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Distinct microbial and faunal communities and translocated carbon in *Lumbricus terrestris* drilospheres

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Abstract

Lumbricus terrestris is a deep-burrowing anecic earthworm that builds permanent, vertical burrows with linings (e.g. drilosphere) that are stable and long-lived microhabitats for bacteria, fungi, micro- and mesofauna. We conducted the first non-culture based field study to assess simultaneously the drilosphere (here sampled as 0–2 mm burrow lining) composition of microbial and micro/mesofaunal communities relative to bulk soil. Our study also included a treatment of surface-applied ^{13}C- and ^{15}N-labeled plant residue to trace the short-term (40 d) translocation of residue C and N into the drilosphere, and potentially the assimilation of residue C into drilosphere microbial phospholipid fatty acids (PLFAs). Total C concentration was 23%, microbial PLFA biomass was 58%, and PLFAs associated with protozoa, nematodes, Collembola and other fauna were 200-to-300% greater in the drilosphere than in nearby bulk soil. Principal components analysis of community PLFAs revealed that distributions of Gram-negative bacteria and actinomycetes and other Gram-positive bacteria were highly variable among drilosphere samples, and that drilosphere communities were distinct from bulk soil communities due to the atypical distribution of PLFA biomarkers for micro- and mesofauna. The degree of microbial PLFA ^{13}C enrichment in drilosphere soils receiving ^{13}C-labeled residue was highly variable, and only one PLFA, 18:1(9c, was significantly enriched. In contrast, 11 PLFAs from diverse microbial groups where enriched in response to residue amendment in bulk soil 0-5 cm deep. Among control soils, however, a significant (^{13}C shift between drilosphere and bulk soil at the same depth (5-15 cm) revealed the importance of *L. terrestis* for translocating perennial rye grass-derived C into the soil at depth, where we estimated the contribution of the recent grass C (8 years) to be at least 26% of the drilosphere soil C. We conclude that *L. terrestris* facilitates the translocation of plant C into

soil at depth and promotes the maintenance of distinct soil microbial and faunal communities that are unlike those found in the bulk soil.

1. Introduction

Soil microbial communities are regulated by resource availability, environmental conditions, and through interactions with higher organisms such as plant roots and soil fauna. Soil fauna influence microbial communities directly through predation and selective grazing, and indirectly by altering soil habitat, transporting microorganisms through soil, and increasing the available surface area of organic matter through comminution (Ingham et al. 1985, Berry 1994, Coleman and Crossley 1996, Seeber et al. 2008). Earthworms, for example, greatly affect soil nutrient cycling by influencing microbial biomass and activity (Parkin and Berry 1999, Tiunov and Scheu 1999, Li et al. 2002; Aira et al. 2009) and therefore are recognised as ecosystem engineers in biogeochemical cycling and soil carbon storage (Bardgett 2005). Several studies have shown that the burrowing activity of anecic earthworms facilitates the translocation of fresh residues into soil and creates distinct habitats (casts, middens, and burrows) whose altered physical and chemical properties can support active and distinct microbial communities (Tiunov et al. 1997, Tiunov and Scheu 1999, Tiunov and Dobrovolskaya 2002, Aira et al. 2009, Nechitaylo et al. 2010).

Lumbricus terrestris is a deep-burrowing anecic earthworm that builds permanent, vertical burrows. Its feeding strategy is to collect surface residues and drag them into the mouths of their burrows, or middens, where residue decomposition is initiated by microorganisms prior to ingestion by the earthworm (Curry and Schmidt 2007). As *L. terrestris* travels along its burrow system, some residues, mucus and casts are deposited along the walls, resulting in a drilosphere enriched in C and N. Microbial growth is stimulated in the drilosphere, which subsequently leads to the growth of eukaryotic grazer populations such as protozoa and nematodes (Tiunov et al. 2001). The turnover of labile substrates, including microbial biomass, results in greater C and N mineralization activities compared to bulk soil environments (Görres et al. 1997, Parkin and Berry 1999, Tiunov and Scheu 1999). There is also evidence that microbial community composition is altered in the drilosphere. For example, populations of nitrifying and denitrifying bacteria are enriched (Parkin and Berry 1999), and the ratio of fungal:bacterial cells may be lower than that of bulk soil (Tiunov and Scheu 2000, Tiunov et al. 2001). One culture-based study described distinct bacterial communities between *L. terrestris* drilosphere and bulk soils, with a dominance of litter-associated *Cellulomonas* and *Promicromonospora* in the drilosphere compared to *Bacillus* and *Streptomyces* in control soil (Tiunov and Dobrovolskaya 2002). Other than these few studies, the drilosphere structure of microbial communities and their grazers is relatively unknown.

Tiunov and Scheu (1999) emphasized that the lifetime of an *L. terrestris* burrow may exceed the lifetime of *L. terrestris* itself. In other words, these burrows are stable and long-lived microhabitats for bacteria, fungi, and micro- and mesofauna, where these organisms are disproportionately affected within a relatively small volume of soil. In order to characterize the community associated with this distinct habitat, we subjected drilosphere and non-drilosphere bulk soils, collected from a grassland field, to phospholipid fatty acid (PLFA) analysis. This non-culture based technique can simultaneously assess relative differences in biomass of bacteria, fungi and fauna (e.g., protozoa and nematodes), as well as broad compositional differences among bacterial community members. In addition, our study included a treatment of surface-applied ^{13}C-

and ^{15}N-labeled plant residue so that we could trace the short-term translocation of residue C and N into the drilosphere, and potentially the assimilation of residue C into drilosphere microbial communities. We hypothesized that soil C and N concentrations, as well as the biomass of bacteria, fungi, and micro/mesofauna would be greater in the drilosphere than in nearby bulk soil due to increased substrate availability from the burrowing activity of *L. terrestris.* We also hypothesized that the burrows support distinct microbial and faunal communities, and thus *L. terrestris* contributes to the spatial heterogeneity of the soil community at the field scale.

2. Materials and Methods

2.1. Experimental design

This study was part of a larger study that examined the effects of *L. terrestris* on residue translocation, and the incorporation of residue C and N into soil and the biomass of other earthworm species. The original study consisted of 15 plots, each 1 m (1 m in size, that were arranged in a randomized complete block design, with 3 plots in each of 5 blocks, in a 1.5-ha field located at the University College Dublin Research Farm, Lyons Estate, Celbridge, Co. Kildare, Ireland (53(18'N latitude and 06(33'W longitude). The field was under set-aside beginning in fall 2001, when it was sown to perennial ryegrass (*Lolium perenne* L.). Set-aside management involved mowing the grass twice annually, with no inorganic or organic fertilizer inputs. Prior to fall 2001, the field was utilized for a series of maize (*Zea mays* L.) trials over a 4-5 year period. The soil is derived from river Liffey alluvium that was deposited on previously lain glacial drift, and is classified as a humic grey or regosol (Lalor 2004). The soil is moderately- to poorly-drained and its texture varies between loam to loamy sand.

Mustard (*Sinapis alba* L. var. Rivona) residues dual-labeled with ^{13}C and ^{15}N were produced in a heated greenhouse at Thornfield, University College Dublin. Mustard plants were grown in pots (3.5 L, height 15 cm, diameter 17 cm) filled with commercial horticultural compost (John Innes No. 2) and approximately 30 seeds were sown into each pot on 2 February 2009. Labeling of plants followed Schmidt and Scrimgeour (2001), and started on 23 February 2009, 21 days after sowing when plants had produced their first set of true leaves and plant height was about 100–120 mm. A dual labeled ^{13}C-^{15}N urea solution was prepared by dissolving 5 g of 99 atom% ^{13}C urea and 100 mg 99 atom% ^{15}N urea in 2 L distilled water. The wetting agent Citowett (BASF, Ludwigshafen, Germany) was also added at 2.5 mL L^{-1}. The labeled solution was applied every two-to-three days to the mustard plants using an ordinary trigger-powered mister ('Spraymist', Hozelock Ltd, Aylesbury, UK). The mister was calibrated to allow delivered volumes to be applied evenly over the plants. Initially, the volume of labeled solution applied per day was 80 mL, but this volume was increased to 200 mL on 2 March and then 400 mL on 9 March to coincide with plant growth. The last day of label application was 13 March, six days prior to harvesting. Because urease enzymes are ubiquitous, including on plant surfaces, we assumed that no strongly labeled urea would be remaining on the plant surface after the six days (Bremner 1995).

Aboveground biomass of the labeled mustard was harvested on 19 March 2009, left intact, thoroughly mixed and stored fresh at 4°C overnight until field application the following day. Five individual mustard plants were oven-dried at 65°C for 24 h to determine average moisture content. On 20 March, 2100 g (fresh mass) of labeled mustard litter was applied to each 90 cm (90 cm core area of all residue-treatment plots (equivalent to 200 g dry mass m^{-2}), and was covered

by coarse plastic mesh to prevent litter loss by wind. The isotopic composition (mean ± SD, n = 5) in the labeled mustard bulk material was –8.5‰ ± 6.0 (1.096 ± 0.007 atom% ^{13}C) and 163‰ ± 89 (0.426 ± 0.032 atom% ^{15}N).

2.2. Soil sampling

Plots were sampled on 28-29 April 2009, when over 90% of the residue biomass had disappeared. Vegetation was clipped to the soil surface and retained for biomass and isotopic analysis. Earthworm burrows with a large surface opening and midden were targeted as potential *L. terrestr*is burrows. Burrows were confirmed to be occupied by *L. terrestris* by injecting approximately 50 mL of a dilute mustard oil irritant (2 mL allyl isothiocyanate dispersed in 40 mL isopropanol [2-propanol], then added to 20 L water and mixed thoroughly) into their burrows with a syringe, and monitoring for expulsion of an *L. terrestris* individual. Confirmed burrows were sampled with a spade by excavating a block of soil (~ 20 cm × 20 cm wide and 20 cm minimum depth) containing the burrow opening in the center. Each soil block was divided into two depth increments, 0-5 cm and 5-15 cm. Drilosphere samples from the 5-15 cm depth increment were obtained using a laboratory spatula to scrape away soil from the 0-2 mm soil layer around the burrow. Non-drilosphere (bulk) soil was collected at least 2 cm away from the burrow at the same depth increment. In addition, non-drilosphere (bulk) soil was collected from the 0-5 cm depth increment for reference comparisons. Soils were placed in polyethylene bags and stored in ice chests for transportation to the laboratory. We were unable to excavate occupied *L. terrestris* burrows in all 15 plots, either because an occupied burrow was absent from a plot, or because a burrow did not extend vertically down through the soil block to a depth of 15 cm. However, samples were successfully obtained from one occupied burrow and associated bulk soil from each of 10 plots, five that were residue amended and five that were not (2 residue treatments (5 replicate plots (3 sample microhabitats = 30 soil samples total).

2.3. Laboratory analyses

Oven-dried samples of perennial ryegrass above-ground biomass were ground with a ball mill and analysed for stable C isotope ratios by continuous-flow isotope ratio mass spectrometry, using a Europa Scientific Roboprep-CN elemental analyser, coupled to a Europa Scientific 20-20 isotope ratio mass spectrometer (Iso-Analytical Ltd, Sandbach, Cheshire, UK). Soil samples were frozen at -20(C, freeze-dried, and ground with a ball mill. A subsample of each soil was analyzed for total C, total N and stable C and N isotope ratios with a Carlo-Erba NA1500 elemental analyser (Milan, Italy) coupled to a VG Isochrom stable isotope ratio mass spectrometer (IRMS) (GV Instruments, Manchester, UK) at the Colorado State University Stable Isotope Laboratory. Stable isotope ratios are expressed in the ?-notation in parts per thousand (‰) according to the equation:

$$? (‰) = ([R_{sample}/R_{reference}] - 1) \times 1000 \quad (1)$$

where R is the ratio of heavier/lighter stable isotope ($^{13}C/^{12}C$ or $^{15}N/^{14}N$) in the sample (R_{sample}) and the reference material ($R_{reference}$). Universal standards Vienna Pee Dee Belemnite and air were used at ^{13}C and ^{15}N standards, respectively.

Phospholipid fatty acids (PLFAs) were extracted from samples following the protocol of Bossio and Scow (1998). PLFAs were analyzed at the University of California Stable Isotope Facility (Davis, CA) on a Thermo gas chromatograph (GC) continuous IRMS system composed

of a Trace GC Ultra gas chromatograph (Thermo Electron Corp., Milan, Italy) with a Varian factorFOUR VF-5ms column (30 m (0.25 mm ID, 0.25 micron film thickness), coupled to a Delta V Advantage IRMS through a GC/C-III interface (Thermo Electron Corp., Bremen, Germany). Peak areas were converted to ng C g^{-1} dry soil based on the concentration of 19:0 internal standard. The (^{13}C values of individual PLFAs were corrected for the addition of the methyl group during transesterification by simple mass balance as follows (Denef et al. 2007):

($^{13}C_{PLFA} = [(N_{PLFA} + 1) (^{13}C_{FAME} - (^{13}C_{MeOH}] / N_{PLFA}$ (2)

Where ($^{13}C_{PLFA}$ is the (^{13}C value of the PLFA after transesterification, N_{PLFA} is the number of C atoms within an individual PLFA, and ($^{13}C_{MeOH}$ is the (^{13}C value of the methanol used for transesterification (-47.65‰ vs. Pee Dee Belemnite on a dissolved organic carbon analyzer interfaced with IRMS at the UC Davis Stable Isotope Facility).

For relative biomass assessment, biomarkers of specific compositional groups were assigned as follows: i14:0, a15:0, i15:0, i16:0, a17:0, and i17:0 for Gram-positive bacteria (Zak et al. 1996, Bossio and Scow 1998); 16:1?7c, 16:1?9c, 18:1?7c, 17:0 cy, and 19:0 cy for Gram-negative bacteria (Paul and Clark 1996, Zak et al. 1996); 10Me16:0, 10Me17:0, and 10Me18:0 for actinomycetes (Zelles 1997); and 18:2?6c and 18:1?9c for fungi (Vestal and White 1989, Paul and Clark 1996, Zak et al. 1996, Bossio and Scow 1998). Because PLFAs with a chain length of 20 are generally found in all eukaryotes, and polyunsaturated versions with animals in particular (Lechevalier and Lechevalier 1988, Zelles 1999), we did not assign these longer chain PLFAs to a particular faunal goup (such as 20:4(6c for protozoa). Rather, we employed PLFAs 20:1(9, 20:4(6, and 20:5(3 as indicators of soil microfauna (protozoa and nematodes) and mesofauna (e.g., Collembola) in general, since combinations of these PLFAs have been detected in nematodes (20:1(9 and 20:4(6; Chen et al. 2001, Ruess et al. 2002) and in protozoa, Collembola and tardigrades (20:4(6 and 20:5(3; Korn 1964, Chamberlain and Black 2005, Rizzo et al. 2010).

2.4. Statistical analyses

Univariate data were analyzed by two-way analysis of variance (ANOVA) in SAS (SAS Institute, Cary, North Carolina). When the main factor “soil microhabitat” (drilosphere, bulk 5-15 cm depth, and bulk 0-5 cm depth) was significant, means were separated by Fisher’s protected least significant different test ($P < 0.05$). Data were pooled when either the residue amendment or microhabitat was not significant. Stable isotope data (soil $?^{13}C$, $?^{15}N$ and individual PLFA $?^{13}C$ ratios) were analyzed by one-way ANOVAs for residue-amended vs. control plots within a microhabitat type, or among microhabitat types within residue-amended or control plots, to avoid data pooling. Community PLFA data were analyzed by Principal Components Analysis (PCA) with the PC-ORD statistical package (MjM Software, Gleneden Beach, Oregon), after normalizing the data as relative mole %. Data were also analyzed by multiple response permutation procedure (MRPP) with Bray-Curtis distance measure in PC-ORD to determine if microbial and micro/mesofaunal PLFA composition differed significantly among drilosphere and bulk soils, and between residue-amended and control soils ($P < 0.05$).

3. Results

The effects of soil microhabitat (5-15 cm drilosphere, 5-15 cm bulk soil, and 0-5 cm bulk soil) and residue amendment on soil C, soil $?^{13}C$ and $?^{15}N$, and community PFLA biomass and composition were independent, with no significant interactions between the two factors. Overall,

the drilosphere was enriched in C and supported a distinct microbial and faunal composition compared to bulk soils. Adding dual-labeled residue resulted in the enrichment of ^{15}N in the drilosphere. Eleven individual PLFAs were significantly enriched in ^{13}C in response to residue amendment; all occurred in the bulk surface soil and only one in the drilosphere.

3.1. Soil C and PLFA composition

Total soil C concentration was significantly greater in drilosphere soil than in bulk soil at the same soil depth (5-15 cm) (Fig. 1). Compared to the two bulk soils, soil C properties in the drilosphere were either intermediate between the 0-5 and 5-15 cm depth increments, or were similar in value to the surface soil. While soil C varied across sampling locations, soil C was not significantly affected by the residue amendment. Values averaged 44.3 g kg^{-1} in control plots and 43.3 g kg^{-1} in residue amended plots.

Total microbial biomass (estimated by the total nmol PLFA g^{-1} soil), biomass of broad microbial groups (gram-positive and gram-negative bacteria, actinomycetes, and fungi), and biomass of faunal PLFAs were greater in the drilosphere than in nearby bulk soil as hypothesized, and greater in the 0-5 cm depth bulk soil than in the 5-15 cm depth bulk soil (Table 1). Biomass estimates in the drilosphere were intermediate between those of the 0-5 and 5-15 cm deep bulk soils, with the exception of the long-chain PLFAs 20:1?9, 20:4(6 and 20:5?3, whose concentrations were as high in the drilosphere soil as in the bulk surface soil.

There were no significant differences in fungal:bacterial PLFA ratio among the three soil microhabitats, indicating no difference in microbial community composition in terms of fungal vs. bacterial biomass (Table 1). However, PCA conducted on all PLFAs resulted in the separation of surface vs. deeper bulk soil communities along PC 1, and drilosphere communities from bulk soil communities along PC 2 (Fig. 2). PLFAs with relatively high eigenvector values and therefore stronger influence on the community patterns are listed in Table 2. Compared to non-drilosphere soil, microbial communities of drilosphere were highly variable and spanned the range of community compositions represented by bulk surface soil (left side of PC 1) and bulk sub-surface soils (right side of PC 1). The drilosphere community was also elevated in saturated PLFA 18:0, monounsaturated PLFAs 19:1 and 20:1(9c, and polyunsaturated PFLAs 20:4(6c and 20:5(3. Surface bulk soils were generally elevated in several monounsaturated PLFA biomarkers for Gram-negative bacteria, as well as fungal biomarker 18:2(6c, whereas the deeper bulk soil was enriched in actinomycetes and Gram-positive bacterial PLFA markers. Community differences were confirmed by MRPP, which found that community PLFA profiles were unique to each of the three soil microhabitats ($P < 0.0001$, $A = 0.227$).

3.2. Stable isotopic ratios of soil and PLFAs

In control plots, (^{13}C was significantly lower in the drilosphere than in the 5-15 cm bulk soil, and lower in the 0-5 cm bulk soil than in the 5-15 cm bulk soil (Fig. 3A). In contrast, (^{13}C values of residue-amended plots were statistically similar among microhabitat types (Fig. 3B). Within 5-15 cm bulk soils, the (^{13}C was significantly higher (-20.30 ± 0.67‰) in control soil than in residue amended soil (-24.76‰ ± 0.62‰). While soil (^{15}N ratios were statistically similar among microhabitats in the control plots (Fig. 3A), residue amendment significantly increased the drilosphere (^{15}N ratio compared to the ratio measured in 5-15 cm bulk soil (Fig. 3B). Residue amendment did not affect soil (^{15}N ratio between surface (0-5 cm) and subsurface (5-15 cm) bulk

soils.

Assimilation of residue-derived ^{13}C into soil communities was evident from the significant upward shift (relative to control soil PLFAs) in the (^{13}C of 11 PLFAs in the bulk surface soil (Fig. 4A) and one PLFA (18:1?9c) in the drilosphere (Fig. 4C). A range of bacterial groups were represented by the ^{13}C-enriched PLFAs in the bulk surface soil, and included PLFAs associated with Gram-negative bacteria (16:1, cy17:0, cy 19:0), Gram-positive bacteria (i15:0, a15:0, i16:0, a17:0), fungi (18:1?9c), 16:0, and 12Me16:0. In contrast four different PLFAs were significantly depleted in ^{13}C at the lower bulk soil depth (i14:0, 14:0, 10Me17:0, and 20:5?3) (Fig. 4B).

4. Discussion

4.1. Microbial and faunal communities

To our knowledge, this is the first non-culture based study to report on the microbial and faunal community of *L. terrestris* drilosphere soil collected from burrow linings (0-2 mm) in the field. We found that *L. terrestris* and plant residue amendment affected soil C, N, and community biomass and composition independently, but the influence of the drilosphere environment was not altered by surface-applied, fresh plant residue made available to *L. terrestris.* This is likely due to drilosphere already being enriched in C and N relative to bulk soil at equivalent depth, so that an additional input of residue-derived C and N did not further enhance the drilosphere effect.

In this study, the drilosphere of *Lumbricus terrestris* was determined to be a C-enriched environment that supported a larger and distinct microbial and faunal community relative to the bulk soil environment at the same depth. Total C concentration was 23%, microbial PLFA biomass was 58%, and biomass of faunal biomarkers were 200-to-300% greater in the drilosphere than in nearby bulk soil. Others have measured anywhere from 9% to more than 100% higher soluble and organic C concentrations in *L. terrestris* drilosphere soil relative to bulk soil (Parkin and Berry 1999, Tiunov and Scheu 1999, Tiunov and Dobrovolskaya 2002). Very few studies have simultaneously examined microbial and micro/mesofauna abundances in earthworm habitats, and in one *L. terrestris* laboratory study, Tiunov et al. (2001) described three times greater microbial biovolume and total nematode density, four times more naked amoeba, and two-to-ten times more flagellates in the drilosphere than in bulk soil. Ciliates were also abundant (2,000 individuals g^{-1} dry soil) in one drilosphere soil, whereas they were not detected in the nearby bulk soil.

Fungi and bacteria are both known to respond favorably to the drilosphere environment, with greater biovolumes of each in *L. terrestris* drilospheres than in bulk soil (Tiunov and Scheu 1999, Tiunov and Dobrovolskaya 2002). However, the drilosphere effect has been shown to be larger on bacteria, with a 200% difference in bacterial biovolumes between drilosphere and bulk soil whereas there was only a 37% effect on fungi (Tiunov and Scheu 1999). The disproportionate responses of bacterial vs. fungal biomass thus resulted in a lower ratio of fungal:bacterial biomass in the drilosphere than in bulk soil. In this study, the ratio of fungal:bacterial PLFAs was equivalent among the soil microhabitats, suggesting that bacterial biomass does not always dominate in *L. terrestris* drilosphere soils. The overall balance between fungal and bacterial components of the microbial community is likely affected not only by substrate availability and quality in the drilosphere, but also by presence of grazing fauna. Tiunov and Scheu (1999) hypothesized that the drilosphere environment is characterized by high grazing pressures from fungivorous fauna which would result in lower fungal:bacterial biomass ratios in drilosphere versus bulk soils. The hypothesis was not proven, however, because in a later study, Tiunov et al.

(2001) found greater numbers of both fungivorous (nematodes) and bacterivorous (nematodes and protozoa) fauna in burrow walls than in bulk soil. Similarly, our data indicate that both fungivorous and bacterivorous fauna were equally affected in the drilosphere, so that the ratio of fungal:bacterial PLFAs remained similar between drilosphere and bulk soils.

We hypothesized that *L. terrestris* drilospheres would support a microbial and faunal community distinct from that of bulk soil, and our hypothesis was supported based on PCA analysis of the community PLFA data. An interesting feature was that the microbial community (consisting of Gram-negative and actinomycetes and other Gram-positive bacteria) was highly variable among drilosphere samples, so that some drilosphere microbial communities were more similar to surface bulk soil communities whereas others were more similar to bulk subsurface communities. Thus, not only does *L. terrestris* contribute to the overall spatial heterogeneity of soil microbial community composition, there is spatial heterogeneity as well among burrows of different individuals. This may be related to the age of the burrow and/or the 'resident' *L. terrestris* itself. Ontogenetic changes in feeding behavior is likely to occur in such large-bodied species, as has been suggested for *Aporrectodea longa* (Schmidt 1999). Also, individuals are known to selectively ingest different proportions of plant material and microbial-rich soil particles (Sampedro et al. 2006). Furthermore, if such variability in microbial community composition occurs among drilospheres of a single species (*L. terrestris*), then it seems likely that greater differences would be observed among drilospheres of different earthworm species.

One characteristic of the drilosphere community that was dramatically different from bulk soil communities was the elevated concentrations and relative proportions of long-chain PLFAs (20:1(9c, 20:4(6c and 20:5(3) to the overall community PLFA biomass. At least two studies have determined that 20:4(6c and 20:5(3 occur within *L. terrestris* tissues, but their contributions to soil PLFAs are minimal or absent (Enami et al. 2001, Sampedro et al. 2006). In addition, *L. terrestris* contains other long-chain PLFAs, including 20:3?6 and 22:5?3 (Sampedro et al. 2006), that were not detected in soils of this study and therefore our assumption is that these PLFAs are predominantly restricted to micro- and mesofauna such as protozoa, nematodes and Collembola. A limitation of the PLFA approach applied to soil fauna is that PLFAs associated with eukaryotes and animals in particular are non-specific and common across taxa (Ruess and Chamberlain 2010), but this has not hindered serious efforts to employ fatty acids to study soil food web structures and trophic interactions (Ruess et al. 2002, Ruess et al. 2005, Ruess and Chamberlain 2010). In this study, we avoided assigning animal PLFAs to specific taxa, but nevertheless, we were able to determine that the drilosphere environment can not only be defined by its greater biomass of heterogeneous microbial communities, but by the presence of greater concentrations of micro/mesofauna PLFAs that are not typical of bulk soils. In the least, the PLFA method may prove useful for course analysis of many soil samples to characterize the spatial variability in faunal biomass and identify biomass "hotspots" that warrant further, detailed analyses of taxonomic composition and trophic structure.

4.2. Soil and PLFA (^{13}C ratios

The original intent of the labeling experiment was to document the role of *L. terrestris* in translocating residue C into soil at depth, by physically moving residue material downward into the burrows, making residue C more available to drilosphere microbial communities. However, our findings did not provide convincing evidence for increased substrate supply in the form of residue C to drilosphere microbial communities, as the ?^{13}C of only one drilosphere PLFA (the

fungal biomarker 18:1?9c) was significantly enriched in ^{13}C in response to residue treatment. In contrast, 11 PLFAs from the bulk surface soil were significantly enriched in ^{13}C in residue-treated plots compared to control plots. This suggests that the major effect of residue C on microbial assimilation activity occurred in the bulk soil underlying the residues. However, the means of all 16 PLFAs shown in Fig. 4C were higher in drilospheres of residue-amended plots versus control plots, but due to high variability among replicate samples, differences were not statistically significant. We propose that access of drilosphere microbial communities 5-15 cm deep to residue C was highly variable, so that it was difficult to detect statistically significant ^{13}C enrichment in microbial PLFAs. This is likely due to the variable feeding and burrowing activities among individual *L. terrestris*, including amounts and quality of substrates ingested and mucus and cast material excreted along the burrow linings. Another consideration is that the residue ^{13}C signature would have been diluted in the PLFA biomass to a greater extent in the drilosphere than in the bulk soil, thus adding to the challenge of detecting significant shifts in microbial PLFA ?^{13}C values in the drilosphere under field conditions. We propose that for future experiments, researchers apply a residue more highly labeled in ^{13}C, and that ?^{13}C measurements be made in the body tissue and casts of the *L. terrestris* that occupied each burrow.

Stable isotope analysis of the control plots yielded interesting results that contrasted with results from the residue-amended plots. Cropping history, for example, was reflected in the control plots, where a significant C isotopic shift was evident between the subsurface and surface soil layers. This shift corresponded to a history of maize, a C4 plant, followed in recent years by perennial ryegrass, a C3 plant with a (^{13}C value of -30.31‰ ± 0.07 SD (n=5). Just as Kramer and Gleixer (2008) found a decreasing influence of a new crop's isotopic signature within increasing soil depth, we found that the lower (^{13}C value (-25.70‰) in the surface soil reflected relatively recent inputs from the perennial ryegrass, whereas the higher (^{13}C value (-20.30‰) in the subsurface was indicative of older organic matter derived from prior C3 and maize (C4) inputs. Interestingly, the (^{13}C value of the drilosphere (-22.92‰) was intermediate, indicating that *L. terrestris* facilitated the incorporation of perennial ryegrass-derived C into soil organic matter at depth in the control plots. By applying a simple two-source mixing model (e.g., Balesdent et al. 1987) with the (^{13}C values of the drilosphere, perennial rye grass, and the bulk subsurface as the reference soil, the average proportion of drilosphere C derived from rye grass was 26%. This figure is only an estimate, but it does provide quantitative evidence that over time (in this case 8 years), *L. terrestris* facilitates the downward movement of plant C into soil at depth by incorporating plant C into casts and depositing C onto the burrow walls. As Don et al. (2008) describe, this feeding and burrowing activity accelerates the vertical translocation of surface C into the subsoil and results in the heterogeneous distribution of young C in the drilosphere and older C in the nearby bulk soil.

In contrast, we were unable to demonstrate that *L. terrestris* facilitated the short-term (40 d) translocation of the surface-applied fresh residues into the subsoil based solely on (^{13}C data from the residue-amended plots. That residue material entered the drilosphere was evident from the strong but variable upward shift in (^{15}N in drilosphere compared to bulk soil within residue-amended plots, but unexpectedly, a corresponding upward shift in (^{13}C did not occur. We detected a lower soil (^{13}C value in residue amended 5-15 cm bulk soil compared to control 5-15 cm bulk soil that we are not able to fully explain. While it is possible that soluble carbon substrates from the surface-applied residues leached into the soil and stimulated the soil microorganisms to degrade older, maize-derived soil organic matter, it is unlikely that the residue amendment rate used (200 g m^{-2}) would induce such a strong change in organic matter at this depth in such a short

time (40 d). Furthermore, the similarity of mean (^{13}C values for most PLFAs in control and amended soil at this depth provides further support that the added plant residues had relatively little affect on microbial C assimilation activity in contrast to the surface soil (Fig. 3B). Thus, the difference in (^{13}C between control and residue amended 5-15 cm bulk soil is likely related to the spatial heterogeneity of the (^{13}C of soil organic matter.

4.3. Summary and Conclusions

Lumbricus terrestris has long been regarded as an ecosystem engineer because of its burrowing activities that alter soil properties, and because of the unique structures (middens, burrow linings, and casts) it creates that are nutrient hotspots and microhabitats that support other organisms. This study is the only one to our knowledge that has applied a non-culture based method to simultaneously examine microbial and micro/mesofaunal community composition from drilosphere soil collected from the field. As hypothesized, the drilosphere contained greater concentrations of soil C (but not N), microbial biomass, and biomass of faunal PLFA biomarkers compared to nearby bulk soil. Community PLFA composition analysis revealed that microbial communities were heterogeneous among individual burrows but overall, communities were distinct from bulk soils due to the significantly higher concentrations of PLFAs indicative of soil fauna such as protozoa, nematodes and Collembola. Thus, *L. terrestris* promotes the maintenance of distinct soil communities that are unlike those found in the bulk soil, and therefore contributes to spatial heterogeneity of the soil community at the field scale.

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Table 1

Concentrations of total microbial PLFAs and PLFA biomarkers (nmol g^{-1} soil) of occupied *Lumbricus terrestris* burrow linings (drilosphere, 5-15 em depth), non-drilosphere bulk soil (5-15 em depth), and bulk surface soil (0-5 em depth). Means(± I SE) were averaged across residue treatments. Within each row, means followed by different letters are statistically significant by analysis of variance and LSD mean separation tests ((0.05).

PLFA	Drilosphere, 5-15 em	Bulk soil, 5-15 em	Bulk so
Total microbial PLFAs	89.4b (8. 7)	56.7c (4 .1)	
Bacteria	43.2b (4.3)	27.7c (2 .1)	
Gram-positive	18.0b (1.9)	12.6c (0.9)	
Gram-negative	25.2b (2.5)	15.1c (1.3)	
Actinomycetes	**4.64b (0.41)**	3.57c (0.16)	6 .49a
Fungi	8.35b (1.05)	4. 77c (0.32)	
Fungal:Bacterial ratio	0.19a (0.01)	0.17a (0.01)	0.19a
Faunal PLFAs			
20,1?9	1.43a (0.23)	0.68b (0. 04)	(0 .11)
20'4 (6	1.13a (0. 30)	0.376b (0.05)	0.98a
20'5?3	1.50a (0. 44)	0. 47b (0.06)	(0. 09)

Table 2

PLFAs with principal components analysis eigenvector values greater than [0.20].

PLFA	Eigenvector 1	PLFA	Eigenvector 2
a15:0	0.208	i15:0	0.355
10Me 16:0	0.216	10Me 16:0	0.240
12Me 16:0	0.279	br19:1	0.337
i17:0	0.279	21:5?3	0.275
2OH 16:1	0.269	18:0	-0.303
10Me 18:0	0.267	19:1	-0.256
cy19:0	0.277	20:1?9	-0.216
16:1?9c/7c	-0.239	20:4?6	-0.200
16:1?7t	-0.252	20:5?3	-0.207
i17:1?7c	0.212		
18:2?6c	-0.213		

Figure Captions

Fig. 1. Total soil C of drilosphere (5-15 cm depth) and non-drilosphere bulk soils (0-5 cm and 5-15 cm depths), averaged across residue treatments. Bars labeled with different letters are significantly different ($P < 0.05$). Error bars represent + 1 standard error.

Fig. 2. Principal components analysis of microbial community PLFAs extracted from the linings of occupied *Lumbricus terrestris* burrows (drilosphere, 5-15 cm depth), non-drilosphere bulk soil (5-15 cm depth), and bulk surface soil (0-5 cm depth) from field plots that were either amended with mustard plant residues or not (Control). The percent variance explained by each axis is shown in parentheses.

Fig. 3. Stable isotopic ratios of carbon and nitrogen of drilosphere (5-15 cm depth) and non-drilosphere bulk soils (0-5 and 5-15 cm depths) from field plots that were non-amended (A) or amended with mustard plant residues (B). Within a residue treatment, bars labeled with different letters are significantly different according to Student's t-tests ($n = 5$). Error bars represent + 1 standard error.

Fig. 4. Carbon stable isotopic ratios ($?^{13}C$) of PLFAs extracted from 0-5 cm depth bulk soil (A) 5-15 cm depth bulk soil (B) and 5-15 cm depth drilosphere soil (C) from Control (black bars) or Residue-amended (gray bars) plots. For a specific PLFA, bars labeled with an asterisk indicate a significant residue effect ($P < 0.05$). Error bars represent + 1 standard error.

Fig. 1

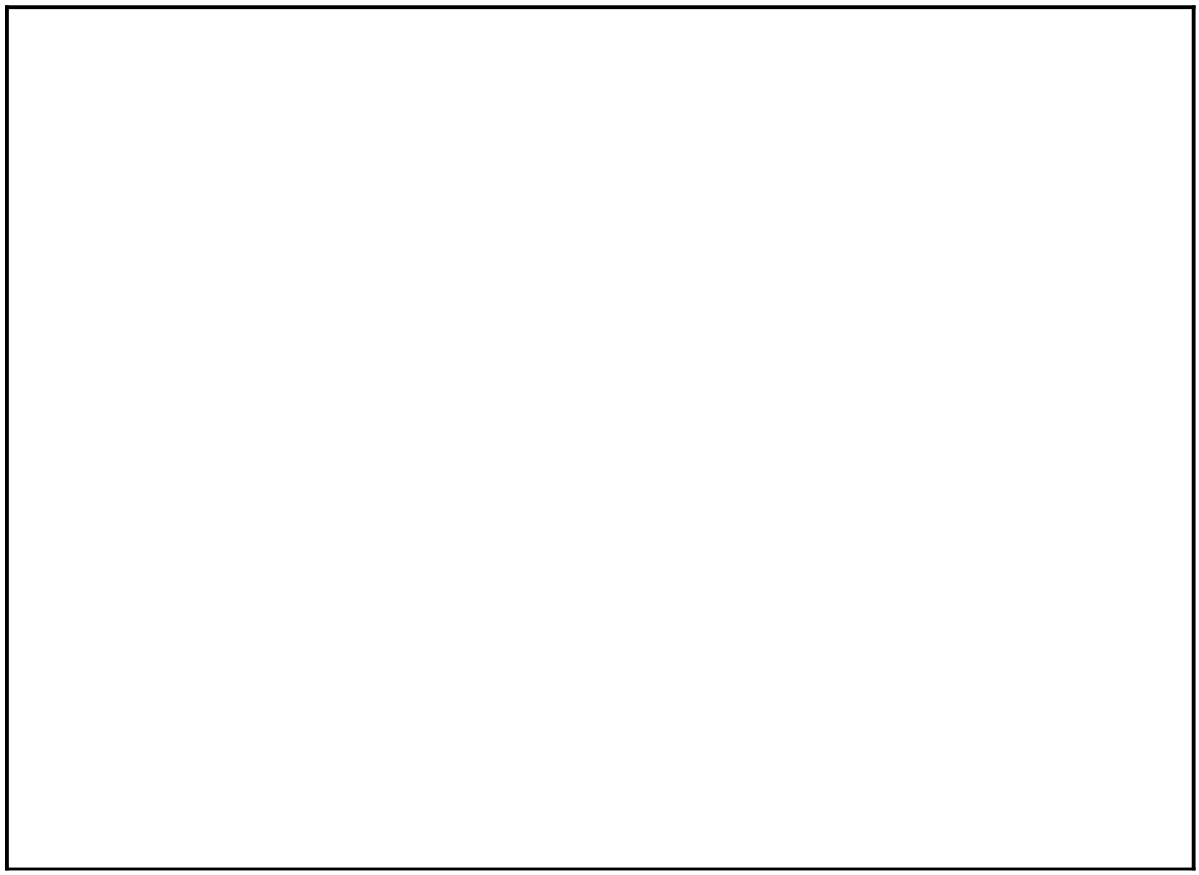
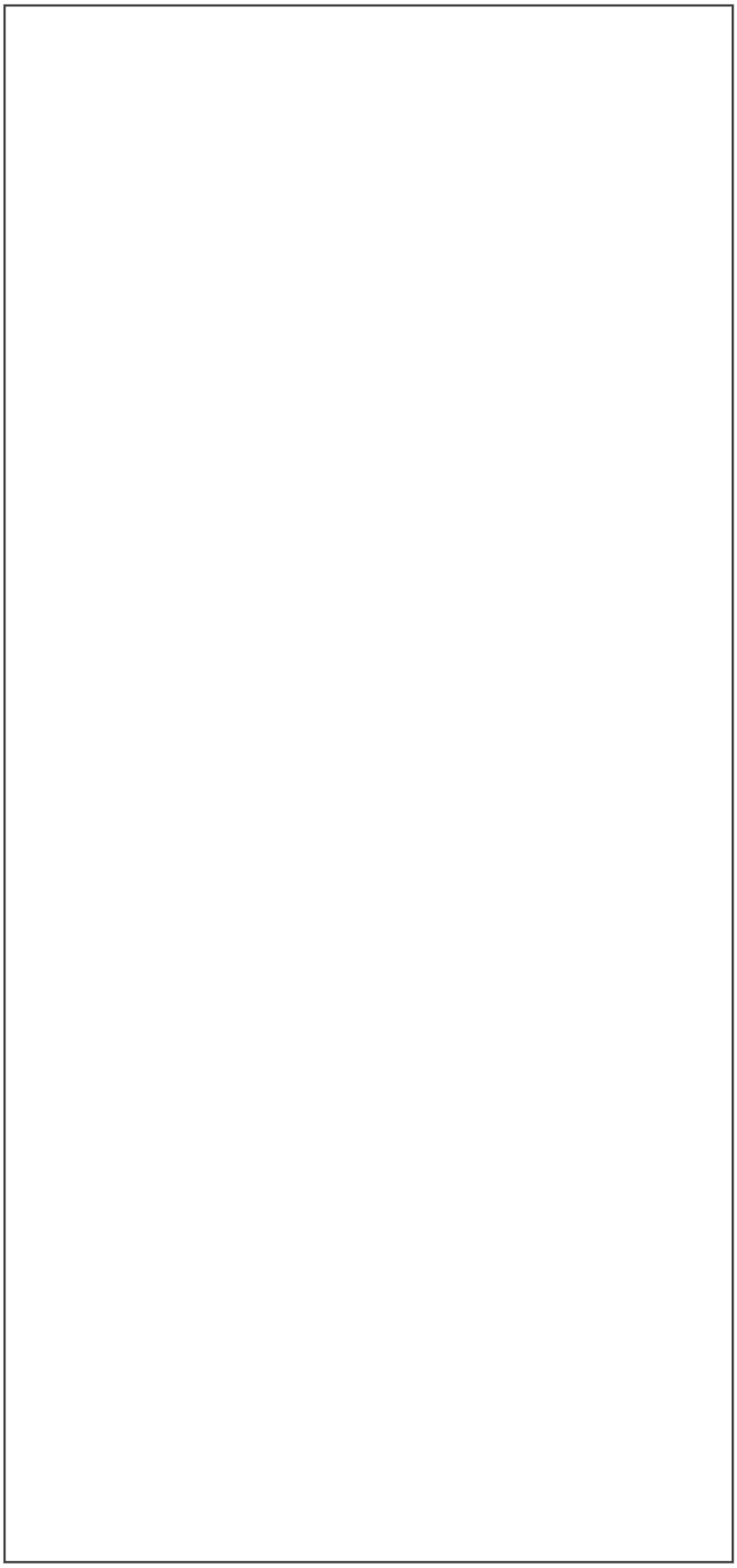
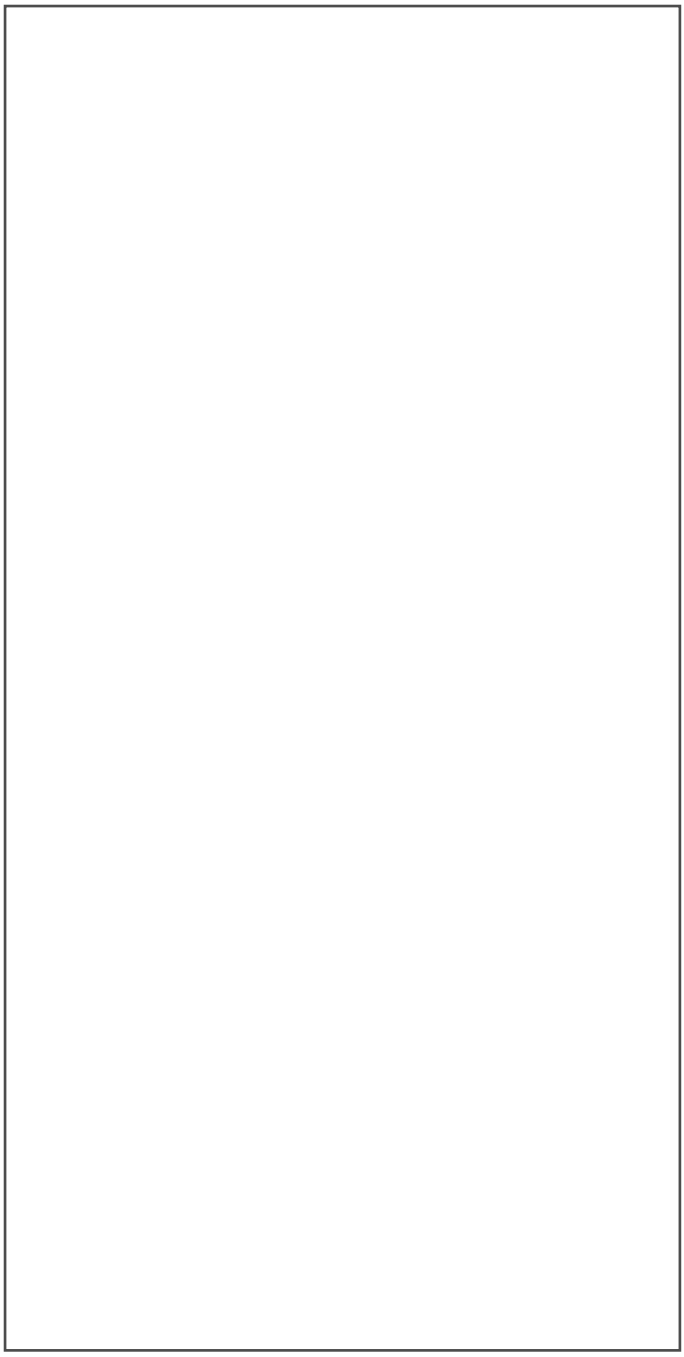


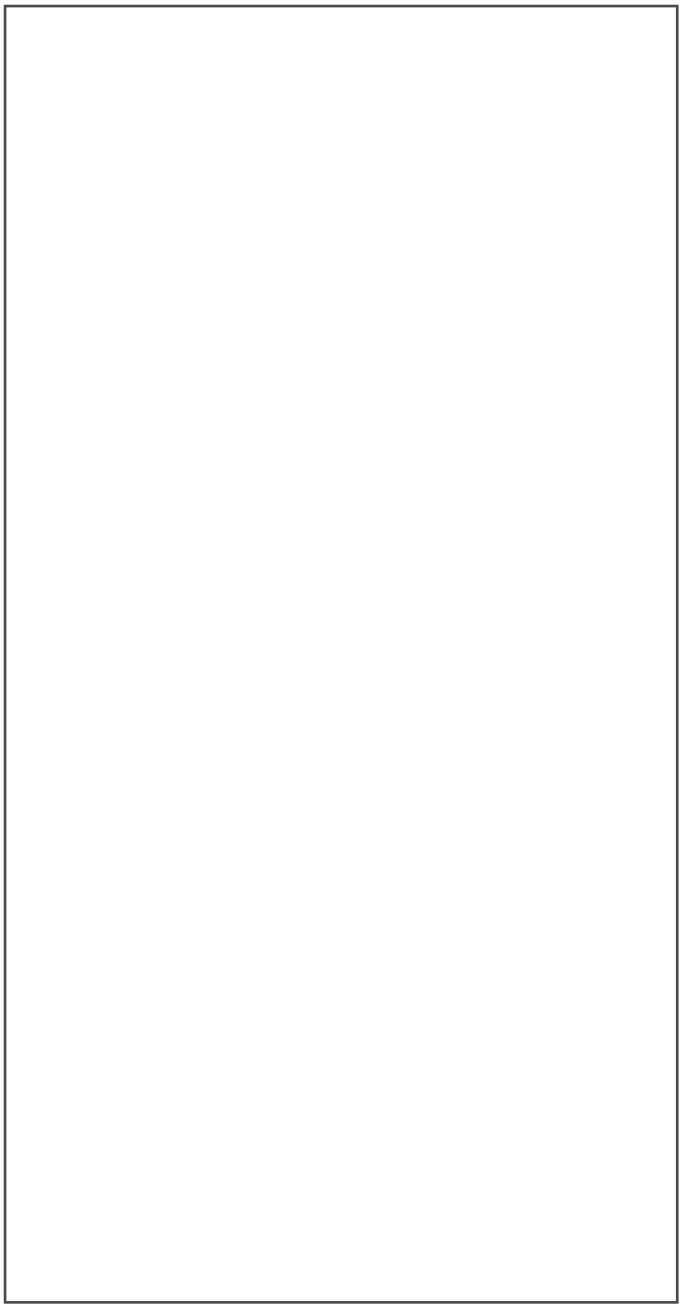
Fig 2

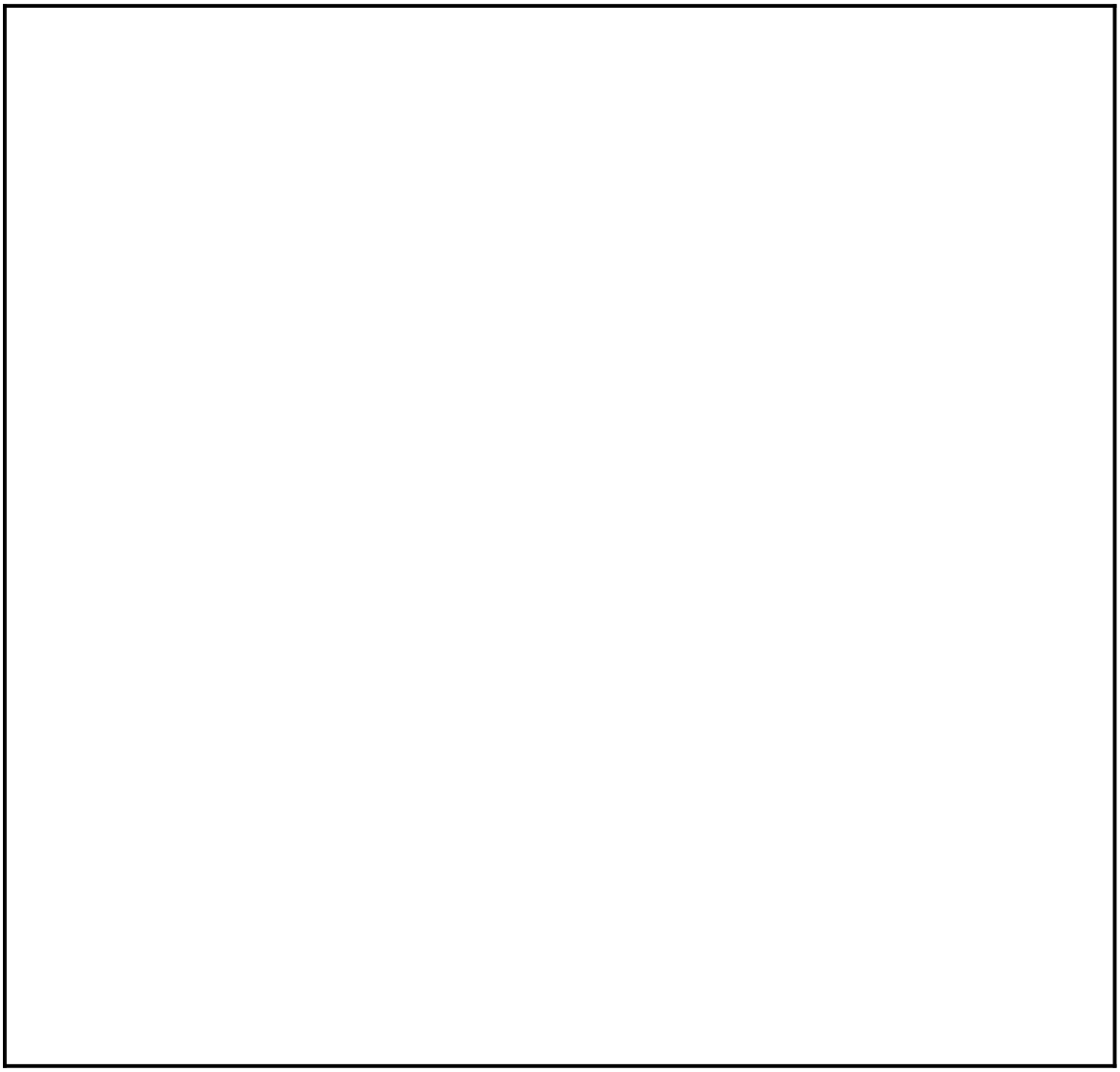
Fig 3

Fig 4









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