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ORIGINAL PAPER

Organic matter composition and the protist and nematode communities around anecic earthworm burrows

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Abstract By living in permanent burrows and incorporating organic detritus from the soil surface, anecic earthworms contribute to soil heterogeneity, but their impact is still understudied in natural field conditions. We investigated the effects of the anecic earthworm *Lumbricus centralis* on fresh carbon (C) incorporation, soil organic matter composition, protists, and nematodes of a Cambisol under grassland. We used plant material labelled with stable isotope tracers to detect fresh C input around earthworm-occupied burrows or around burrows from which the earthworm had been removed. After 50 days, we sampled soil (0–10 cm depth) in concentric layers around the burrows, distinguishing between drilosphere (0–8 mm) and bulk soil (50–75 mm). *L. centralis* effectively incorporated fresh C into the drilosphere, and this shifted soil organic matter amount and chemistry: total soil sugar content was increased compared to unoccupied drilosphere and bulk soil, and the contribution of plant-derived sugars to soil organic matter was enhanced. Earthworms also shifted the spatial distribution of soil C towards the drilosphere. The total abundance of protists and nematodes was only slightly higher in earthworm-occupied drilosphere, but strong positive effects were found for some protist clades (e.g. *Stenamoeba* spp.). Additional data for the co-occurring anecic earthworm species *Aporrectodea longa* showed that it incorporated fresh C less than *L. centralis*, suggesting that the two species may have different effects on soil C distribution and organic matter quality.

Keywords Decomposition · Lumbricidae · Nematodes · Protists · Soil organic matter · Stable isotope tracers

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Introduction

Anecic earthworms forage at the soil surface for organic detritus which they bring inside their vertical burrows and translocate large amounts of organic matter belowground (Hale et al. 2005; Nuutinen 2011). Together with the earthworms' excreta, the soil near the burrows forms the 'drilosphere', a microhabitat that acts as a hotspot of many edaphic processes, such as C and N incorporation (Andriuzzi et al. 2013; Fahey et al. 2013), mineralization (Don et al. 2008), and nitrification and denitrification (Parkin and Berry 1999). These functions are associated with, and partly driven by, enhanced biological activity. For instance, the soil around burrows of *Lumbricus terrestris* supports bacterial communities that can respond rapidly to the earthworm-mediated input of fresh organic detritus (Tiunov and Scheu 1999).

Considering their widespread presence, anecic earthworms can be a major driver of soil heterogeneity. However, few studies have analysed the quantity and composition of the soil organic matter (SOM) in the drilosphere (Szlavecz 1985; Don et al. 2008), and none tested the effects of anecic earthworms experimentally. This is the first knowledge gap that we address here, as both the quantity and the quality of organic matter are important to biochemical cycling in soil.

Some studies have shown that protists and nematodes can reach higher abundance in *L. terrestris* middens and burrows compared to bulk soil, and significant shifts in community composition have been reported for nematodes (Görres et al. 1997; Maraun et al. 1999; Tiunov et al. 2001a; Tao et al. 2009). However, most studies have been performed on repacked soil in laboratory conditions, and very few have investigated nematodes and protists around natural burrows in the field. In a rare example of the latter, Stromberger et al. (2012) analysed phospholipid fatty acids inside and outside the drilosphere of *L. terrestris*, showing this microhabitat hosts' distinct microfaunal communities. However, the markers used in that study were not specific enough to discriminate between eukaryotic groups, for instance, protists and nematodes, and thus no conclusion on their relative abundance could be made (Stromberger et al. 2012). Hence, the second knowledge gap we address is how the small-scale heterogeneity in SOM quality and quantity driven by earthworm activity may influence protist and nematode community composition.

We performed a field experiment in a temperate grassland soil to test the effects of anecic earthworms on drilosphere soil biochemistry and on abundance and community composition of protists and nematodes associated with naturally formed burrows. Traditionally, the drilosphere around burrows has been delimited as a 2-mm-thick layer, but this has recently been shown to be an underestimation for the anecic *L. terrestris* (Andriuzzi et al. 2013). We focused on *L. centralis* (Bouché), which is morphologically and ecologically similar to the widespread *L. terrestris*. In addition, we were able to sample soil around the burrows of a co-occurring species, *Aporrectodea longa* (Ude), also traditionally classified as anecic. We provided plant material enriched in C stable isotopes (^{13}C) around the surface openings of natural burrows occupied by one of the two target species or from which the earthworm had been removed. We measured the incorporation of fresh surface-derived C and analysed the composition of sugars in the SOM within a radius of 8 mm around burrow walls (drilosphere) and 5 cm away (bulk soil); we focused on non-cellulosic sugars as they provide indications on the relative contribution of fresh and decomposed organic matter (see methods for details). Finally, we assessed the effects of earthworm presence (*L. centralis*) on abundance of protists, in total, and divided into morphologically determined clades, and on nematodes, in total, and divided into families and feeding groups. We tested the hypotheses that the drilosphere is a hotspot of incorporation of fresh organic matter from the soil surface; that it supports higher abundance of protists and nematodes than bulk soil; and that earthworm activity is necessary to maintain this status as a soil biochemical and biological hotspot.

Materials and methods

Experimental design and data collection

The field experiment was performed between April and May 2013 in Lusignan, France (46°25′12.91″ N, 0°07′ 29.35″ E). The site is part of the SOERE ACBB (http://www.soere-acbb.com/), which investigates the impact of agricultural management on soil biogeochemical cycles and biodiversity. Soil type is Cambisol with a silty-loamy texture (Chabbi et al. 2009), no $CaCO_3$, pH 6.4, organic C 14.0 g kg^{-1} soil and N 1.6 g kg^{-1} soil. In 2005, a mixture of three grass species was sown (*Lolium perenne*, *Festuca arundinacea* and *Dactylis glomerata*), with the addition of 120 kg N ha^{-1} $year^{-1}$. Grass was mown at 5-cm height and removed 2 days before the experimental set-up.

Plant material enriched in ^{13}C was produced in a greenhouse at the University College Dublin (Ireland). Two-week-old maize seedlings (*Zea mays*) were labelled using the urea leaf-feeding method of Schmidt and Scrimgeour (2001), by daily spraying with a ^{13}C-enriched urea solution (97 atom% ^{13}C). To further boost ^{13}C labelling, the seedlings were enclosed for 2 (non-consecutive) days in commercially available transparent polyethylene bags that can be hermetically sealed; by inserting a thin tube under a sealable cap, acid (35 % HCl) was injected into a vial containing 99 % ^{13}C sodium bicarbonate just before sealing, releasing ^{13}C-enriched CO_2. Labelling lasted 10 days (8 days urea, 2 days sodium bicarbonate). The maize shoots were harvested and cut into 5-

mm fragments, which were thoroughly mixed and transported fresh to the field site after 2 days. We recognize that this material (from young, green plants, high in N) does not represent actual litter or residue from senescent plants, but comparable material is available to soil animals through agronomic practices such as green manure, mulching and cover cropping.

The openings of anecic earthworm burrows (n=28), made by either *L. centralis* or *A. longa*, were located by visual inspection of the soil surface in a 400 m^2 area, and an experimental design based on Andriuzzi et al. (2013) was applied. A minimum distance of 1 m between burrows was deemed sufficient for independence of replicates. To establish an exclusion treatment to test earthworm presence effects, the resident anecic earthworms were removed from nine burrows, which later received labelled material, using 50 ml of a 0.2 % *v*/*v* allyl-isothiocyanate solution (AITC) as in Stromberger et al. (2012). The solution was injected with a syringe and did not touch the burrows' walls in the top 10 cm of soil. Nevertheless, to account for the potential confounding effects of AITC and earthworm removal, anecic earthworms were removed by the same procedure from another nine burrows and then re-introduced after rinsing with water. Additionally, occupied burrows (n=4) were chosen as isotopic controls; that is, they were not given the labelled maize in order to measure the natural isotope abundance.

All burrows, including the isotopic controls, were rinsed with 0.5 L of water to wash down AITC and avoid water addition biases. The burrows from which the anecic earthworms were successfully removed are hereafter referred to as 'unoccupied burrows', but smaller invertebrates were active around them, e.g. smaller earthworms and slugs were observed during sampling. In an 8-cm radius around each burrow opening, 10±0.2 g of the labelled maize were placed on the soil surface and fixed with a metal mesh cage (0.5 cm mesh size) to prevent displacement by wind and rain.

Sample collection

After 50 days, mesh cages were removed. No maize material was detected above the earthworm-occupied burrows, while fragments were still found above unoccupied burrows. Dilute AITC was injected into the burrows to expel resident earthworms as described above, and intact soil blocks (15×15 cm, 10 cm deep) containing individual burrows were excavated with a knife. Earthworm presence/removal or burrow structure could not be ascertained in some samples, reducing the sample size (see "Stable isotope ratio and non-cellulosic sugar analyses" and "Biotic analyses").

Shortly after collection, the soil blocks were transported to a laboratory. Using a mini spatula, four concentric layers (0–2, >2 to 4, >4 to 8 and 50–75 mm) were taken around each burrow. Samples were sub-divided for chemical (SOM and stable isotopes) and biotic analyses (abundance of protists and nematodes); due to the different requirements of soil amount and difficulty in separating some layers in some samples, not all analyses could be performed. After weighing, earthworms were fixed in pure ethanol, and caudal segments were cut, dissected and freeze-dried (unlabelled controls n=2 *L. centralis*, n=2 *A. longa*; labelled n=5 *L. centralis*, n=2 *A. longa*).

Stable isotope ratio and non-cellulosic sugar analyses

Soil samples for chemical analyses were oven-dried at 50 °C for 24 h. A sub-set was analysed for stable isotope ratios (isotopic controls n=3 replicates×4 concentric layers; isotopically labelled n=7×4 unoccupied, n=4×4 *L. centralis*, n=2×4 *A. longa*). Oven-dried soil and freeze-dried earthworm samples were powdered in a ball mill (Mixer Mill MM 200, Retsch, Haan, Germany), weighed into tin capsules and sent to Iso-Analytical Ltd. (Cheshire, UK) for the analysis of stable isotope C and N ratios, and total C and N concentrations with an Elemental Analyser–Isotope Ratio Mass Spectrometer (Europa Scientific 20-20). Ethanol preservation is assumed to have only minor effects on intact tissue ^{13}C signatures (Sarakinos et al. 2002). Isotopic values are expressed in the δ-notation in parts per thousand (‰). The maize shoots were highly and consistently enriched in ^{13}C ($\delta^{13}C$=3432.9±2.8 ‰, n=3) compared to plant material in the C_3-dominated grassland site (−31‰<$\delta^{13}C$<−29‰, Sanaullah et al. 2010).

The three layers considered as drilosphere following Andriuzzi et al. (2013) (hereafter designated as 0–2, 2–4 and 4–8 mm) were pooled to obtain sufficient material for SOM analysis (labelled only n=4 unoccupied, n=4 *L. centralis*, n=4 *A. longa*). Neutral non-cellulosic sugars were analysed using gas chromatography after hydrolysis (Rumpel and Dignac 2006). Briefly, 1 g of soil was hydrolysed using 10 ml of 4 M TFA at 110 °C for 4 h, and sugar monomers were transformed into acid alditols. The monosaccharides were recovered and analysed with a HP 6890 gas chromatograph equipped with a flame ionization detector and a silica capillary column (BPX 70, 60-m long, 0.32-mm internal diameter, 0.25-mm film thickness). The following temperature program was used: from 200 °C to 250 °C at 3 °C min^{-1}, isothermal for 15 min. The total sugars were calculated as the sum of all individual monosaccharides. The ratio of (galactose+mannose) to (arabinose+xylose), or GM/AX, was used as an indicator of sugar origin: the lower the ratio, the larger the contribution of fresh plant-derived material.

Biotic analyses

Protists and nematodes in drilosphere (0–8 mm) and bulk soil were extracted from *L. centralis* burrows (n=4 replicates×2 microhabitats) and unoccupied burrows (n=5×2). The soil samples for extraction of protists were kept at 4 °C and sent

to the University of Cologne (Germany) in a cooled Styrofoam box within 48 h of collection. Nematodes were extracted from another set of soil samples (1 g each) shortly after collection, by mixing the soil with water (10 ml), heating at 60 °C for 2 min and then adding formaldehyde (final concentration 4 %), before shipping to SRUC (Edinburgh, UK).

The protists were enumerated using a modified version of the liquid aliquot method (LAM) according to Butler and Rogerson (1995), with slight modifications as described in Geisen et al. (2014a), and identified with an inverted microscope (Nikon Eclipse TS100) at ×100 and ×200 magnifications. Protists were determined to morpho-group level according to Lee et al. (2000), Smirnov and Brown (2004), Smirnov et al. (2011) and Jeuck and Arndt (2013). Naked amoebae were identified up to genus level according to the most recent phylogeny, and individual genera were subsequently grouped into different higher taxonomic levels (Smirnov et al. 2011). The total numbers of flagellates and amoebae per gramme dry weight soil were calculated from the cumulative abundances in microtiter plates and the respective dilution of soil.

The nematodes were counted at ×40 magnification to estimate abundance, identified to family level and allocated to feeding groups: bacterial feeding, fungal feeding, plant parasites, plant-associated (i.e. feeding on root hairs and mycorrhizal hyphae), omnivores and predators (Yeates et al. 1993). Due to low numbers, omnivores and predators were merged, and fungal feeding and plant-associated nematodes were combined into hyphal feeding (the latter also reflecting uncertain trophic classification of plant-associated nematodes found, e.g. *Tylenchus*–see Yeates et al. 1993).

Statistical analyses

To verify the efficacy of the stable isotope tracers, a linear mixed-effect model on soil $\delta^{13}C$ around earthworm-occupied burrows was fitted, using burrow identity as random effect and labelled plant material presence as fixed effect, and subjected to a marginal Wald *F*-test (suitable to unbalanced designs). Means and standard errors of earthworm body $\delta^{13}C$ were calculated separately for labelled and unlabelled specimens (the large difference in variance prevented a direct statistical comparison).

Subsequent analyses were restricted to burrows provided with the labelled plant material. The interactive effects of *L. centralis* (present vs removed) and microhabitat (drilosphere layers vs bulk soil) on soil $\delta^{13}C$, C and N contents, and C/N ratios were analysed in linear mixed-effect models with burrow as random effect. AITC use was included as factor to test for undesired biases, but none were detected. To account for potential body size effects, the analyses were repeated with earthworm weight as covariate, and Spearman's correlation coefficient between earthworm weight and body $\delta^{13}C$ was calculated. The effects of *L. centralis* presence on the GM/AX ratio and total sugar content of SOM in the drilosphere (0–8 mm) were analysed with general linear models. We report data for *A. longa* but do not include them in the main statistical analyses because of the low number of suitable samples for this species.

The effects of *L. centralis*' presence and microhabitat (drilosphere vs bulk soil) on abundance (individuals g^{-1} soil) of nematodes, in total and for each feeding group, and of protists, in total and for taxonomic groups, were tested in linear mixed-effect models with burrow as random effect. The potential differences in the taxonomic composition of protists and nematodes between soil around occupied and unoccupied burrows were visualized with non-metric multidimensional scaling ordination (NMDS), based on a Bray-Curtis dissimilarity matrix generated on the abundance of nematode families or protist groups, and tested with non-parametric multivariate analysis of variance (npMANOVA), which compared the observed data with 1000 random permutations.

Results are reported as mean±standard error (SE) with associated *p*-values estimated from the models; post hoc multiple comparisons were carried out with Tukey HSD when appropriate (≥10 pairwise comparisons). Homogeneity and normality assumptions were checked by visual inspection of the residuals. The variance explained by the mixed-effect models was estimated as conditional R^2 (which includes the random effect, i.e. accounts for variability between burrows) and marginal R^2 (only the fixed effects) following Nakagawa and Schielzeth (2012). Analyses were done in R 2.15.0 (R Development Core Team 2013), using libraries "nlme" (Pinheiro et al. 2013) and "vegan" (Oksanen et al. 2012).

Results

Incorporation of fresh C into soil and earthworm tissue

Soil that had received the labelled plant material was significantly enriched in ^{13}C compared to the isotopic controls (0–10-cm depth, all concentric layers pooled as follows: $\delta^{13}C = -25.86 \pm 0.56$‰ vs -27.88 ± 0.07‰, $p < 0.01$). Around the burrows under labelled material, average soil $\delta^{13}C$ was higher in *L. centralis* drilosphere soil (0–8 mm) than unoccupied drilosphere soil (-23.43 ± 1.02‰ vs -27.25 ± 1.37‰, $p = 0.03$), pointing to a larger incorporation of plant-derived C (Fig. 1). The mixed-effect model explained almost 60 % of the total variance (conditional $R^2 = 0.57$), but less than half as much once the influence of the burrow random effect was removed (marginal $R^2 = 0.22$), indicating that the variability between burrows was important. The few available *A. longa* burrows ($n = 2$) had lower soil $\delta^{13}C$ values than *L. centralis* burrows (-27.08 ± 0.31‰ in the drilosphere).

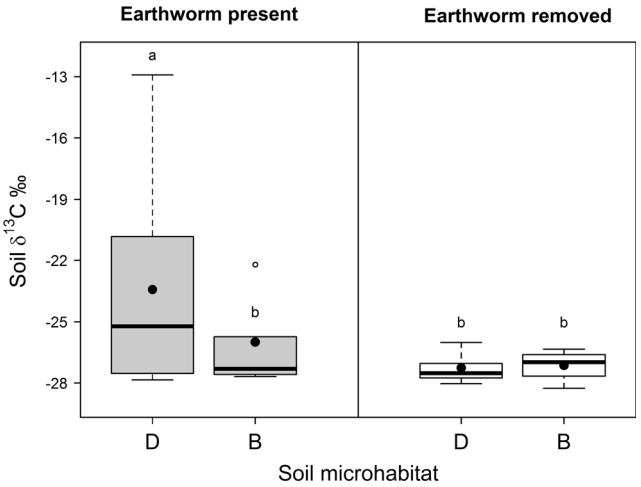

Fig. 1 C stable isotope ratios in soil (0–10-cm depth) around earthworm burrows occupied by *L. centralis* (n=4) and burrows from which the earthworm had been expelled 50 days before sampling (n=7), in the drilosphere (*D*, 0–8 mm around burrow walls) and in bulk soil (*B*, 50–75 mm around burrow walls). Maize fragments labelled with ^{13}C were placed near the burrow entrances at the start of the experiment. The *thick lines* inside the boxes are the medians, the *black dots* are the means, and the *error bars* outside the boxes delimit the interquartile range. *Different letters* mark significant differences ($p \leq 0.05$)

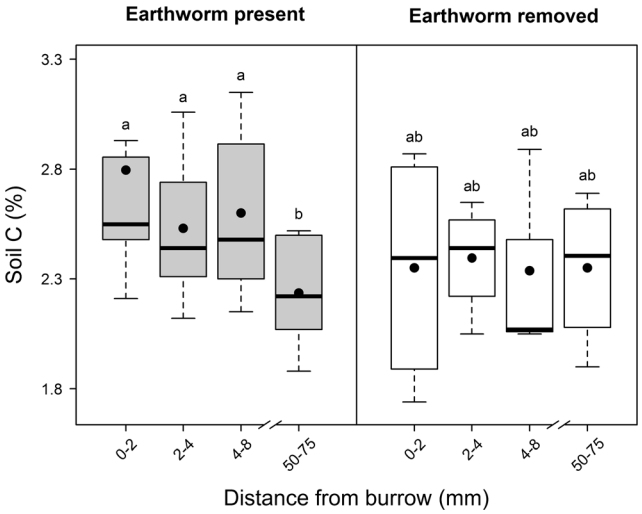

Fig. 2 Total C content in soil (0–10 cm depth) around burrows with a resident *L. centralis* (n=4) and burrows from which the earthworm had been removed (n=7). The *thick lines* and the *dots* inside the boxes are medians and means, respectively, and the *error bars* delimit the interquartile range. *Different letters* mark significant differences ($p \leq 0.05$)

As expected, *L. centralis* specimens from the burrows under labelled maize had on average higher $\delta^{13}C$ (1.67±11.85 ‰) than isotopic control specimens (−21.00±0.13‰), but there was a very high variability. This indicates that some earthworms had fed on the labelled maize more than others; this did not depend on their body size (Spearman's r=0.3, p=0.69). Tissue $\delta^{13}C$ of the *A. longa* specimens (n=2) from labelled burrows was even more variable, ranging from −20.95 to 57.63‰.

Soil C and SOM chemistry

There was no overall difference in total C content between bulk soil and unoccupied *L. centralis* drilosphere (all layers 2.55±0.14 % and 2.36±0.11 % respectively, p>0.10). However, earthworm presence shifted the spatial distribution of C around burrows (Fig. 2): when a worm was resident, the total soil C was significantly higher in the drilosphere than in surrounding soil ($p \leq 0.05$, Tukey HSD), whereas there was no difference around unoccupied burrows (p>0.10). Again, the variability between burrows was high and had a substantial influence on the observed patterns (conditional R^2=0.64, marginal R^2=0.16). The earthworm effects on soil C were not driven by differences in individual body size, because adding individual weight as a covariate did not change the outcome (the covariate was non-significant, and R^2 increased only by 2 %). Like total C content, soil C/N ratio was significantly (p<0.01) higher in drilosphere than bulk soil when *L. centralis* was present (11.18±0.19 vs 10.04±0.11, p<0.01), whereas no such difference was found around unoccupied burrows (10.56±0.18 vs 10.62±0.03). Soil N content itself was neither affected by earthworm presence nor did it differ between drilosphere layers and bulk soil (both in the range 0.17–0.30 %).

The total sugar content in SOM (Fig. 3) was clearly higher (p<0.01) around *L. centralis* burrows (15.1±1.2 mg g^{-1} soil) than around unoccupied burrows (9.2±1.6 mg g^{-1} soil). The presence of a resident earthworm resulted in a higher relative contribution of plant-derived sugars to the SOM sugar pool, as revealed by the lower GM/AX ratio (0.56±0.04*L. centralis*, 0.82±0.06 unoccupied, p<0.01, Fig. 3). The burrows with *A. longa* had total sugar content (10.3±1.6 mg per g of soil) more similar to that of unoccupied burrows than burrows of *L. centralis*, and the same was observed for the GM/AX ratio (0.75±0.06).

Effects of earthworm activity on protists and nematodes

The total abundance of protists was not significantly influenced by the presence of *L. centralis* or the soil microhabitat (drilosphere vs bulk soil), although the highest numbers were recorded in the earthworm-occupied drilosphere (Fig. 4). NMDS ordination and npMANOVA (Supplementary material) did not reveal any potential difference in overall taxonomic composition between soil microhabitats. However, there were interactive effects of earthworm presence and microhabitat on some abundant clades within higher taxonomic clades, viz. the supergroup Amoebozoa and the phylum Cercozoa (Fig. 4 and Supplementary material).

In particular, monotactic amoebae of the amoebozoan class Tubulinea were more abundant in drilosphere than bulk soil around unoccupied burrows (p=0.02), whereas they had

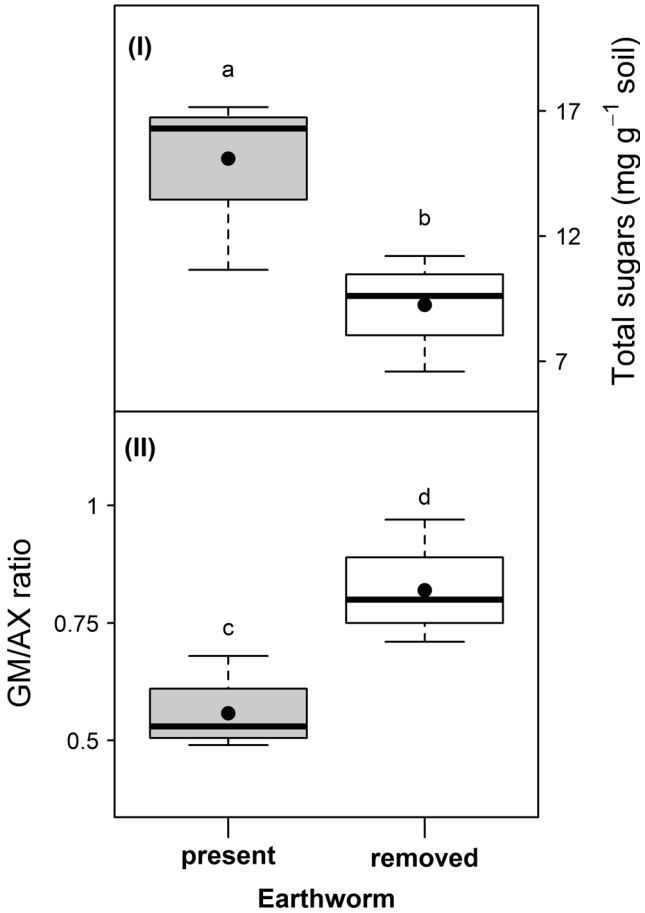

Fig. 3 Total sugar content (*I*) and GM/AX ratio (*II*) in soil organic matter around burrows either occupied by *L. centralis* (n=4) or with earthworm removed (n=4). GM/AX=(galactose+mannose) / (arabinose+xylose); the lower the ratio, the higher the relative contribution of plant-derived sugars vs microbial-derived sugars, indicating less decomposed organic matter. The *thick lines* and the *dots* inside the boxes are medians and means, respectively, and the *error bars* delimit the interquartile range. *Different letters* mark significant differences ($p \leq 0.05$)

similar abundances in the two microhabitats around occupied burrows ($p>0.10$, Fig. 4). Within the amoebozoan class Discosea, amoebae of the genus *Stenamoeba* were more than twice as abundant in the drilosphere with *L. centralis* than in bulk soil ($p=0.01$, Fig. 4), whereas an opposite pattern occurred around unoccupied burrows ($p=0.01$). Amongst the amoeban classes, the larger-bodied *Variosea* were the least responsive (Fig. 4). Flagellates of the order Glissomonadida, from supergroup Cercozoa, were more abundant in the drilosphere than in bulk soil if the earthworm was present ($p=0.04$), while Cercozoa on the whole were more abundant in drilosphere than in bulk soil, whether the earthworm was present or not ($p<0.01$, Supplementary material).

The abundance of nematodes was not markedly affected by earthworm presence or soil microhabitat (drilosphere vs bulk soil $p>0.10$, mixed-effect models), but, similarly to protists, the highest numbers were found in the drilosphere around earthworm-occupied burrows (Fig. 4). There were no recognizable patterns in taxonomic composition (Supplementary material), whether the analysis included rare families or not (absent from 75 % or more of the samples). Most nematodes were hyphal feeders, plant parasites or bacterial feeders (Supplementary material), with Tylenchidae (52.0±4.9 %) and Cephalobidae (13.3±1.5 %) as the most represented families.

Discussion

Anecic earthworms maintain hotspots of fresh incorporated C

We confirmed that the drilosphere (in 0–10-cm depth) of the anecic earthworm *L. centralis* is much thicker than traditionally presumed (at least 8 mm as compared to 2 mm), as was shown previously for *L. terrestris* in intact soil in the field (Andriuzzi et al. 2013). The added plant material was highly enriched in ^{13}C and indicated higher incorporation of surface C in the drilosphere than in bulk soil. Such C translocation might have been due to mobilization of soluble litter compounds (Gaillard et al. 2003), excretion of earthworm casts along the burrow walls, and possibly the activity of other invertebrates in and around the burrow, for instance microarthropods (Chamberlain et al. 2006). Whatever the mechanisms involved, the presence of active *L. centralis* played a key role, as demonstrated by the lower soil ^{13}C enrichment around burrows from which the resident earthworm had been removed a priori (Fig. 1). In this study we focused on topsoil, which is where most soil biological activity occurs, but the activity of anecic earthworms also affects deeper layers (Zaller et al. 2013).

As a result of earthworm-driven incorporation of fresh organic material, both the quantity and the quality of SOM were modified: soil in the drilosphere controlled by *L. centralis* had higher total sugar content and lower GM/AX ratio than in the drilosphere surrounding vacant burrows (Fig. 3). A larger proportion of the plant-derived sugars xylose and arabinose in the SOM sugar pool (= lower GM/AX) is diagnostic of a higher contribution of recently incorporated plant material, whereas a larger share of microbial sugars such as galactose and mannose (= higher GM/AX) is linked to older and more stabilized organic matter (Spielvogel et al. 2008). All our samples had relatively low GM/AX ratios (<1) compared to the bulk soil analysed in other studies (Spielvogel et al. 2008; Rumpel et al. 2010), indicating a high relative abundance of fresh plant-derived sugars. This is not surprising, as burrows made by anecic earthworms are preferential pathways of detritus incorporation into soil, and the unoccupied burrows we sampled had been earthworm-free for only 50 days.

Despite the latter fact, earthworm presence led to a concentration of C in the drilosphere compared to the corresponding

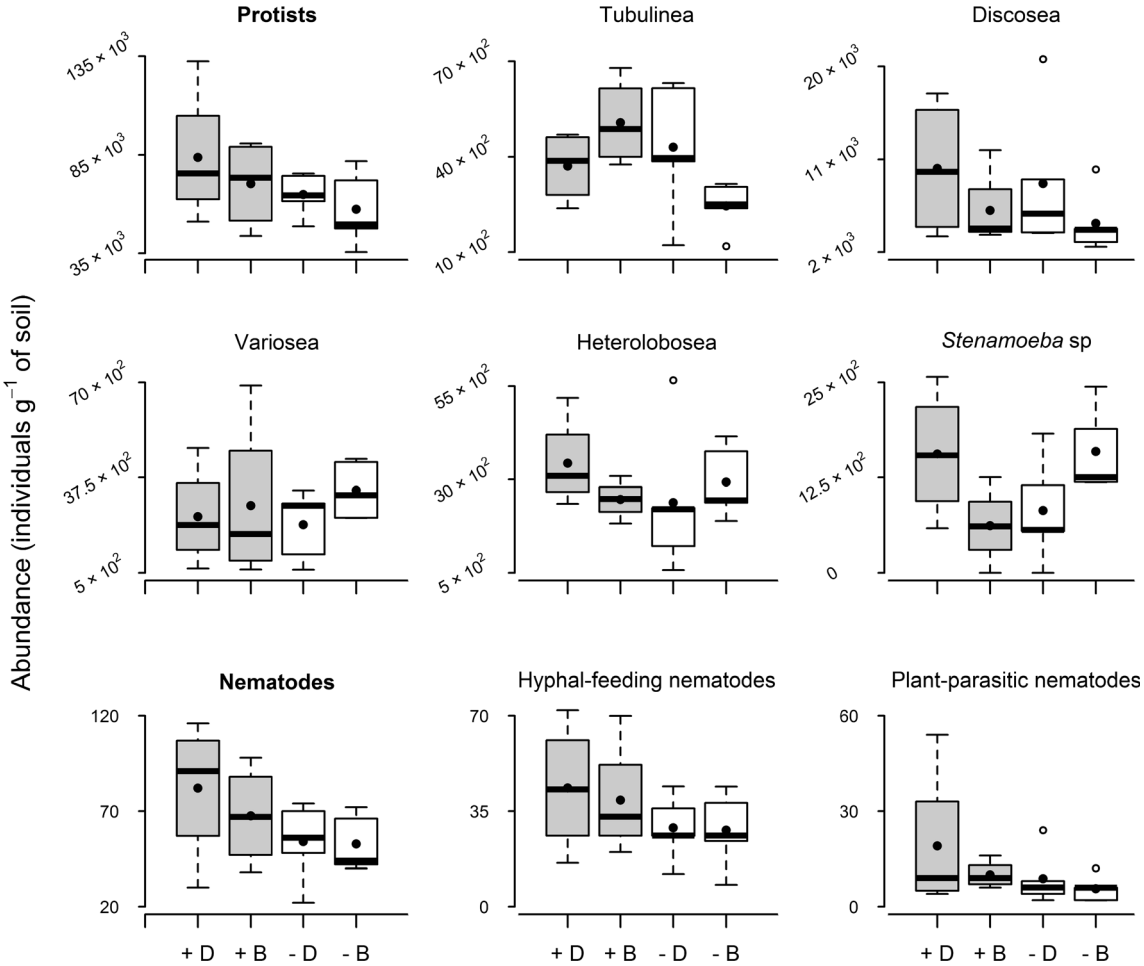

Fig. 4 Abundance of soil protists (*top* and *middle rows*) and nematodes (*bottom row*) in soil around burrows occupied by *L. centralis* (+, *grey boxes*) or from which the earthworm had been removed 50 days before sampling (–, *white boxes*), in drilosphere (*D*; 0–8 mm around burrow walls) and bulk soil (*B*; 50–75 mm around burrow walls). For protists, abundance is shown for the entire community, the four classes of amoebae and the highly represented genus *Stenamoeba* (class Discosea). For nematodes, abundance is shown for the entire community and the two most abundant trophic groups. The *thick lines* in the boxes are the medians, the *black dots* are the means, and the *error bars* delimit the interquartile range

bulk soil, as recorded in other studies on anecic earthworm burrows (Don et al. 2008; Stromberger et al. 2012; but see Tiunov et al. 2001b). Notably, this did not occur around burrows from which the earthworms had been removed. While 50 days without a resident earthworm might have been enough for some C losses (e.g. through DOC leaching), a more likely explanation is that the earthworms mediated larger inputs of C from the labelled plant material to the soil, as indicated by both ^{13}C and SOM data (Figs. 1 and 3). Indeed, the drilosphere around isotopic control burrows, which were occupied by *L. centralis* but were not given the labelled plant material, had somewhat lower soil C concentrations (24.8± 0.8 g kg^{-1} soil, n=4) than the labelled earthworm-occupied drilosphere (26.4±1.3 g kg^{-1} soil, n=4). This earthworm-mediated input of fresh organic matter into soil can affect the microbial community in the drilosphere, leading to faster turnover and higher enzymatic activity (Tiunov and Scheu 1999; Uksa et al. 2015) and to changes in community composition, e.g. possibly enhancing bacterial compared to fungal saprotrophs (Dempsey et al. 2013).

The earthworms occupying the burrows under labelled maize were only weakly enriched in ^{13}C, and the individual variability in isotopic signature was high. Given the strong ^{13}C enrichment of the plant material, this high variability is surprising. Although a complete disappearance of the maize was observed above the burrows with anecic earthworms (suggesting their important role in litter degradation) and *L. centralis* effectively incorporated plant material belowground (Figs. 1 and 3), some individuals apparently assimilated little or negligible amounts of the labelled plant material. This could be partly explained by individual differences in consumption, meaning that some earthworms fed on the plant material for a shorter time than others. In fact, even though anecic earthworms may transport very fresh leaves inside their burrows (Griffith et al. 2013), they preferably feed on them only after they are more or less decayed and mixed with mineral soil

(Doube et al. 1997). Another possibility is that due to the short labelling period, the stable isotope tracer in the maize fragments was mostly in highly labile fractions, which could have been lost during decomposition before residues were ingested by earthworms. The variation in earthworm body size was not influential, as individual weight was not a significant predictor in the statistical analyses.

Protists and nematodes around anecic earthworm burrows

Protists and nematodes attained higher average abundance in the presence of *L. centralis*, but burrow-to-burrow variability was high and resulted in a substantial overlap. We did not detect clear community-wide differences between drilosphere and bulk soil, although the highest abundance of both groups was measured in earthworm-occupied drilosphere (Fig. 4). Earthworms may provide protists and nematodes with advantageous conditions in the drilosphere through nutrient enrichment, stable soil moisture and higher microbial activity, and possibly enhanced pore space (Görres and Amador 2010). Moreover, earthworms feed on protists (e.g. naked amoebae) and nematodes (Dash et al. 1980; Bonkowski and Schaefer 1997), and so they might regulate their community composition and abundance also via direct trophic effects.

In a laboratory experiment with *L. terrestris* and two types of leaf litter, nematodes were consistently more abundant in the drilosphere (0–4 mm) than outside, while for protists the same occurred under litter from *Tilia cordata* but not *Fagus sylvatica* (Tiunov et al. 2001a). In our study, the trophic structure of the soil nematode community was little affected by earthworm presence and soil microhabitat, whereas there were clearer effects on the taxonomic composition of protists. For instance, Cercozoa and in particular Glissomonadida, a dominant group of bacterial-feeding soil flagellates (Howe et al. 2009), were more numerous in the drilosphere, whether *L. centralis* was present or not (Supplementary material). Also the abundance of *Stenamoeba* spp., a species-rich genus within the eukaryotic supergroup Amoebozoa (Geisen et al. 2014b), was higher in drilosphere than in corresponding bulk soil when *L. centralis* was present but was depressed in the drilosphere when the burrow was abandoned (Fig. 4). This finding corresponds with the increased numbers of amoebae of *Vannella* morphotypes (which previously included *Stenamoeba*) in *L. terrestris* middens reported by Anderson and Bohlen (1998). Desiccation sensitive protists such as *Stenamoeba* (Geisen et al. 2014a) might have especially benefitted from the moisture conditions in the active drilosphere, characterized by frequent mucus secretion by the earthworm. The enriched microfauna in *Lumbricus* burrows may contribute to fast nutrient cycling by grazing on microbes and thereby influencing microbial turnover, enzymatic activity and community composition, as well as through the release of nutrients otherwise locked in microbial cells (Bonkowski 2004). However, still far too little is known on how changes in the protist community composition may affect the rest of the soil food web.

Do anecic earthworm species have similar effects on soil biogeochemistry?

Our results obtained from real, natural earthworm burrows highlight the variability and the difficulties associated with field-based experiments compared to laboratory approaches (Kampichler et al. 2001). Issues with validating earthworm presence (or removal) in target burrows and with the collection of the required amount of soil for the various analytical methods impeded a rigorous statistical comparison of *A. longa* and *L. centralis*. Nevertheless, our data suggest that *A. longa* incorporated less surface organic matter into soil than *L. centralis*, so that its drilosphere was more comparable to bulk soil or soil around unoccupied burrows (e.g. in soil $\delta^{13}C$ and GM/AX ratio). If confirmed, this would mean that the two anecic species had specific effects on soil biogeochemical and biological heterogeneity under the given field conditions due to different effect traits related to the incorporation of surface organic matter. Such difference would be consistent with traditional niche theory (Leibold 1995), according to which a strong functional overlap of two co-occurring species must lead to the competitive exclusion of one of them. In fact, although soil is a highly heterogeneous environment where competing species may co-occur at small scales (Amarasekare 2003)–the so-called enigma of soil animal diversity (Anderson 1975)–trait differences between co-occurring anecic earthworm species have been found. In particular, *A. longa* feeds more extensively on highly mineralized SOM than do *Lumbricus* spp., the latter showing a stronger preference for fresher detritus (Briones et al. 2005; Thakuria et al. 2010). Also, the burrowing behaviour of *A. longa* appears somewhat intermediate between that of endogeic earthworms, which dig transient channels below the soil surface, and true anecic earthworms (Bastardie et al. 2005). But it should be noted that *A. longa* and other anecic species are remarkably flexible in their diet (Schmidt 1999; Thakuria et al. 2010; Griffith et al. 2013), as they tune their behaviour according to environmental conditions (e.g. availability of surface residues).

Conclusions

The anecic earthworm *L. centralis* incorporates fresh organic C into soil around its burrows, altering both the quantity and the chemical quality of the soil C pool in this microhabitat. Soil around burrows that did not have a resident earthworm during the experiment (50 days) was biochemically more

similar to soil beyond earthworm influence than to earthworm-occupied drilosphere soil, indicating that for biogeochemical effects to be persistent, the earthworm has to be present. While earthworm presence had small effects on the overall abundance of nematodes or protists in the drilosphere, it stimulated some phylogenetic clades of protists. Additional data on the co-occurring anecic earthworm *A. longa* hinted at the possibility that distinct species in the anecic functional group may differ in their effects due to niche differentiation, in particular, in terms of incorporation of surface detritus belowground.

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