard. For the low concentration 5.125 μl of each of the Asn, Asp, Gln, Gln water stock solution (100 ppm) was spiked per 1 g of soil DW to obtain final concentration of 0.05 $\text{pmol } \mu\text{l}^{-1}$ ($\approx 3.5 \mu\text{mol kg}^{-1}$ soil DW). For the high concentration 125 μl of each of the Asn, Asp, Gln, Gln water stock solution (100 ppm) was spiked per 1 g of soil DW to obtain final concentration of 1 $\text{pmol } \mu\text{l}^{-1}$ ($\approx 70 \mu\text{mol kg}^{-1}$ soil DW). For the recovery of $^{15}\text{NH}_4$, 4.4 g of soil was adjusted to 50% of WHC and 26.2 μl of the NH_4Cl water stock solution (1000 ppm) was spiked on soil to obtain concentration of 1.8 $\text{pmol } \mu\text{l}^{-1}$ ($\approx 126 \mu\text{mol kg}^{-1}$ soil DW). The concentration of each analyte used for spiking the soil was calculated previously and depends on the approximate concentration known to occur naturally in the soil samples. Recovery experiments were prepared in five replicates. Spiked soil samples were extracted and analyzed as described before. The analyte recovered was calculated by subtracting the analyte concentration in a blank soil (water spiked soil) from the analyte concentration in a spiked soil and then the analyte recovered was calculated as percentage of analyte added (Table 3).

Table 3 Recovery rates and LOD for amino acids and ammonium. The data for recovery are presented as the mean recovery % of six replicates $\pm$ RSTDEV.

Compound	Recovery (%)		
	Low spike ^a	High spike ^b	LOD ($\text{pmol } \mu\text{l}^{-1}$)
Asn	78 (± 19)	97 (± 2.7)	0.003
Asp	74 (± 5.4)	79 (± 3.7)	0.005
Gln	96 (± 23)	84 (± 6)	0.005
Glu	90 (± 4)	87 (± 7.3)	0.001
NH_4	-	120 (± 6.1)	0.001

^a Recovery rates (%) for amino acids were calculated by spiking the soil with 0.05 $\text{pmol } \mu\text{l}^{-1}$ of respective amino acid standard

^b Recovery rates (%) for amino acids were calculated by spiking the soil with 1 $\text{pmol } \mu\text{l}^{-1}$ of respective amino acid standard and for ammonium by spiking with 1.8 $\text{pmol } \mu\text{l}^{-1}$

Calculations and statistical analysis

No $^{13}\text{C}_4^{15}\text{N}_2$ -Asn, $^{13}\text{C}_4^{15}\text{N}$ -Asp, ^{15}N -Glu, $^{13}\text{C}_5^{15}\text{N}$ -G and $^{15}\text{NH}_4$ were detected in the control soil, therefore no corrections for the naturally abundant labeled compounds were made when calculating the degradation kinetics of $^{13}\text{C}_4^{15}\text{N}_2$ -Asn and the resulting concentrations of amino acids closely related to its metabolism.

Three different kinetic models were applied to estimate $^{13}\text{C}_4^{15}\text{N}_2$ -Asn dissipation kinetics (RStudio software version 0.99.46);

(1) single first-order (SFO), where

$$C = C_0 \exp^{-kt} \quad (1)$$

in which C is the amount of amino acid present at a time t , k is the rate constant describing the degradation process, and C_0 is the amount of amino acid at time 0,

(2) first-order multi-compartment (FOMC), where

$$C = C_0 \left(\frac{t}{\beta} + 1 \right)^{-\alpha} \quad (2)$$

in which C is the amount of amino acid present at a time t , C_0 is the amount of amino acid at time 0, β is parameter determined by the variation in k values, and α is a positional parameter,

(3) double first-order in parallel (DFOP), where

$$C = C_1 \exp^{-k_1 t} + C_2 \exp^{-k_2 t} \quad (3)$$

in which C is the amount of amino acid present at a time t , C_1 is the mount of amino acid at time 0 in the first compartment, k_1 is the rate constant for degradation in the first compartment, C_2 is the mount of amino acid at time 0 in the second compartment, k_2 is the rate constant for degradation in the second compartment (Etzerodt et al., 2008). The DT_{50} value was also calculated which provides estimated time needed for 50% dissipation of an active substance under field conditions. For SFO kinetics DT_{50} corresponds to the half-life of the compound concerned (Etzerodt et al., 2008). Goodness of fit was evaluated by visual inspection of the curve, evaluations of residuals, degrees of freedom (DF) adjusted R^2 , and confidence intervals.

Title: Dissipation kinetics of asparagine in soil measured by compound-specific analysis with metabolite tracking.

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Electronic Supplementary Material 2

Asn concentration in soil

The concentration of unlabeled Asn in the soil was on average equal to $0.307 (\pm 0.04) \mu\text{mol kg}^{-1}$ soil DW, both in the low (100 μM) and high (1000 μM) treatments (Fig. 1). This concentration remained constant over duration of the $^{13}\text{C}_4^{15}\text{N}_2$ -Asn dissipation study and no significant differences were observed between different sampling times ($p > 0.05$).

Fig. 1 Concentration ($\mu\text{mol kg}^{-1}$ soil DW) of unlabeled Asn in soil after spiking with $^{13}\text{C}_4^{15}\text{N}_2$ -Asn at either 100 μM (a) or 1000 μM (b) and sampled after 0, 10, 20, 30, 40, 50, 60, 90, 110, 140 and 180 min. Blank represents the Asn concentration in soil spiked with MilliQ water. Bars correspond to mean concentrations (n=5) and error bars are standard deviations.