

Hotspots and hot moments of amino acid N in soil: Real-time insights using continuous microdialysis sampling

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Soil Biology and Biochemistry

DOI: 10.1016/j.soilbio.2018.12.026

Published: 01/04/2019

Peer reviewed version

Cyswllt i'r cyhoeddiad / Link to publication

Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA): Hill, E., Jones, D. L., Paterson, E., & Hill, P. (2019). Hotspots and hot moments of amino acid N in soil: Real-time insights using continuous microdialysis sampling. *Soil Biology and* Biochemistry, 131, 40-43. https://doi.org/10.1016/j.soilbio.2018.12.026

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- 2 microdialysis sampling
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14	Declarations of Conflict of Interest: None	
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16	Article type:	Short Communication

17 ABSTRACT Protein hotspots in soil, such as those associated with decaying soil fauna or plant litter, may produce ephemeral patches of disproportionately high soil nutrients. These 18 hotspots may occur at the macro and microscale in close proximity to plant roots, however, 19 20 the likely concentration of soluble products produced in these hotspots remains poorly understood. To address this, we buried two contrasting biomass residues in soil, namely 21 earthworm (Lumbricus terrestris) and clover (Trifolium repens). Their transformation to 22 23 amino acids, NH_4^+ and NO_3^- was monitored continually over 6 days using microdialysis. All treatments showed greater soluble nitrogen (N) concentrations compared to the unamended 24 25 controls. The highest concentrations of both amino acids (12.9 mM after 12 h) and NH₄⁺ (45.3 mM after 6 h) were generated in the vicinity of decomposing earthworm. In 26 comparison, dried clover residues yielded 2.7 mM of amino acids at 6 h. After 12 h, amino 27 28 acid and NH₄⁺ concentrations in both earthworm and dried clover treatments showed a steep 29 decline, returning close to background levels (<20 µM). Through the use of microdialysis we are able to show that soil nutrient hotspots may provide nearby roots with concentrations of 30 31 amino acids and NH₄⁺ several orders of magnitude higher than found in the bulk soil solution.

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Keywords: Dissolved organic nitrogen; DON; Mineralization; Plant-microbial competition; Proteases
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Amino acids (AAs) and oligopeptides are the most abundant and first quantitativelysignificant protein breakdown products to be directly available as nitrogen (N) sources for plants and soil microbes (Sauheitl et al., 2009; Farrell et al., 2011; Warren, 2013; Moran-Zuloaga et al., 2015). Consequently, transformation of proteins to AAs is a major factor limiting N availability in soil and competition between plant roots and soil microbes for AAs is fierce (Jones and Kielland, 2002; Bardgett et al., 2003; Jan et al., 2009; Hill et al., 2011; Hill and Jones, 2018). The concentration of AAs in soil appears to be a key determinant in 42 the outcome of competition, with higher concentrations probably favouring plant AA-N capture (Jones et al., 2005). Therefore establishing true concentrations of AAs in soil is 43 crucial to our understanding of plant N acquisition, and N cycling in soil. Typically, total free 44 AA concentrations in contrasting ecosystems have been reported to remain fairly constant at 45 $23 \pm 5 \mu$ M, with the concentration of individual AAs typically ranging from 0.1-5.0 μ M 46 (Jones et al., 2009). These measurements in the bulk soil reflect the balance between slow 47 48 rates of AA production and, rapid microbial AA consumption. However, biogeochemical 'hotspots', ephemeral patches which yield disproportionately high nutrient levels relative to 49 50 the surrounding soil matrix, may supply high quantities of AAs to nearby roots (McClain et al., 2003; Schimel and Bennett, 2004; Jones et al., 2005; Kuzyakov and Blagodatskaya, 51 2015). 52

Using plant and soil fauna residues and microdialysis, we measured concentrations of protein breakdown products which may realistically occur close to roots. Microdialysis is a membrane-based sampling technique which offers non-invasive measurement of the soil solution phase, allowing probes to be positioned in close spatial proximity to samples and therefore yielding a high spatial resolution. We hypothesised that AAs generated from soil hotspots of protein breakdown transiently occur at concentrations greatly exceeding those found in measurements of bulk soil solution.

Microdialysis probes were single use, 100 kDa cut-off, 10 mm membrane length, 0.5
mm membrane diameter, 2.6 μL membrane internal volume and inlet internal volume of 1.4
μL (CMA Microdialysis, Torshamnsgatan, Sweden). Probes with a 100 kDa membrane pore
size have been shown by other researchers to recover a great fraction of amino acids,
compared to 20 kDa probes (Buckley et al., 2017). Probes were calibrated for relative
recovery using amino acid (L-alanine), NH4Cl and KNO₃ standard solutions of 0.1, 0.25, 0.5,
0.75, 1 and 25 mM (Inselsbacher et al., 2011; Lange, 2012). Probes (*n* = 8) were positioned

67 in standard solutions at room temperature, and perfused with ultra-pure deionised-water (5 μ L min⁻¹). Aliquots of dialysate were collected at 30 min intervals during a 120 min 68 perfusion period. Total amino acids in dialysate were measured fluorometrically according to 69 70 Jones et al. (2002) while NH4⁺ and NO3⁻ were determined colorimetrically according to Mulvaney (1996) and Miranda et al. (2001), respectively. Relative recovery was found to be 71 $14.5 \pm 5.5\%$ of the supplied standard solution, irrespective of N-form, standard solution 72 73 concentration or time. The relative recovery was later used to estimate actual concentrations of target analytes in the soil; although we cannot completely exclude some difference 74 75 between standard solution calibration and measurements in soil solution. Collection vials were weighed before and after perfusion to ensure that there was no net loss or gain of water 76 due to transmembrane flux or leakage. 77

Soil (0-15 cm) was collected from the Ah horizon of an agricultural grassland at
Henfaes Agricultural Research Station, N. Wales, UK (53°23'N, 4°01'W, 19 m.a.s.l). The
sandy clay loam textured soil is classified as a Eutric Cambisol (FAO), derived from postglacial alluvial deposits. The main properties of the soil and added protein sources are shown
in Table S1.

Soil was sieved to 2 mm, homogenised and added to 1.5 ml microcentrifuge tubes, 83 each tube receiving ca.1.5 g of field-moist soil. Three protein sources were added to the soil 84 in a 1:10 w/w protein source to soil ratio as follows: a) fresh foliage of Trifolium repens L. 85 86 (clover), b) dried foliage of T. repens, and c) fresh necromass of the earthworm, Lumbricus terrestris L., with soil controls receiving no protein addition. All treatments were replicated 87 three times. These protein sources, added by weight, represent different levels of N input, 88 89 however our hypothesis is predicated on a greater understanding of realistically occurring hotspots in the soil, rather than a direct comparison between treatments. Protein sources were 90 added to the centre of each tube. Each replicate was sealed using gas-permeable film 91

(Parafilm M[®], Bemis Inc., USA) to restrict water loss by evaporation. Probes were positioned 92 to a depth of 10 mm in the centre of the tube, using a syringe introducer and perfused with 93 ultra-pure deionised water, at a continuous rate of 5 μ L min⁻¹, using a multi-channel syringe 94 pump (NE-1200 Multi-Phaser, New Era Pump Systems Inc., NY, USA) over a sampling 95 period of 144 h. Samples were taken at hourly intervals for the first 6 h, then every 6 h 96 thereafter (samples at 30 h were contaminated during storage and therefore omitted from 97 98 analysis). Samples were analysed for amino acids and inorganic N as described above and for pH and electrical conductivity (EC) with standard microelectrodes. Outliers were identified 99 100 using Grubbs' test (9 of 1008 measurements were removed). Data were analysed using a mixed two-way ANOVA with a Tukey HSD post-hoc test (SPSS v22; IBM, New York, 101 USA), with a $p \le 0.05$ cut-off for statistical significance. 102

103 Amino acid concentrations in controls did not exceed a mean concentration of 0.018 mM at any point during the experiment. Initially, levels of AA's were significantly higher in 104 all treatments within the first 4 h following protein addition (Fig. 1). We attribute this to 105 immediate loss of soluble components e.g. from damaged cells. All treatments had greater (p 106 ≤ 0.05) AA and NH₄⁺ concentrations than controls during the majority of sampling intervals. 107 Amino acids in dried clover and earthworm treatments showed a sharp rise in concentration 108 after ~4 h, with those in dried clover rising to 2.7 mM after 6 h and to 12.9 mM in the 109 earthworm treatment after 12 h, before sharply decreasing and levelling off after ~72 h. In 110 111 contrast, fresh clover showed initially low AA concentrations (lowest mean concentration of 0.006 mM at 12 h), with only slightly higher concentrations than the control at some time 112 points until ~54 h, thereafter reaching a peak of 0.099 mM at ~72 h, which was conversely 113 114 the time at which the other treatments returned to levels comparable with controls. In comparison to the air-dried clover which was dead at the point of addition to the soil, it is 115 likely that the fresh clover leaves underwent slow autolysis and death after excision. During 116

this autolysis, leaf protein and amino acids are catabolised internally for energy production (with plant cell walls probably remaining largely intact) and are less likely to be lost to the soil (Marella et al., 2017). The single highest concentration of AAs was observed in the earthworm treatment at 6 h, with one replicate of 29.3 mM of AAs, though this was identified as an outlier.

Concentrations of NH₄⁺ in the control treatment did not exceed 0.21 mM during the 122 123 entirety of the experiment. In contrast, in the earthworm treatment, concentrations peaked at 45.3 mM at 12 h within the same replicate as the greatest AA concentration. Generally, 124 125 however, ammonium concentrations in other treatments were low in comparison to the earthworm, reaching peak mean concentrations of 2.67 mM and 1.71 mM for the dried and 126 fresh clover respectively, with significantly ($p \le 0.05$) greater concentrations than the control 127 128 observed at every time point for dried clover, and from 24 h onwards for fresh clover (Fig. 2). Interestingly, mean nitrate concentrations of all treatments remained low throughout 129 the sampling period (< 1 mM) with the highest concentration of 0.71 mM seen in the 130 earthworm treatment at 6 h. We observed no clear relationship between pH and treatment 131 and/or time (Fig. S1), however, EC was significantly higher in the earthworm and dried 132 clover treatments than the control during the majority of sampling times (12-144 h) (Fig. S2). 133

The temporal pattern of our results suggests that the rise in levels of free AA's is 134 matched by increased microbial consumption and growth that rapidly lowers the 135 136 concentration. This suggests that a distinction should be made between concentration hotspots (where breakdown rate is high and exceeds microbial demand leading to 137 accumulation in solution) and flux hotspots (where breakdown rate is high but is matched by 138 139 microbial demand and where little change in solution concentration occurs). Likewise, microbial demand may have become saturated earlier on in the sampling period due in part to 140 adding N at a higher concentrations (despite being the same weight) in earthworm and dried 141

clover treatments than that of fresh clover. However, hotspots occurring naturally within the
soil would reflect this, yielding a range of concentrations from the macroscale, down to very
fine microscales.

Results indicate that transformation of proteins to amino acids in ephemeral hotspots 145 of protein addition may provide orders of magnitude greater levels of amino acids than 146 previously measured in bulk soil solution (Jones et al., 2002; Jones et al., 2005; Hill et al., 147 148 2011). Further, although our experiment used relatively large fragments, it seems likely that hotspots occur at a much finer scale, for instance in the vicinity of dead microbial cells or 149 150 damaged root cells in the detritusphere, as hypothesised by other researchers (Marschner et al., 2012; Kuzyakov and Blagodatskaya, 2015). As a consequence of this, it is therefore 151 probable that the form and quantity of N acquired by plants varies considerably on a fine 152 153 temporal and spatial scale.

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156 Acknowledgments

This work was supported by the Biotechnology and Biological Sciences Research Council; and the Natural Environment Research Council [Grant number NE/M009106/1], by a Soils Training and Research Studentships (STARS) grant to Elliot J. Hill. STARS is a consortium consisting of Bangor University, British Geological Survey, Centre for Ecology and Hydrology, Cranfield University, James Hutton Institute, Lancaster University, Rothamsted Research and the University of Nottingham.

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235 Figure legends

Figure 1. Temporal dynamics of soil solution amino acid concentrations in response to the

- addition of protein-rich hotspots (dead earthworm (A), dried (B) or fresh clover leaves (C)) to
- soil and measurement by microdialysis. Values represent means + SEM (n=4).

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240	Figure 2. Temporal dynamics of soil solution ammonium (A) and nitrate concentrations (B)
241	in response to the addition of protein-rich hotspots (dead earthworm, dried or fresh clover
242	leaves) to soil and measurement by microdialysis. Values represent means + SEM ($n=4$).
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Soil parameters	Values
Soil moisture (%)	31.47 ± 0.22
рН	5.33 ± 0.04
EC (μ S cm ⁻¹)	45.62 ± 3.25
Total carbon (%)	2.55 ± 0.18
Total nitrogen (%)	0.28 ± 0.02
Nitrate (N mg kg ⁻¹ oven dry soil)	0.872 ± 0.10
Ammonium (N mg kg ⁻¹ oven dry soil)	3.94 ± 1.02

Table 1. Physiochemical properties of the A-horizon of the Eutric Cambisol (Means + SEM).

Table 2. Total C & N ratios of protein additions (Means + SEM).

	Carbon (%)	Nitrogen (%)
Clover	48.6 ± 0.2	4.81 ± 0.06
Earthworm	43.73 ± 3.78	9.72 ± 1.03