WILEY

A Virtual Event Designed For The Masses

Now Available On-Demand!

Scale up your research and translate your results more rapidly and simply than ever before. Welcome to vLC-MS.com - the event for Orbitrap Exploris mass spectrometers and much more!

Tune in to:

- Explore the LC-MS portfolio and meet the expanded Orbitrap Exploris MS system in our staffed Exhibit Hall.
- Learn from mass spectrometry experts, such as Professor Alexander Makarov himself, about Orbitrap mass spectrometry technology and the applications it enables.
- Browse posters and short presentations in our application area.

Event Highlights:

Prof. Alexander Makarov

Dr. Christian Münch

Thomas Moehring

vLC-MS.com

REGISTER NOW

ThermoFisher SCIENTIFIC

RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2008; **22**: 1643–1652
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.3455

RCM

Enhancing the understanding of earthworm feeding behaviour via the use of fatty acid $\delta^{13}C$ values determined by gas chromatography-combustion-isotope ratio mass spectrometry†

Jennifer A. J. Dungait[1], Maria J. I. Briones[2], Roland Bol[3] and Richard P. Evershed[1]*

[1]Organic Geochemistry Unit, Bristol Biogeochemistry Research Centre, University of Bristol, School of Chemistry, Cantocks Close, Bristol BS8 1TS, UK
[2]Departmento Ecología y Biología Animal, Facultad de Biología, Universidad de Vigo, 36310 Vigo, Spain
[3]Cross-Institute Programme for Sustainable Soil Function (Soil-CIP), Institute of Grassland and Environmental Research, North Wyke, Okehampton EX20 2SB, UK

Received 24 January 2008; Accepted 25 January 2008

Litter-dwelling (epigeic) *Lumbricus rubellus* and soil-dwelling (endogeic) *Allolobophora chlorotica* earthworms were observed aggregating under C_3 ($\delta^{13}C = -31.3‰$; $\delta^{15}N = 10.7‰$) and C_4 ($\delta^{13}C = -12.6‰$; $\delta^{15}N = 7.5‰$) synthetic dung pats applied to a temperate grassland ($\delta^{13}C = -30.3‰$; $\delta^{15}N = 5.7‰$) in an experiment carried out for 372 days. Bulk $\delta^{13}C$ values of earthworms collected from beneath either C_3 or C_4 dung after 28, 56, 112 and 372 days demonstrated that (i) *L. rubellus* beneath C_4 dung were significantly ^{13}C-enriched after 56 days ($\delta^{13}C = -23.8‰$) and 112 days ($\delta^{13}C = -22.4‰$) compared with those from C_3 dung treatments (56 days, $\delta^{13}C = -26.5‰$; 112 days, $\delta^{13}C = -27.0‰$), and (ii) *A. chlorotica* were 2.1‰ ^{13}C-enriched ($\delta^{13}C = -24.2‰$) relative to those from C_3 dung ($\delta^{13}C = -26.3‰$) treatments after 372 days. Bulk $\delta^{15}N$ values did not suggest significant uptake of dung N by either species beneath C_3 or C_4 dung, but showed that the endogeic species (total mean $\delta^{15}N = 3.3‰$) had higher $\delta^{15}N$ values than the epigeic species (total mean $\delta^{15}N = 5.4‰$). Although the two species exhibited similar fatty acid profiles, individual fatty acid $\delta^{13}C$ values revealed extensive routing of dietary C into body tissue of *L. rubellus*, but minor incorporation into *A. chlorotica*. In particular, the direct incorporation of microbial biomarker fatty acids ($iC_{17:0}$, $aC_{17:0}$) from ^{13}C-labelled dung *in situ*, the routing of dung C into *de novo* synthesised compounds ($C_{20:4\omega6}$, $C_{20:5\omega3}$), and the assimilation of essential fatty acids ($C_{18:1\omega9}$, $C_{18:1\omega7}$, $C_{18:2\omega6}$, $C_{18:3\omega3}$) derived from dung, were determined.

Earthworms are important agents of organic matter (OM) decomposition in terrestrial ecosystems, accelerating the rate of comminution and dispersal through their feeding activities. They are broadly classified into three functional groups according to their feeding behaviour: 'epigeic' types feed on litter with little soil ingested; 'endogeic' geophagous species ingest mineral soil and associated OM; and 'anecic' earthworms form burrows in soil and feed on surface litter.[1,2] Numerous studies of the feeding habits of earthworms rely on direct observations of the feeding activity, microbiological analyses of gut contents, palatability tests, recording ingestion and consumption rates and measurements of the growth rates of worms raised on different substrates.[3] However, the use of such techniques to identify the diet of soil invertebrates are not always reliable as they can detect only recent trophic interactions[4,5] and are resource- and time-consuming.[6] Therefore, the feeding preferences of individual earthworm species are still not well understood.[7]

The application of stable isotope techniques is particularly important in soil zoology since many processes, including trophic relationships between animals, are hard to study *in situ*.[5] Recent applications of bulk stable C and N isotope determinations at natural abundance levels have been used to explore the feeding ecology of earthworms in grassland systems.[8–11] However, Neilson *et al.*[10] suggested that the relationship between feeding ecologies and bulk isotope signatures is perhaps more complex than can be explained on simple trophic grounds and requires consideration of the inherent variability of habitat diversity. Gearing[12] reviewed sources of variability in bulk $\delta^{13}C$ values of diet including inhomogeneity in a single food source, selective assimilation of foodstuffs, differences between major organic fractions and seasonal differences in the quality of food. Briones and Schmidt[9] also suggested that changes in bulk stable isotope

*Correspondence to: R. P. Evershed, Organic Geochemistry Unit, Bristol Biogeochemistry Research Centre, School of Chemistry, Cantocks Close, Bristol BS8 1TS, UK.
E-mail: r.p.evershed@bristol.ac.uk
†Presented at the annual meeting of the Stable Isotopes Mass Spectrometry Users' Group (SIMSUG), 20–22 June, 2007, hosted by the Institute for Research on the Environment and Sustainability (IRES) and the School of Civil Engineering and Geosciences, Newcastle University, UK.

values may be due to physiological changes or variations in body biochemical composition. Furthermore, bulk stable isotopic determinations provide average values of a wide range of individual biochemical components, each of which will have characteristic isotopic compositions. Isotopic determinations of these biochemical components provide additional information regarding the assimilation and biosynthesis of specific compounds from an organism's diet.[13]

Analysis of earthworm lipids has revealed a wide range of fatty acids (FA) presumed to be principally derived from dead plant material supplemented by living and dead microorganisms present in ingested soil.[14–18] However, conclusions on the provenance of dietary constituents can only be determined by labelling specific components; recent reviews of stable isotope applications in earthworm ecology have recommended the isotopic analysis of biochemical fractions and specific compounds.[7–11] Albro *et al.*[14] demonstrated that the routing of ^{14}C-*n*-hexadecanoic acid ($C_{16:0}$) into a range of biosynthesised free FA was comparatively rapid compared with other lipids of *Lumbricus terrestris*. Recent *in vitro* studies in soil invertebrate ecology have shown the potential of applying compound-specific isotopic methodologies to the investigation of dietary C recruitment into the FA[13,19–23,27] and cholesterol[19] of Collembola. FA have proven invaluable since their isotopic values rapidly reflect the diets or substrates utilised by consumer organisms.[13,20,21,24–27] For example, in Collembola, FA have been shown to exhibit rapid turnover following a C_3 to C_4 diet switch (half-life 1.5–3.6 days) compared with other lipid types, i.e. sterols (half-life 5.6–5.8 days).[19] Further compound-specific stable isotope analyses of Collembola offered a choice of differently labelled diets in laboratory mesocosms confirmed that *Folsomia candida* and *Proisotoma minuta* preferred nematodes to fungi.[26] Ruess *et al.*[27] fed Collembola with isotopically different fungal and nematode diets in order to gain insight into C fractionation and transfer of FA in the food chain. Beyond these applications, compound-specific isotope studies of soil invertebrates are rather scarce.

The role of earthworms in the decomposition and dispersal of dung is widely acknowledged.[7] Many kinds of animal dung are highly attractive and nutritious food sources for earthworms.[28,29] The tendency for a range of different species of earthworms to aggregate in and under dung pats has frequently been observed,[30–32] coincident with increases in earthworm biomass.[28,33] Pot experiments have revealed that dung was eaten by earthworms at a rate of 10 mg DW g^{-1} body weight $days^{-1}$,[34] and, based on gut contents, Guild[35] and Hendriksen[36,37] reported that earthworms in pastures may ingest from 10–27 t cow dung $ha^{-1}a^{-1}$, thereby demonstrating a fundamental role for earthworms in the decay and disappearance of cow pats;[32] however, very little is known about the specific biochemical constituents of dung utilised by earthworms. Different earthworm species also show preferences for different types and ages of dung.[38]

In this investigation we combine bulk and compound-specific stable isotope methods to explore the feeding behaviour of earthworms under dung pats *in situ*. We hypothesised that earthworms would display trophic niche differentiation with respect to the resource presented as dung pats in a grassland environment that would be indicated by: (i) contrasting bulk δ^{13}C and δ^{15}N values; (ii) differences in FA profiles between species; and (iii) δ^{13}C values indicative of dung C incorporation into FA by *de novo* biosynthesis or direct incorporation. The results show that application of compound-specific stable isotope methodologies to earthworm ecology provides new insights into food selection behaviour by earthworms and their role in cycling of C and N from dung in grassland soils.

EXPERIMENTAL

Field site

A field experiment was established in April 2002 on a previously grazed C_3 grassland (Little Burrows plot) at IGER-North Wyke, Devon, UK (50°45′N and 4°53′W). The site had been ploughed and reseeded 3 years prior to the start of the experiment. The soil is of the Halstow series of non-calcareous pelocols, with a brownish clay loam or silty clay A horizon (0–17 cm) and a mean pH of 6.6 (0–5 cm horizon). The area had a mean annual temperature of 10.5°C and total annual precipitation of 1269 mm (April 2004 to March 2005).

Experimental design and sampling

Full details of natural abundance ^{13}C-labelled dung production and field application are given elsewhere.[39] In brief, two cows were switched from a C_3 *Lolium perenne* silage diet to a C_4 *Zea mays* silage diet at the IGER-Trawsgoed Research Farm, to produce C_3-labelled ($\delta^{13}C = -31.3 \pm 0.1$‰; $\delta^{15}N = 10.7 \pm 0.4$‰) and C_4-labelled ($\delta^{13}C = -12.6 \pm 0.3$‰; $\delta^{15}N = 7.5 \pm 1.4$‰) dung. Dung pats (1.5 kg, fresh weight, $n = 112$) and controls (no dung; $n = 28$) were applied to a C_3 grassland plot ($\delta^{13}C = -30.3 \pm 1.3$‰; $\delta^{15}N = 5.7 \pm 2.0$‰) at IGER-North Wyke in a fully randomised 7×20 experimental design on 22 April 2002. The dung residues, controls and soil beneath (0–5 cm) were destructively sampled after 28, 56, 112 and 372 days. *L. rubellus* and *A. chlorotica* were observed to aggregate below the cow pats on each sampling occasion. These species have contrasting feeding behaviour (*L. rubellus* = epigeic; *A. chlorotica* = endogeic).[3,40] All specimens were collected from within or beneath dung residues, dissected, gutted to remove unassimilated soil OM, and freeze-dried prior to analysis.

Bulk stable isotope analysis

Freeze-dried earthworm tissues were ground with a mortar and pestle using liquid N_2. Air-dried (30°C) soils, vegetation and dung were ground in a ball mill. Vegetation samples were taken 1 week prior to commencement of the field experiment. An aliquot was measured into a tin capsule and weighed. Triplicate δ^{13}C (‰), δ^{15}N (‰), % total organic C (TOC) and % total N (TN) values were determined at IGER-North Wyke by continuous flow-isotope ratio mass spectrometry (CF-IRMS) using a Europa 20-20 mass spectrometer (Crewe UK) after combustion to pure N_2 and CO_2. Reference standards were run every ten samples. The 1σ values were ±0.1‰ for the determination of δ^{13}C and δ^{15}N.

DOI: 10.1002/rcm

Fatty acid analysis

Total lipids were extracted from whole earthworm tissue according to an adapted method of Krauss *et al*.[41] The earthworms were lipid-extracted with dichloromethane (DCM)/hexane (2:1 v/v) at 60°C for 6 h, then again at room temperature overnight. The bound lipid components were released by saponification (1 ml 0.5 M methanolic NaOH, 1 h, 80°C), followed by acidification and extraction into diethyl ether. Total lipid extracts were separated into two fractions, 'acid' and 'neutral', using a bonded aminopropyl solid-phase extraction cartridge (500 mg sorbent, 2.8 mL eluent capacity; Phenomenex, Torrance, CA, USA). Extracts dissolved in DCM/isopropanol (2:1 v/v) were slowly flushed through a cartridge pre-eluted with hexane. After further elution with DCM/isopropanol (2:1 v/v, 8 mL), the collected 'neutral' fraction was removed and the cartridge slowly flushed with 2% (v/v) acetic acid in diethyl ether (8 mL) thereby eluting the 'acid' fraction. Solvent was removed from both the fractions under a gentle stream of N_2. FA were methylated using 14% (v/v) BF_3-MeOH solution (100 μL; Aldrich, Poole, UK; 1 h, 80°C) and extracted with chloroform to give fatty acid methyl esters (FAMES) for gas chromatographic (GC) analysis. The samples were stored dry under N_2 at −20°C prior to analysis. FA compositions are reported as a percentage of total FA. FA structures are represented by two numbers separated by a colon. The number before the colon indicates the C chain length and the number after corresponds to the number of double bonds. The position of the first double bond is counted from the methyl end of the molecule and prefixed by the symbol ω. Methyl branching on the first (iso) or second (anteiso) C is designated by *i* or *a* prefixes, respectively.

FAMES were analysed on a 5890 Series II gas chromatograph (Hewlett Packard, Palo Alto, CA, USA). Sample introduction was via an on-column injector, and the detector was a flame ionisation detector. Separation of FAMEs was achieved using a Factor Four VF23MS column (60 m × 0.32 mm i.d., 0.15 μm film thickness; Varian, Palo Alto, CA, USA). The carrier gas was H_2 (10 psi head pressure), and the oven temperature was programmed from 50°C (1 min isothermal) to 150°C at 15°C min^{-1}, from 150°C to 240°C at 4°C min^{-1}, and held at 240°C for 20 min isothermal.

FAMES were identified using a Trace GC-MS system (ThermoFinnigan, San Jose, CA, USA). The column and temperature program details were the same as those described above except that He was used as the carrier gas. The interface was set to the maximum oven temperature, the ion source was set to 30°C below this and the quadrupole mass spectrometer scanned the range of *m/z* 50–650 at 1.7 scans s^{-1}. Data were acquired and processed using Excalibur data system version 1.2 (ThermoFinnigan).

The $\delta^{13}C$ values of FAMES were determined using a Varian 3400 gas chromatograph attached to a Delta S IRMS instrument (Finnigan MAT, Bremen, Germany) (electron ionisation, 100 eV, 1 mA electron energy, 3 Faraday cup collectors *m/z* 44, 45 and 46, CuO/Pt Finnigan MAT Mark I combustion interface maintained at 850°C). Water removal was via a Nafion membrane. The GC conditions were the same as those described above except that He was the carrier gas. Samples were calibrated against reference CO_2 of known isotopic composition, introduced directly into the source three times at the beginning and end of every run. Each sample was run in duplicate to ensure reliable mean $\delta^{13}C$ values. FAME reference standards were run after every four samples.

$\delta^{13}C$ values obtained for the FAMES were corrected for the addition of the extra C using the mass balance equation shown in Eqn. (1). The $\delta^{13}C$ value for the BF_3-MeOH (−34.1‰) was determined by bulk CF-IRMS.

$$n_{cd}\delta^{13}C_{cd} = n_c\delta^{13}C_c + n_d\delta^{13}C_d \quad (1)$$

where n = number of C atoms, c = underivatised compound, d = derivatising agent, and cd = derivatised compound of interest.

Statistical analyses

As samples were taken at dissimilar time intervals, the effects of time, treatment and time*treatment on the outcome of $\delta^{13}C/\delta^{15}N$ values, TOC and TN (%) were estimated using the REML (Restricted Maximum Likelihood) procedure (Genstat version 7.0). All values were based on replicates of $n = 3$. The standard deviation of the mean ($\pm 1\sigma$) is given for each mean value.

Bulk $\delta^{13}C$ and $\delta^{15}N$ values, and fatty acid $\delta^{13}C$ values of earthworms from beneath C_3 dung pats were used as a control at each date to account for the trophic effect of rumination on dung values compared with bulk soil values, and to allow for changes in the biochemistry of earthworm tissues over the year.

RESULTS

Bulk $\delta^{13}C$ and $\delta^{15}N$ determinations

The mean $\delta^{13}C$ and $\delta^{15}N$ values of vegetation sampled at the site 1 week prior to commencing the field experiment were -29.8 ± 0.4‰ and 1.6 ± 0.7‰, respectively, and the values for the soil were $\delta^{13}C = -30.3 \pm 1.3$‰; $\delta^{15}N = 5.7 \pm 2.0$‰. Thus, the $\delta^{15}N$ value of the soil was ~4‰ enriched compared with the vegetation, while the $\delta^{13}C$ value of the soil was significantly similar to that of the vegetation. Bulk $\delta^{13}C$ values of the 0–1 cm soil horizon increased significantly under the C_4 dung pats from -28.5 ± 0.3‰ to -25.0 ± 1.3‰ after 56 days, returning to values of -31.4 ± 0.7‰ after 372 days.[49] No significant difference was seen between the $\delta^{13}C$ values of the control and of the C_3 dung-treated soils.

Figure 1 shows that the tissue of all earthworms associated with C_3 and C_4 dung were ^{13}C-enriched by 2.0–7.6‰ and 2.7–9.7‰, respectively, compared with the bulk control soil. The tissues of *L. rubellus*, the epigeic earthworm, associated with C_4 dung were particularly ^{13}C-enriched after 112 days, with bulk $\delta^{13}C$ values of -22.4 ± 1.5‰, compared with -27.0 ± 0.1‰ for similar species under C_3 dung; the endogeic species *A. chlorotica* sampled beneath either C_3 or C_4 dung type displayed similar $\delta^{13}C$ values of -25.7 ± 0.3‰ and -25.2 ± 1.0‰, respectively. However, at 372 days the $\delta^{13}C$ values of the endogeic species were significantly ^{13}C-enriched ($\delta^{13}C = 24.2 \pm 1.4$‰).

The bulk $\delta^{15}N$ values of earthworms (Fig. 1) indicate that endogeic worms were utilising a more ^{15}N-enriched food source than epigeic species for most of the experiment. The

DOI: 10.1002/rcm

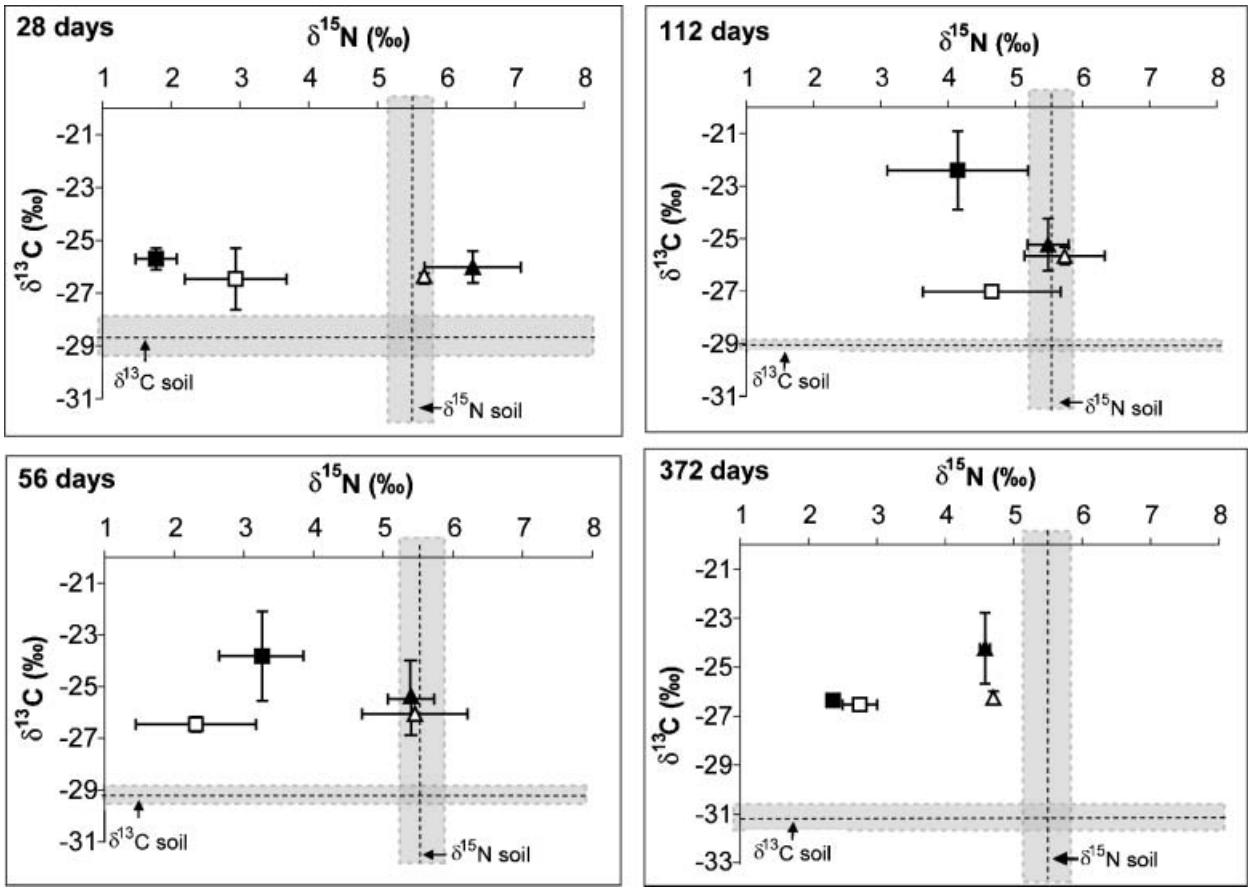

Figure 1. Bulk $\delta^{13}C$ and $\delta^{15}N$ values of *Lumbricus rubellus* collected from beneath C_3 (□) and C_4 (■) dung pats and *Allolobophora chlorotica* collected from beneath C_3 (△) and C_4 (▲) dung pats after 28, 56, 112 and 372 days, plotted with control soil (0–1 cm) bulk mean $\delta^{13}C$ and $\delta^{15}N$ values; shaded region = $\pm 1\sigma$.

$\Delta^{15}N$ value between the C_3 and C_4 dung was 3.2‰, which is a larger $\Delta^{15}N$ value than that between the mean $\delta^{15}N$ values for the epigeic and endogeic earthworms; however, there was no significant difference between $\delta^{15}N$ values for earthworms collected beneath either dung type.

Fatty acid distributions

Total FA were extracted from tissues of *L. rubellus* and *A. chlorotica*. Twenty-eight FA were identified (Fig. 2), and these were common to both earthworms beneath either C_3 or C_4 dung. The abundance of the major FA (>5%) in both species was notably similar (Table 1); *n*-octadecanoic acid ($C_{18:0}$) accounted for a maximum of ca. 40% in both species after 372 days, with maximum abundances of $C_{16:0}$ (ca. 9%) also attained by this date. Similarly, maximum contributions of *Z*-9-octadecanoic acid ($C_{18:1_{\omega}9}$) were determined after 56 days: $10.5 \pm 4.0\%$ for *L. rubellus*, and $11.5 \pm 7.3\%$ for *A. chlorotica*. However, this trend did not apply to all FA; for example, maximum abundances of bacterial *n*-heptadecanoic acid ($C_{17:0}$) were $8.1 \pm 4.0\%$ after 56 days for the epigeic species, and $9.7 \pm 0.7\%$ after 372 days for the endogeic species. Polyunsaturated fatty acids (PUFA) eicosa-5,8,11,14-tetraenoic acid ($C_{20:4_{\omega}6}$) and eicosa-5,8,11,14,17-pentaenoic acid ($C_{20:5_{\omega}3}$) were below detection limits in both dung and soil (Fig. 3). However, total PUFAs ($C_{18:3_{\omega}6}$, $C_{18:3_{\omega}3}$, $C_{20:2_{\omega}6}$, $C_{20:3_{\omega}6}$, $C_{20:4_{\omega}6}$, $C_{20:4_{\omega}3}$, $C_{20:5_{\omega}3}$, $C_{22:4_{\omega}6}$, $C_{22:5_{\omega}3}$) contributed a maximum of ca. 50% and 30% of the total FA of *L. rubellus* and *A. chlorotica*, respectively, after 28 days. This declined over the experimental period to around 30% of these values after 372 days, and appeared to be compensated for by increases in the major saturates, $C_{18:0}$ and $C_{16:0}$. The bacterial component of earthworm FA, i.e. *i*/*a*$C_{15:0}$, $C_{15:0}$, *i*/*a*$C_{16:0}$, *i*/*a*$C_{17:0}$, $C_{17:0}$, *i*/*a*$C_{18:0}$,[61,67] were comparatively consistent, contributing around 20% of the relative abundance of total FA in both species at each sample date. Octadeca-9,12-dienoic acid ($C_{18:2_{\omega}6}$) accounted for <13% of the relative abundance of the *L. rubellus* FA, and ca. 4% of *A. chlorotica* FA after 56 days.

Fatty acid compound-specific $\delta^{13}C$ determinations

Individual FA $\delta^{13}C$ values for *L. rubellus* after 28 days (Fig. 4(a)) show that $C_{16:0}$, *i*$C_{17:0}$, *a*$C_{17:0}$ and octadeca-9,12,15-trienoic acid ($C_{18:3_{\omega}3}$) were 1.5–7.6‰ more ^{13}C-enriched in the earthworms under the C_4 dung than those under the C_3 dung. Significantly, bulk $\delta^{13}C$ values showed no difference between FA of earthworms from C_3 and C_4 dung-treated plots at this time (Fig. 1). After 56 days (Fig. 4(b)), all *L. rubellus* FA apart from $C_{18:2_{\omega}6}$ and $C_{20:4_{\omega}6}$ and $C_{20:5_{\omega}3}$ had increased $\delta^{13}C$ values of 2.8–5.7‰ under C_4 dung compared with *L. rubellus* under C_3 dung. Maximum bulk $\delta^{13}C$ values were determined after 112 days for the epigeic species

DOI: 10.1002/rcm

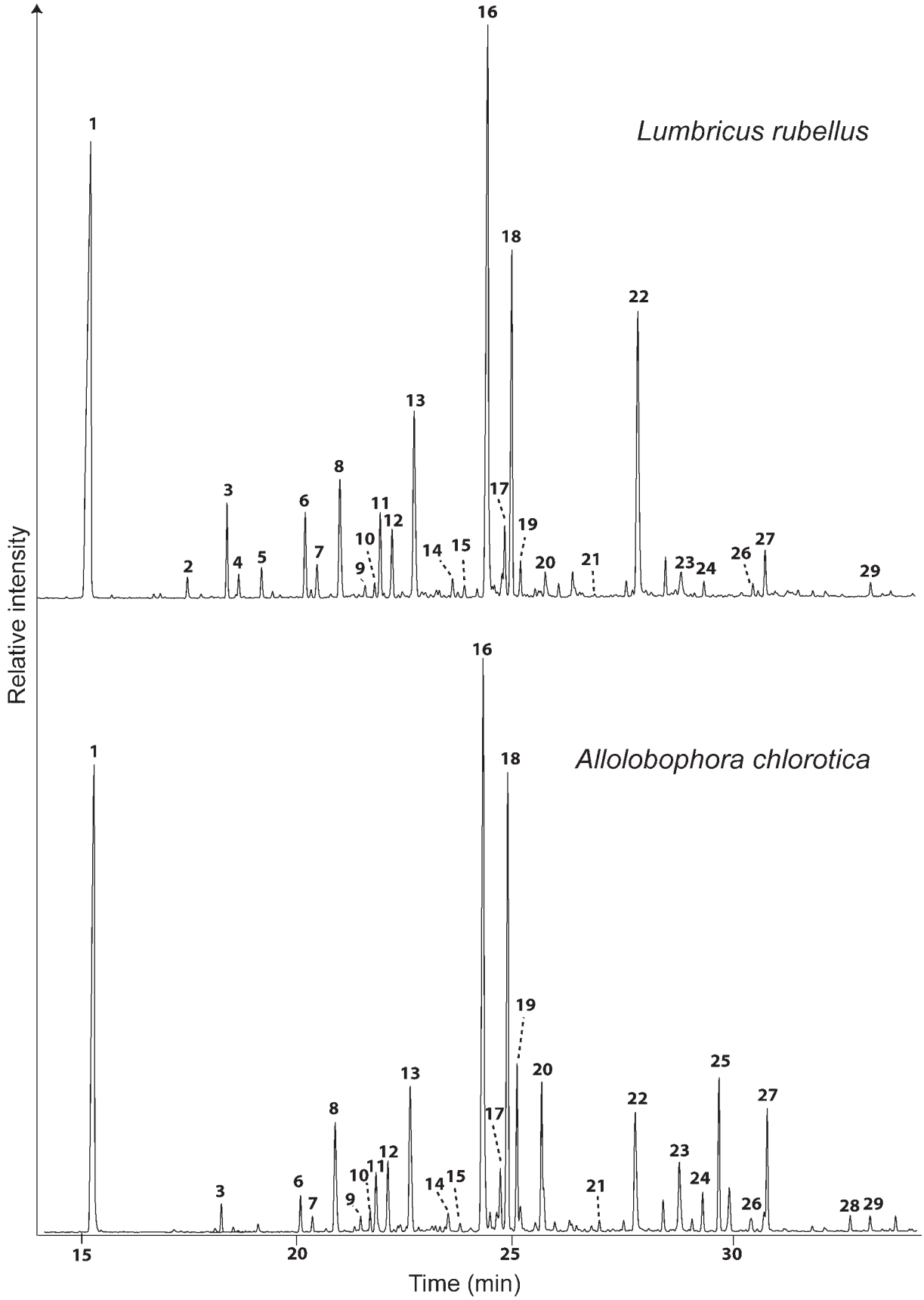

Figure 2. Partial TIC chromatograms of *Lumbricus rubellus* and *Allolobophora chlorotica* fatty acid methyl esters collected from C_4 dung-treated plots after 56 days. Key: 1 = i.s. (*n*-nonadecane); 2. $C_{14:0}$; 3. $iC_{15:0}$; 4. $aC_{15:0}$; 5. $C_{15:0}$; 6. $iC_{16:0}$; 7. $aC_{16:0}$; 8. $C_{16:0}$; 9. $C_{16:1_{\omega}9}$; 10. $C_{16:1_{\omega}7}$; 11. $iC_{17:0}$; 12. $aC_{17:0}$; 13. $C_{17:0}$; 14. $iC_{18:0}$; 15. $aC_{18:0}$; 16. $C_{18:0}$; 17. $C_{18:1_{\omega}11}$; 18. $C_{18:1_{\omega}9}$; 19. $C_{18:1_{\omega}7}$; 20. $C_{18:2_{\omega}6}$; 21. $C_{18:3_{\omega}6}$; 22. $C_{18:3_{\omega}3}$; 23. $C_{20:2_{\omega}6}$; 24. $C_{20:3_{\omega}6}$; 25. $C_{20:4_{\omega}6}$; 26. $C_{20:4_{\omega}3}$; 27. $C_{20:5_{\omega}3}$; 28. $C_{22:4_{\omega}6}$; 29. $C_{22:5_{\omega}3}$.

DOI: 10.1002/rcm

Table 1. FA compositions of *Lumbricus rubellus* and *Allolobophora chlorotica* associated with dung pats after 28, 56, 112 and 372 days. FA compositions are mean percentages of total FA $\pm 1\sigma$. All individual FA present at >5% abundance are shown

	Time (days)							
	Lumbricus rubellus				*Allolophobora chlorotica*			
FA	28	56	112	372	28	56	112	372
$iC_{16:0}$	1.4 ± 0.7	2.8 ± 0.5	2.9 ± 1.8	1.4 ± 1.5	1.6 ± 0.9	2.2 ± 1.5	2.6 ± 1.0	1.8 ± 0.7
$C_{16:0}$	3.5 ± 1.4	5.7 ± 0.5	5.5 ± 2.3	8.5 ± 3.5	6.4 ± 3.6	6.3 ± 1.8	8.4 ± 3.4	8.7 ± 2.7
$iC_{17:0}$	1.8 ± 0.1	3.0 ± 0.8	2.9 ± 1.2	3.3 ± 0.5	2.6 ± 1.0	2.8 ± 0.8	3.5 ± 0.7	3.1 ± 1.0
$aC_{17:0}$	1.1 ± 0.2	2.4 ± 0.8	2.2 ± 1.0	2.4 ± 1.1	2.6 ± 1.2	3.1 ± 0.6	4.2 ± 1.4	3.4 ± 0.7
$C_{17:0}$	5.8 ± 1.4	8.1 ± 0.5	7.6 ± 3.2	6.6 ± 4.7	7.6 ± 2.6	6.7 ± 2.5	9.0 ± 2.7	9.7 ± 0.7
$C_{18:0}$	16.1 ± 6.4	31.9 ± 6.0	26.5 ± 8.8	42.3 ± 17.5	28.2 ± 11.7	25.1 ± 5.8	30.6 ± 13.3	42.6 ± 17.3
$C_{18:1_{\omega}9}$	8.8 ± 3.4	10.5 ± 4.0	6.2 ± 7.1	7.3 ± 7.4	6.8 ± 4.6	11.5 ± 7.3	9.6 ± 7.1	7.2 ± 2.0
$C_{18:1_{\omega}7}$	1.0 ± 0.8	0.8 ± 0.6	1.0 ± 1.1	4.1 ± 6.8	2.6 ± 2.3	3.6 ± 4.4	3.3 ± 3.8	6.5 ± 9.2
$C_{18:2_{\omega}6}$	4.0 ± 1.2	12.7 ± 5.2	1.6 ± 0.8	0.9 ± 0.8	1.3 ± 4.6	4.1 ± 1.2	3.1 ± 1.4	2.4 ± 2.0
$C_{18:3_{\omega}3}$	8.2 ± 4.5	0.4 ± 1.8	6.7 ± 6.8	4.8 ± 2.1	4.2 ± 2.6	3.7 ± 1.4	2.0 ± 2.3	2.1 ± 0.8
$C_{20:4_{\omega}6}$	8.1 ± 3.4	1.6 ± 2.1	4.2 ± 2.7	0.4 ± 0.4	7.3 ± 5.7	4.5 ± 3.9	3.8 ± 3.1	1.3 ± 1.8
$C_{20:5_{\omega}3}$	11.6 ± 7.2	2.7 ± 0.4	3.7 ± 1.2	1.8 ± 1.8	6.6 ± 6.4	5.5 ± 4.0	1.8 ± 1.5	2.4 ± 3.5

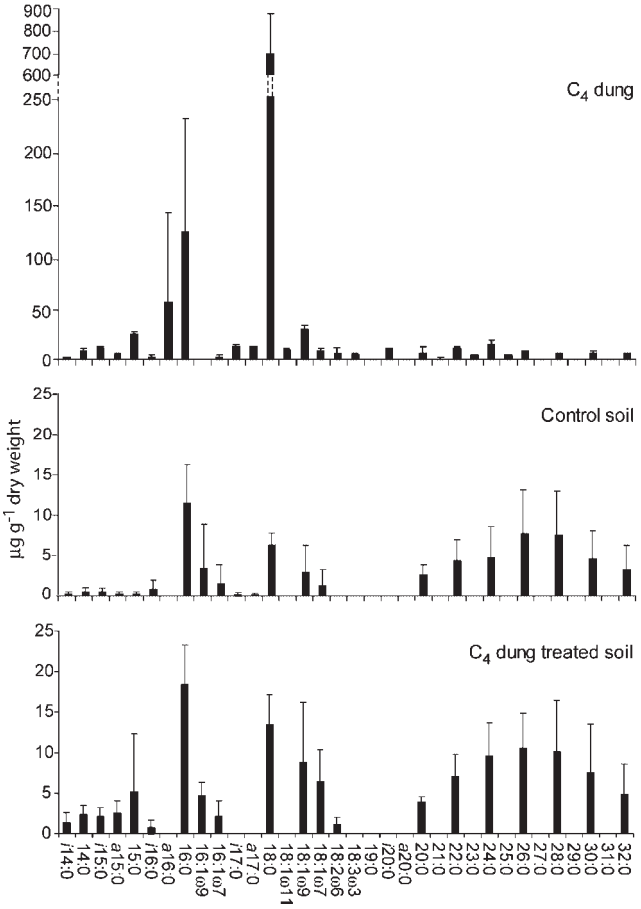

Figure 3. Relative abundance distributions of free fatty acids extracted from fresh C_4 dung, and control and C_4 dung-treated soils (0–1 cm) after 56 days.[49]

under C_4 dung, and this coincided with FA $\Delta^{13}C_{C3\text{-}C4}$ values of 5.1–11.1‰, showing significant incorporation of dung C into all FA of *L. rubellus* aggregating under C_4 dung pats, compared with those under C_3 dung pats (Fig. 4(c)). However, after 372 days (Fig. 4(d)) there was no significant difference between *L. rubellus* FA from either C_3 or C_4 dung-treated plots.

In comparison with the epigeic species, endogeic *A. chlorotica* from C_4 dung-treated plots showed minor ^{13}C-enrichment of FA, relative to those in C_3 plots, after 28, 112 and 372 days, and no effect after 56 days. The ^{13}C-enrichment of $C_{18:3_{\omega}3}$ ($\delta^{13}C = -27.6 \pm 0.8$‰) was determined after 28 days (Fig. 4(e)); $C_{16:0}$ ($\delta^{13}C = -25.5 \pm 1.1$‰), $C_{18:1_{\omega}9}$ ($\delta^{13}C = -29.4 \pm 0.2$‰) and $C_{18:3_{\omega}3}$ ($\delta^{13}C = -27.7 \pm 0.9$‰) after 112 days (Fig. 4(g)). However, unlike *L. rubellus*, significant ^{13}C-enrichments were observed in *A. chlorotica* sampled from beneath C_3 dung pats in $C_{18:1_{\omega}9}$ ($\Delta^{13}C_{C4\text{-}C3} = 3.7$‰) after 28 days, and $aC_{17:0}$ ($\Delta^{13}C_{C4\text{-}C3} = 1.9$‰) and $C_{18:0}$ ($\Delta^{13}C_{C4\text{-}C3} = 1.8$‰) after 372 days (Fig. 4(h)).

DISCUSSION

Bulk $\delta^{15}N/\delta^{13}C$ values of earthworms

Bulk $\delta^{15}N$ and $\delta^{13}C$ values demonstrated that, over the course of the experiment, epigeic and endogeic earthworm species displayed trophic niche differentiation when feeding on dung (Fig. 1). Endogeic species in both C_3 and C_4 dung-treated soils were consuming N that was significantly more ^{15}N-enriched than were the epigeic species. This agrees with the findings of previous natural abundance $^{13}C/^{15}N$ bulk stable isotope studies of earthworm ecology,[8,10,11,42–44] which suggest that endogeic earthworms consume ^{15}N-enriched N compared with epigeic litter-feeders due to progressive ^{15}N-enrichment with on-going mineralisation with increasing soil depth.[43,45] Although both C_3 and C_4 dung were comparatively ^{15}N-enriched relative to bulk soil, and C_3 dung was 3.2‰ more ^{15}N-enriched than C_4 dung, there was no overall significant difference between bulk $\delta^{15}N$ values for earthworms associated with either treatment. This indicates that the majority of earthworms were either using an N source other than dung, or that the N in their tissues was in a stable pool; the long life-span and slow tissue N turnover of earthworms makes them less sensitive to short-term changes in substrate availability.[44] Nevertheless, our results show that the tissues of the epigeic earthworm, *L. rubellus*, were

DOI: 10.1002/rcm

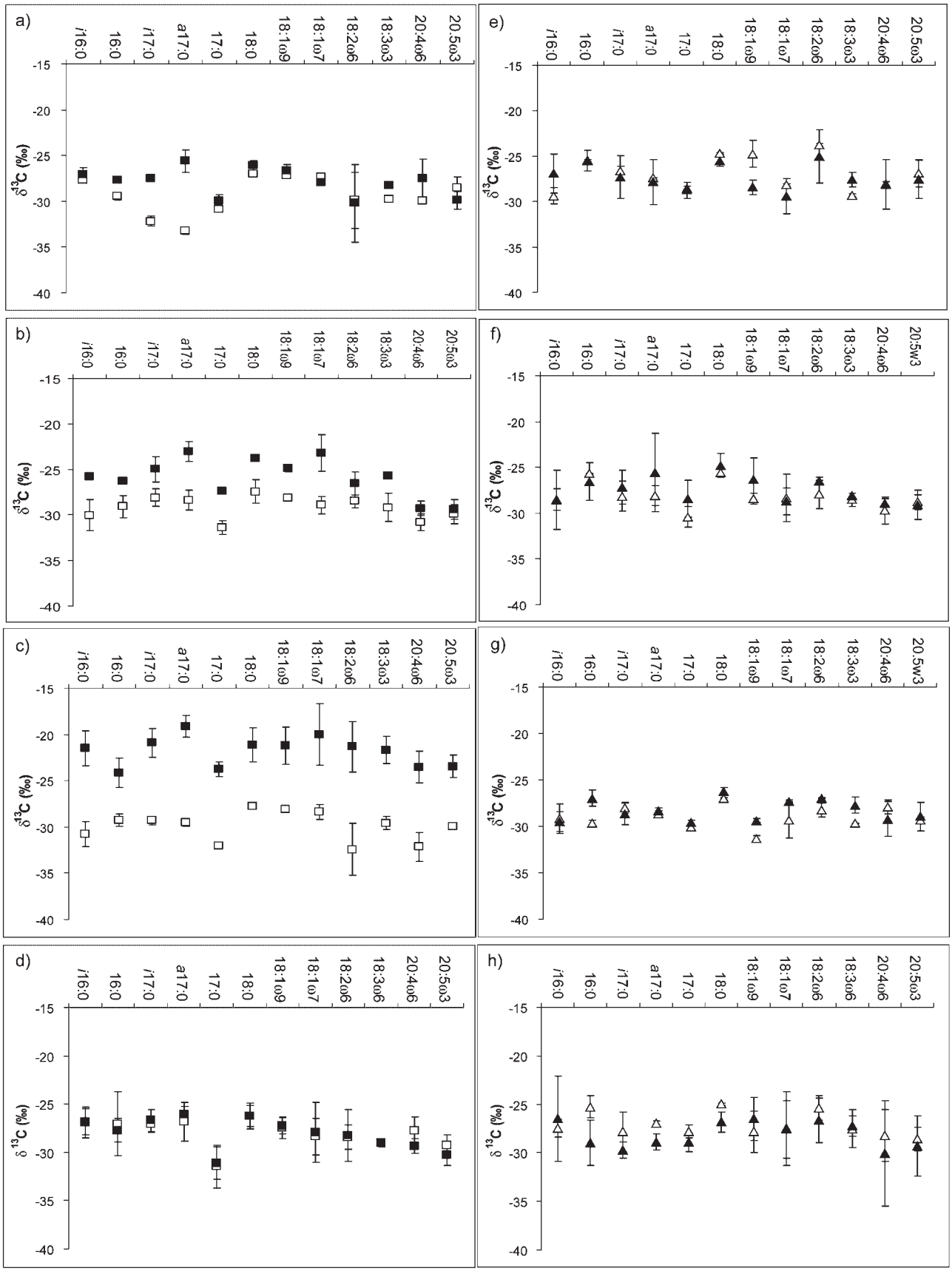

Figure 4. $\delta^{13}C$ values for individual fatty acids extracted from *Lumbricus rubellus* (a–d) and *Allolobophora chlorotica* (e–h) body tissues after 28 (a, e), 56 (b, f), 112 (c, g) and 372 days (d, h). Key: *L. rubellus* C_3 (□), C_4 (■); *A. chlorotica* C_3 (△), C_4 (▲).

DOI: 10.1002/rcm

comparatively ^{15}N-enriched after 112 days in both C_3 and C_4 dung-treated plots. As this coincided with significant enrichment in $\delta^{13}C$ values in this species in the C_4 dung-treated plots (see below) this provides additional, though tentative, evidence for the utilisation of dung by this litter-feeding species. However, the rate of incorporation and turnover of dung N in earthworm tissues needs to be further explored to understand the full significance of these findings.

L. rubellus and *A. chlorotica* sampled from C_3 dung-treated soils displayed bulk $\delta^{13}C$ values ranging between −26 and −27‰ at all sample times (Fig. 1). These $\delta^{13}C$ values were consistently ^{13}C-enriched compared with bulk soil, vegetation and C_3 dung, beyond the previously observed 1‰ trophic enrichment.[46] This implies that the majority of earthworm body tissue C is derived from a ^{13}C-enriched component of OM, e.g. the carbohydrate fraction,[47] and incorporated into tissues with slow turnover rates that are relatively unaffected by short-term fluctuations of the bulk $\delta^{13}C$ value of soil OM, i.e. collagenous cuticle. We found that the mean $\delta^{13}C$ values of individual monosaccharide components of untreated control soils were 6‰ more enriched in ^{13}C than bulk soil,[48] and monosaccharides from C_3 dung were up to 9‰ more ^{13}C-enriched than bulk C_3 dung.[49] Active incorporation of C_4 dung was indicated by significantly elevated $\delta^{13}C$ values of the tissues of epigeic *L. rubellus* after 56 days (bulk $\delta^{13}C = -23.8 \pm 1.6$‰) and 112 days (bulk $\delta^{13}C = -22.4 \pm 1.5$‰), and endogeic *A. chlorotica* after 372 days (bulk $\delta^{13}C = -24.2 \pm 1.4$‰). These findings suggest a temporal difference in the use of dung C by different earthworm species and functional groups. Interestingly, unexpected increases were seen in soil monosaccharide $\delta^{13}C$ values in C_4 dung-treated soils after 372 days,[48] which correlated with bulk ^{13}C-enrichment in *A. chlorotica* tissues. This suggests a link between endogeic geophagy and the reintroduction of recalcitrant dung-derived C to the surface soil horizons that had previously been sequestered in the soil matrix. However, additional feeding experiments with specifically labelled dietary components are required to further explore this phenomenon, and to determine the contribution of substrates with varying stability and abundance to earthworm biochemistry. Other factors such as starvation, diet shift, diet quality, and the consumer's developmental stage can also significantly affect the isotopic signatures in the consumer's tissue. Thus, experiments evaluating the trophic shift of the species studied, which ideally also address their specific life history traits affecting stable isotope signatures, are needed to correctly interpret stable isotope data gathered in the field.[4]

Fatty acid relative abundances

A predominance of $C_{20:4_{\omega}6}$ and $C_{20:5_{\omega}3}$, with significant $C_{18:2_{\omega}6}$ and $C_{18:3_{\omega}3}$, was observed in earthworms by Hansen and Czochanska,[16] and Sampedro *et al.*[18] observed that $C_{18:0}$, $C_{18:1_{\omega}7}$, $C_{18:1_{\omega}9}$, $C_{18:2_{\omega}6}$, $C_{20:4_{\omega}6}$ and $C_{20:5_{\omega}3}$ were the most abundant FA in the anecic earthworm *L. terrestris*. In this study, the most abundant FA in *L. rubellus* and *A. chlorotica* were $C_{18:0}$, $C_{18:1_{\omega}9}$, $C_{17:0}$ and $C_{16:0}$ (Table 1). These new data show differences in FA % composition between species sampled *in vivo*, which varied with time. This demonstrates that FA incorporation and biosynthesis differs according to FA type between species, and may be due to seasonal variations in food quality and availability[16] or adaptive responses to environmental conditions, such as temperature.[50]

A wide variety of FA ranging from $C_{10:0}$ to $C_{32:0}$ have been identified in earthworms.[16–18,51–53] Based on FA profiles, it has been proposed that earthworms derive their FA directly from plant material and microorganisms, while others are biosynthesised *de novo*.[15,16] Recently, Sampedro *et al.*[18] and Sampedro and Whalen[17] found that diverse bacterial and fungal-derived FA, which earthworms cannot biosynthesise *de novo*, were present in the gut and body walls, and storage lipids. Therefore, there are three pathways of dung C assimilation into earthworm FA: (i) direct incorporation of dung FA; (ii) secondary incorporation via ingestion of microorganisms that have previously used dung C for biosynthesis; and (iii) ingestion of dung constituents and *de novo* biosynthesis of FA using products of digestion.

Fatty acid $\delta^{13}C$ values

In this study, evidence for each of the above pathways is provided by the occurrence of specific FA in earthworm body tissues from C_4 dung-treated plots that have higher $\delta^{13}C$ values than those from C_3 dung-treated plots. Significant differences were determined between $\delta^{13}C$ values of FA in both earthworm species. The FA of *L. rubellus*, the litter-feeding species, in the C_4 dung-treated plots showed more extensive dung C incorporation than *A. chlorotica*, the geophagous species (Fig. 4). The $\delta^{13}C$ values of *L. rubellus* (Figs. 4(a)–4(d)) corresponded closely to bulk $\delta^{13}C$ values (Fig. 1), suggesting major incorporation of dung C into all FA after 56 and 112 days when bulk dung C incorporation into soil was highest. Maximum dung C incorporation after 56 days was caused by heavy rainfall[39] which would probably have promoted earthworm activity in general and also enhanced access to dung C, especially for the geophagous species; however, no ^{13}C-enrichment in *A. chlorotica* FA was determined after 56 days (Fig. 4(f)). This suggests that the *A. chlorotica* sampled were predominantly ingesting and assimilating indigenous C due to their geophagous feeding habits. Endogeic earthworms are more common in the top 10 cm of soil,[54,55] but can burrow up to 30 cm depth.[56]

Maximum abundances of $C_{20:4_{\omega}6}$ and $C_{20:5_{\omega}3}$ were observed in both epigeic and endogeic earthworm species after 28 days (Table 1). $C_{20:4_{\omega}6}$ and $C_{20:5_{\omega}3}$ have been described as typical soil microfauna FA, and have been identified in nematodes[57] and Collembola.[25,26] However, these polyunsaturates were not evident in the dung or soil (Fig. 3), indicating *de novo* biosynthesis in both *L. rubellus* and *A. chlorotica*. A similar conclusion was drawn by Chamberlain and Black[25] when considering Collembola FA compositions. Elevated $\delta^{13}C$ values of $C_{20:4_{\omega}6}$ and $C_{20:5_{\omega}3}$ of *L. rubellus* in C_4 dung-treated plots after 112 days (Fig. 4(c)) indicated utilisation of relatively stable constituents of the dung for fatty acyl lipid biosynthesis in late summer. At the same time, significant ^{13}C-enrichment was determined in all other FA in *L. rubellus* tissues, which coincided with maximum bulk

DOI: 10.1002/rcm

$\delta^{13}C$ values for the species (Fig. 1), indicating major incorporation of dung C into earthworm tissues *per se*. However, significant bulk ^{13}C-enrichment in *A. chlorotica* was evident after 372 days when no ^{13}C-enrichment was determined in any FA. This indicates that alternative storage or structural biochemical components of the earthworm body tissues with slow turnover had incorporated significant dung-derived C in the C_4 dung-treated plots. Therefore, further analysis of the contributing biochemical components to bulk ^{13}C-enrichment in *A. chlorotica* body tissues after 372 days is required. Significantly, ^{13}C-enrichment was observed in $aC_{17:0}$ and $C_{18:0}$ in *A. chlorotica* from the C_3 dung-treated plots after 372 days, in addition to $C_{18:1_{\omega}9}$ after 28 days, suggesting migration of the endogeic species between differently treated plots. Experiments with stained and radio-labelled (^{60}Co) worms showed that epigeic species move at $\sim$2 m month^{-1}, anecic species move at between 0.5 and 3.6 m month^{-1}, and endogeic species at between 1.7 and 3.1 m month^{-1}, and speed of movement varies between fresh burrowing activity, 2–10 cm h^{-1}, and use of existing burrows, 35–56 cm h^{-1}.[58,59] However, Mather and Christensen[60] observed that Lumbricid species were able to travel up to several metres a night during resource-seeking activity.

Bacterial FA derived from phospholipids of cell membranes, i.e. $i/aC_{15:0}$, $C_{15:0}$, $i/aC_{16:0}$, $i/aC_{17:0}$, $C_{17:0}$ and $i/aC_{18:0}$, do not occur in any other organisms and can be used as biomarker compounds.[61] These components constituted a significant proportion of body tissue FA in both *L. rubellus* and *A. chlorotica* (Table 1). Earthworms may specifically consume dung OM because it creates a 'hot spot' for microbial activity. Lattaud *et al*.[62] asserted that most of the enzyme activity in the earthworm gut is due to ingested microflora, while Pokarzhevskii *et al*.[63] proposed that earthworms are 'ecosystemivorous' and rely on consumption of lower level organisms for resources such as enzymes and amino acids. Odd-chain and methyl-branched FA are only biosynthesised by bacteria.[18] Therefore, evidence for the direct uptake and incorporation of bacterial FA derived from C_4 dung is indicated by the elevated $\delta^{13}C$ values of methyl-branched odd-chain FA, e.g. $iC_{17:0}$ ($\delta^{13}C = -27.5 \pm 0.2$‰) and $aC_{17:0}$ ($\delta^{13}C = -25.6 \pm 1.2$‰), in earthworms sampled from C_4 dung-treated plots, i.e. *L. rubellus* tissues after 28, 56 and 112 days, and $aC_{17:0}$ ($\delta^{13}C = -27.0 \pm 0.3$‰) in *A. chlorotica* tissues in C_3 dung-treated plots after 372 days (Fig. 4). These bacterial FA may derive: (a) directly from extant colonies or gut bacterial debris from the dung; (b) from soil microbial biomass that used dung C as a resource; or (c) from the mutualistic gut microbial population within the earthworm. The very depleted $\delta^{13}C$ values of $iC_{17:0}$ and $aC_{17:0}$ in *L. rubellus* tissues after 28 days (Fig. 4(a)), -32.2 ± 0.6‰ and -33.2 ± 1.2‰, respectively, may indicate direct incorporation of bacterial FA from C_3 dung; the $\delta^{13}C$ values of these FA were -37.0 ± 1.3‰ and -37.6 ± 1.1‰ in the C_3 dung compared with -27‰ and -31‰ in the control (no dung) soil.[49] The aforementioned depleted values are within the reported range for phospholipid fatty acids (PLFA), e.g. Boschker *et al*.[64] showed that specific bacterial membrane lipids from a mixed culture were ^{13}C-depleted by about 4–6‰ relative to their substrate.

Earthworms are also unable to biosynthesise unsaturated FA $C_{18:1_{\omega}9}$, $C_{18:1_{\omega}7}$, $C_{18:2_{\omega}6}$,[18] and studies of other invertebrates have shown they are incapable of biosynthesising $C_{18:3_{\omega}3}$;[65] therefore, these are essential FA (EFA) for earthworms. $C_{18:1_{\omega}9}$ is the most abundant monoenoic acid in grassland plant aerial vegetation, roots and soil,[66] and was a significant component of C_4 dung (Fig. 3). Therefore, the ^{13}C-enrichment in $C_{18:1_{\omega}9}$ from *L. rubellus* after 56 and 112 days, and in *A. chlorotica* after 112 and 28 days (in the C_3 dung-treated plots), strongly indicates the direct incorporation of dung C despite the potential for dilution of the $\delta^{13}C$ value of the FA by indigenous sources. The mean $\delta^{13}C$ value for free mono-, di- and triunsaturated $C_{18:0}$ in the dung was -16.8 ± 0.26‰, and -30.32 ± 2.0‰ for similar unsaturated FA in the control soil (56 days; 0–1 cm[49]). $C_{18:3_{\omega}3}$ is also a plant FA, found in lower abundance than other polyunsaturated FA in dung (Fig. 3), but significant ^{13}C-enrichment was determined in $C_{18:3_{\omega}3}$ extracted from *L. rubellus* tissues after 28, 56 and 112 days and in *A. chlorotica* after 28 and 112 days suggesting a dung-derived source.

$C_{18:1_{\omega}7}$ is described as a gram-positive bacterial biomarker PLFA.[61] It is found in much lower abundances in the earthworm tissues than the methyl-branched and odd-chain biomarker FA (Table 1). Although this FA was a negligible component of dung (Fig. 3), it was ^{13}C-enriched after 56 and 112 days in *L. rubellus* from C_4 dung-treated plots. Therefore, its incorporation is evidence of secondary incorporation of dung C that has been metabolised by soil bacteria prior to ingestion. Like $C_{18:1_{\omega}7}$, ^{13}C-enriched $C_{18:2_{\omega}6}$ was only found in *L. rubellus* from C_4 dung-treated plots after 112 days, suggesting that the epigeic earthworm was actively foraging in dung-treated plots at this time. $C_{18:2_{\omega}6}$ is commonly described as a fungal biomarker PLFA, based on the work of Frostegard and Baath,[67] and earthworms have been shown to express a preference for OM inoculated with fungal species,[68–70] perhaps because fungi produce abundant EFA. However, this diunsaturate is also a significant component of plant FA,[71] and was found at a concentration of $\sim$1600 μg g^{-1} in both C_3 and C_4 dung.[49] Therefore, there are multiple potential sources of this FA for earthworms in grazed grassland systems.

CONCLUSIONS

We have provided evidence for trophic niche differentiation in two earthworm species with contrasting functional ecologies, exploiting dung as a dietary resource in experiments that lasted for more than 1 year. We have also confirmed that compound-specific stable isotope analysis complements and enhances bulk $^{13}C/^{15}N$ isotope values in investigations of the functional ecology of earthworms, providing extra information on the routing of dietary C into body tissues. In particular, we have determined the direct incorporation of microbial biomarker fatty acids (FA) from ^{13}C-labelled dung *in situ*, the routing of dung C into *de novo* synthesised compounds and the assimilation of essential FA derived from dung. Thus, we have presented novel insights into the role of earthworms in the cycling of C from cow dung in agroecosystems.

DOI: 10.1002/rcm

Acknowledgements

This study was undertaken while JAJD was in receipt of a BBSRC/IGER CASE PhD studentship. The production and supply of C_3- and C_4-labelled cow dung were managed by Roger Evans at IGER-Aberystwyth, and the use of the NERC Life Sciences Mass Spectrometry facilities at the University of Bristol is gratefully acknowledged.

REFERENCES

1. Scheu S, Schlitt N, Tiunov A, Newington J, Jones H. *Oecologia* 2002; **133**: 254.
2. Bouché MB. *Ecol. Bull.* 1977; **25**: 122.
3. Briones MJI, Bol R, Sleep D, Sampedro L, Allen D. *Rapid Commun. Mass. Spectrom.* 1999; **13**: 1300.
4. Traugott M, Pazmandi C, Kaufmann R, Juen A. *Soil Biol. Biochem.* 2007; **39**: 1023.
5. Tiunov A. *Biol. Bull.* 2007; **34**: 395.
6. Gunn A, Cherrett JM. *Pedobiologia* 1993; **37**: 303.
7. Curry JP, Schmidt O. *Pedobiologia* 2007; **50**: 463.
8. Briones MJI, Bol R. *Pedobiologia* 2003; **47**: 560.
9. Briones MJI, Schmidt O. In *Recent Research Developments in Crop Science*, vol. **1**, Pandalai S (ed). Research Signpost: Trivandrum, 2004; 11–26.
10. Neilson R, Boag B, Smith M. *Soil Biol. Biochem.* 2000; **32**: 1053.
11. Schmidt O, Curry JP, Dyckmans J, Rota E, Scrimgeour CM. *Pedobiologia* 2004; **48**: 171.
12. Gearing JN. In *Carbon Isotope Techniques*, Coleman DC, Fry B (eds). Academic Press: San Diego, 1991; 201–218.
13. Evans CJ, Evershed RP, Black HIJ, Ineson P. *Anal. Chem.* 2003; **75**: 6056.
14. Albro PW, Corbett JT, Schroeder JL. *Biochem. Cell Biol.* 1993; **71**: 220.
15. Albro PW, Schroeder JL, Corbett JT. *Lipids* 1992; **27**: 136.
16. Hansen RP, Czochanska Z. *J. Sci. Food Agric.* 1975; **26**: 961.
17. Sampedro L, Whalen JK. *Appl. Soil Ecol.* 2007; **35**: 226.
18. Sampedro L, Jeannotte R, Whalen JK. *Soil Biol. Biochem.* 2006; **38**: 2188.
19. Chamberlain PM, Bull ID, Black HIJ, Ineson P, Evershed RP. *Oecologia* 2004; **139**: 325.
20. Chamberlain PM, Bull ID, Black HIJ, Ineson P, Evershed RP. *Soil Biol. Biochem.* 2006; **38**: 1275.
21. Chamberlain PM, Bull ID, Black HIJ, Ineson P, Evershed RP. *Soil Biol. Biochem.* 2006; **38**: 1146.
22. Haubert D, Langel R, Scheu S, Ruess L. *Pedobiologia* 2005; **49**: 229.
23. Haubert D, Haggblom MM, Langel R, Scheu S, Ruess L. *Soil Biol. Biochem.* 2006; **38**: 2004.
24. Ruess L, Schutz K, Haubert D, Haggblom MM, Kandeler E, Scheu S. *Ecology* 2005; **86**: 2075.
25. Chamberlain PM, Black HIJ. *Comp. Biochem. Phys. B* 2005; **140**: 299.
26. Chamberlain PM, Bull ID, Black HIJ, Ineson P, Evershed RP. *Soil Biol. Biochem.* 2005; **37**: 1608.
27. Ruess L, Tiunov A, Haubert D, Richnow HH, Haggblom MM, Scheu S. *Soil Biol. Biochem.* 2005; **37**: 945.
28. Lowe CN, Butt KR. *Pedobiologia* 2005; **49**: 401.
29. Marhan S, Scheu S. *Pedobiologia* 2005; **49**: 239.
30. Schmidt O, Doherty P, Curry J. Dyckmans J. In *8th International Symposium on Earthworm Ecology*, Krakow, Poland, 2006.
31. Svendsen JA. *J. Anim. Ecol.* 1957; **26**: 411.
32. Holter P. In *Earthworm Ecology*, vol. **5**, Satchell JE (ed). Chapman & Hall: London, 1983; 49–57.
33. Hughes MS, Bull CM, Doube BM. *Biol. Fert. Soils* 1994; **18**: 241.
34. Barley K. *Aust. J. Agr. Res.* 1959; **10**: 179.
35. Guild WJM. In *Soil Zoology*, Kevan DKM (ed). Butterworths Publications Ltd.: London, 1955; 83–98.
36. Hendriksen NB. *Pedobiologia* 1991; **35**: 65.
37. Hendriksen NB. *Biol. Fert. Soils* 1991; **11**: 170.
38. Scown J, Baker G. *Eur. J. Soil Biol.* 2006; **42**: S310.
39. Dungait JAJ, Bol R, Evershed RP. *Isot. Environ. Health* 2005; **41**: 3.
40. Briones MJI, Bol R, Sleep D, Sampedro L. *Pedobiologia* 1999; **43**: 675.
41. Krauss M, Wilcke W, Zech W. *Environ. Sci. Technol.* 2000; **34**: 4335.
42. Neilson R, Boag B. *Pedobiologia* 2003; **47**: 1.
43. Briones MJI, Bol R, Sleep D, Allen D, Sampedro L. *Soil Biol.Biochem.* 2001; **33**: 1673.
44. Schmidt O, Ostle NJ. *Appl. Soil Ecol.* 1999; **12**: 7.
45. Nadelhoffer KF, Fry B. *Soil Sci. Soc. Am. J.* 1988; **52**: 1633.
46. Deniro MJ, Epstein S. *Geochim. Cosmochim. Acta* 1978; **42**: 495.
47. Schweizer M, Fear J, Cadisch G. *Rapid Commun. Mass Spectrom.* 1999; **13**: 1284.
48. Dungait JAJ, Bol R, Evershed RP. *Org. Geochem.* submitted.
49. Dungait JAJ. *PhD thesis*, University of Bristol, Bristol, 2005; 252.
50. Petersen SO, Holmstrup M. *Soil Biol. Biochem.* 2000; **32**: 1787.
51. Lovern JA. *Biochem. J.* 1940; **34**: 709.
52. Cerbulis J, Wight Taylor M. *Lipids* 1969; **4**: 363.
53. Hansen RP, Czochanska Z. *Lipids* 1974; **9**: 825.
54. Jégou D, Cluzeau D, Wolf HJ, Gandon Y, Trehen P. *Biol. Fert. Soils* 1997; **26**: 116.
55. Capowiez Y, Pierret A, Moran C. *Biol. Fert. Soils* 2003; **38**: 301.
56. Bastardie F, Capowiez Y, Cluzeau D. *Appl. Soil Ecol.* 2005; **30**: 34.
57. Ruess L, Haggblom MM, Zapata EJG, Dighton J. *Soil Biol. Biochem.* 2002; **34**: 745.
58. Mazaud D, Bouché B. In *Soil Biology as Related to Land Use Practices*, Dindal D (ed). EPA: Washington DC, 1980; 687–701.
59. Bastardie F, Capowiez Y, Renault P, Cluzeau D. *Biol. Fert. Soils* 2005; **41**: 320.
60. Mather JG, Christensen O. *Pedobiologia* 1992; **36**: 51.
61. Zelles L. *Biol. Fert. Soils* 1999; **29**: 111.
62. Lattaud C, Locati S, Mora P, Rouland C, Lavelle P. *Appl. Soil Ecol.* 1998; **9**: 189.
63. Pokarzhevskii AD, Zaboyev DP, Ganin GN, Gordienko SA. *Soil Biol. Biochem.* 1997; **29**: 559.
64. Boschker HTS, de Brouwer JFC, Cappenberg TE. *Limnol. Oceanogr.* 1999; **44**: 309.
65. Dadd RH. *J. Nutr.* 1980; **110**: 1152.
66. Bull ID, Nott CJ, van Bergen PF, Poulton PR, Evershed RP. *Soil Biol. Biochem.* 2000; **32**: 1367.
67. Frostegard A, Baath E. *Biol. Fert. Soils* 1996; **22**: 59.
68. Moody SA, Briones MJI, Piearce TG, Dighton J. *Soil Biol. Biochem.* 1995; **27**: 1209.
69. Manna MC, Jha S, Ghosh PK, Acharya CL. *Bioresource Technol.* 2003; **88**: 197.
70. Bonkowski M, Griffiths BS, Ritz K. *Pedobiologia* 2000; **44**: 666.
71. Van Soest PJ. *The Nutritional Ecology of the Ruminant*. Cornell University Press: Ithaca, NY, 1994.

DOI: 10.1002/rcm