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Short communication

Ageing effects of casts of *Aporrectodea caliginosa* on soil microbial community structure and activity

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ABSTRACT

We studied the ageing of casts of the earthworm species *Aporrectodea caliginosa* in the field and their effects on soil microbial community structure and activity in a grassland soil. For this purpose, we sampled casts and the bulk soil (below and surrounding casts), 1 and 2 months after deposition of the casts. Discriminant and cluster analyses separated casts from soil samples, and revealed significant differences in microbial community structure in aged casts. There were no differences between soil below casts and surrounding soil samples. The overall microbial biomass and microbial activity were higher in casts than in soils. The same was true for the main groups of soil microbial communities, bacteria and fungi. Ageing of casts did not decrease the overall microbial biomass, and there was an unexpected increase in fungal biomass; however, ageing clearly decreased microbial activity. The effects of *A. caliginosa* on soil microbial communities in the grassland were strong, and contrary to our expectations, persisted over time. In fact, our data indicates that casts seem to function as isolated hot spots of microbial biomass and activity, with minimal interactions with soil.

1. Introduction

Earthworm casting represents one of the most important processes in turning over soil horizons and, in temperate zones, cast production ranges between 36 and 108 Mg ha^{-1} year^{-1} (Lavelle and Spain, 2001), thus constituting a key process to be considered in biogeochemical cycles. Earthworms, therefore, greatly alter the physical and chemical properties of their environment through their continuous burrowing, feeding and casting activities (Edwards, 2004). By doing so, they enhance decomposition through direct digestion of soil organic matter and this changes the availability of resources and populations of soil organisms, which may be selectively ingested (reviewed in Hättenschwiler et al., 2005).

Endogeic earthworms generally deposit casts on the surface of the soil or inside their galleries. Earthworm casts deposited on the surface of the soil, such as those of *Aporrectodea caliginosa*, undergo natural changes in the field, including ageing and a gradual disappearance, which would imply their reincorporation into the soil and re-ingestion. Thus, casts from *A. caliginosa*, microbiologically active and nutrient enriched (Aira et al., 2003), are released onto the soil, and their nutrient contents and microbial communities are slowly reduced through natural ageing processes (Aira et al., 2005). It is therefore possible that the huge amount of surface casts produced may have a later effect on the soil by the downward movement of casts particles or stimulation of soil microorganisms during ageing, and this may extend the effects of earthworms on microorganisms to the soil below the casts.

In the present study, we explored the cast ageing and their effects on microbial community structure (phospholipid fatty acid profiles) and microbial activity (fluorescein diacetate hydrolysis) in a grassland soil inhabited by the endogeic earthworm species *A. caliginosa*. For this purpose, we sampled casts and the soil below and surrounding them (bulk soil) 1 and 2 months after deposition of the casts. We questioned whether *A. caliginosa* modifies the soil microbial community only through casting processes (formation and ageing), and if these effects are extended to the soil below the casts during the ageing process.

2. Materials and methods

2.1. Experimental design

The study was carried out in a grassland ecosystem in Vigo, NW Spain; casting is strongly seasonal at the site, with two peaks occurring in spring and autumn. Cast density at the site prior to the experiment was 83 ± 13 casts m^{-2}. We sampled earthworms using the formalin-extraction method (area 0.5 m^2, n = 10; Raw, 1959) in early autumn; the only earthworm species found was *A. caliginosa*. The population density at the time of sampling was 136 ± 12 earthworms m^{-2} (mean biomass, 63 ± 5 g m^{-2}). In early

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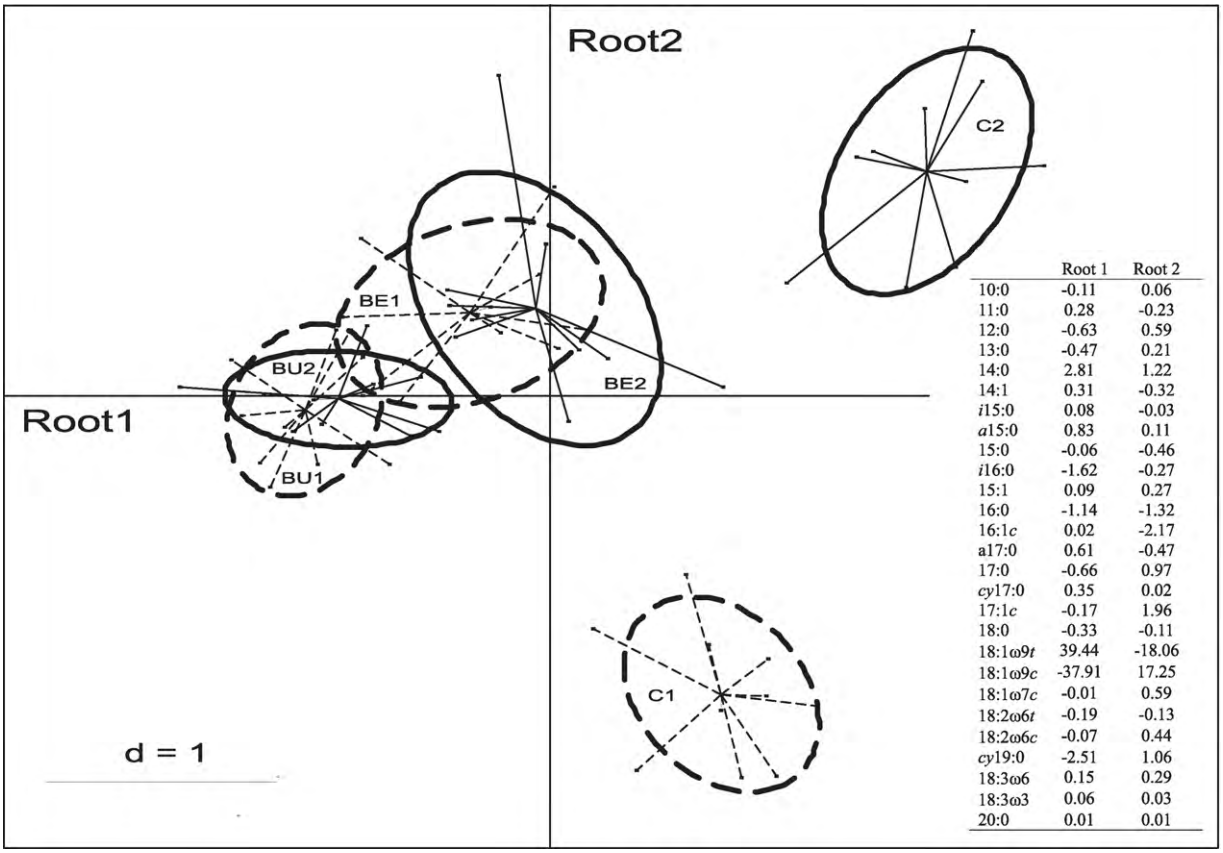
autumn, the grassland was carefully examined for earthworm activity and 75 newly deposited casts (there were not there the day before) were randomly selected and flagged by inserting a labeled eppendorf tube into the ground beside each cast. We took samples destructively 1 and 2 months later. We chose these sampling intervals because during the first month of ageing we did not find any modifications of several microbial biomass and activity indicators (Aira et al., 2005), which slowly decreases after that. At the sampling time, we randomly collected 10 casts and their corresponding soil below and surrounding bulk soil (distance <20 cm) samples; we collected soil samples to a depth of 5 cm with a 5 cm-diameter corer sampler. In this way, we completely removed casts from soil, and then took soil below samples; after carefully checking them we did not find any buried cast. Samples were sieved (2 mm mesh) and analyzed for their microbial community structure (PLFA profiles) and activity (fluorescein diacetate hydrolysis). Fresh casts showed a slightly higher organic matter content than soil (18 ± 2 and 15 ± 1%, respectively); main differences between fresh casts and soil were in $N–NH_4^+$ content (70 ± 2 and $10 \pm 3\ \mu g\ g^{-1}$ dw, respectively), with no differences in other parameters like dissolved organic nitrogen and carbon.

2.2. Analytical methods

The moisture content of fresh samples of casts was determined after drying at 105 °C for 24 h. Microbial communities were assessed by phospholipid fatty acid (PLFA) analysis. Total lipids were extracted from 2 g of freeze-dried soil samples with methanol and chloroform (1:2, v:v). The mixture was then filtered and evaporated under a stream of N_2 gas. The total lipidic extract was then dissolved with chloroform (3× 1 mL). Lipids were separated into neutral, glyco- and phospholipids in silicic acid columns (Strata SI-1 Silica (55 μm, 70 A), 500 mg/6 mL), with chloroform, acetone and methanol, respectively. The fraction containing phospholipids was evaporated under a stream of N_2 and redissolved in 500 μL of methyl-*tert*-butyl ether. One hundred microliters of this solution was placed in a 1.5 mL vial with 50 μL of the derivatizating agent (trimethylsulfonium hydroxide, TMSH), and the mixture was vortexed for 30 s and allowed to react for 30 min before adding 10 μL of nonadecanoic acid methyl ester as an internal standard. The chromatographic conditions have been described elsewhere; to identify and quantify the fatty acid methyl esters, retention times and mass spectra were compared with those obtained for known standard mixtures or pure PLFAs (Aira et al., 2009).

The PLFAs used as biomarkers were those described in the literature (Frostegård and Bååth, 1996). Total microbial biomass was determined as the sum of all extracted PLFAs expressed as $\mu g\ g^{-1}$ dry weight. The abundance of each of the different microbial groups (bacteria and fungi) was determined by the abundance of specific biomarkers commonly used for these groups. The sum of PLFAs considered to be predominantly of bacterial origin (15:0 and 17:0) was divided into Gram-positive bacteria (G^+) PLFA (i14:0, i15:0, a15:0, i16:0, i17:0 and a17:0) and Gram-negative (G^-) PLFAs (16:1c, cy17:0, 17:1c, 18:1ω7c and cy19:0) (Frostegård and Bååth, 1996). The G+/G– ratio was calculated by independently summarising these PLFAs. Fungi were determined from the relative concentration of 18:2ω6c PLFA (Frostegård and Bååth, 1996).

Microbial activity of the samples was assessed by hydrolysis of fluorescein diacetate (FDA). Fresh samples (2 g) were incubated for 20 min at 30 °C after the addition of 15 mL of 60 mM phosphate



	Root 1	Root 2
10:0	-0.11	0.06
11:0	0.28	-0.23
12:0	-0.63	0.59
13:0	-0.47	0.21
14:0	2.81	1.22
14:1	0.31	-0.32
*i*15:0	0.08	-0.03
*a*15:0	0.83	0.11
15:0	-0.06	-0.46
*i*16:0	-1.62	-0.27
15:1	0.09	0.27
16:0	-1.14	-1.32
16:1*c*	0.02	-2.17
a17:0	0.61	-0.47
17:0	-0.66	0.97
*cy*17:0	0.35	0.02
17:1*c*	-0.17	1.96
18:0	-0.33	-0.11
18:1ω9*t*	39.44	-18.06
18:1ω9*c*	-37.91	17.25
18:1ω7*c*	-0.01	0.59
18:2ω6*t*	-0.19	-0.13
18:2ω6*c*	-0.07	0.44
*cy*19:0	-2.51	1.06
18:3ω6	0.15	0.29
18:3ω3	0.06	0.03
20:0	0.01	0.01

Fig. 1. Discriminant function analysis performed on 27 PLFAs of bacterial and fungal origin from samples of casts (C), soil below casts (BE) and bulk soil (BU), 1 and 2 months after deposition of the casts (numbers 1 and 2 and dashed and solid lines, respectively), which are labeled inside their 95% inertia ellipses. The 10 points inside each inertia ellipse are the 10 replicates per treatment (*d* is the scale of the root 1 and root 2 axes). The table gives the values of canonical correlations of each PLFA used in the analysis with the two roots. Root 1 represents 54.7% of the variance and Root 2 represents 28.5% of the variance.

Table 1
ANOVA for total, bacterial, Gram-positive, Gram-negative and fungal PLFAS and fluorescein diacetate (FDA) hydrolysis.

	Sample		Time		Sample × time	
	$F_{2,54}$	P	$F_{1,54}$	P	$F_{2,54}$	P
Total PLFAs	6.08	0.004	3.01	0.089	0.65	0.525
Bacteria PLFAs	5.09	0.009	0.47	0.497	1.21	0.303
Gram-positive bacteria PLFAs	3.34	0.042	0.01	0.899	0.33	0.716
Gram-negative bacteria PLFAs	4.09	0.022	1.02	0.315	2.32	0.107
Fungal PLFAs	6.93	0.002	0.59	0.443	3.96	0.024
FDA hydrolysis	12.34	0.000	73.25	0.000	2.13	0.128

buffer (pH 7.6) and 0.2 mL of a 1000 $\mu g\,mL^{-1}$ solution of FDA prepared in acetone. The reaction was stopped by adding 15 mL of chloroform and methanol (2:1, v:v). Samples were then centrifuged and the aqueous phase filtered. The absorbance of the filtrates was measured within the next 50 min at 490 nm with a Bio-Rad Microplate Reader 550 (Adam and Duncan, 2001).

2.3. Statistical analysis

Data were analyzed by ANOVA (aov function), with the type of sample (cast, soil below cast and bulk soil) and time (1- and 2-month-old samples) as the main factors. Data were transformed prior to analysis, to meet ANOVA assumptions (normality and homogeneity of variances). Thus, total PLFAs, bacterial and Gram-negative PLFAs were log-transformed, whereas Gram-positive, fungal and fungal to bacterial PLFAs ratio were transformed with the boxcox function in the MASS library. Moisture content was included as a covariate in all analyses and did not affect any of the variables. Significant differences in the main effects were further analyzed by paired comparisons, with the Tukey HSD test. In order to analyze the underlying effect of the type of sample and time on the soil microbial community, data from the PLFA analysis were subjected to discriminant analysis with the discriminant function from the ade4 library. The Monte-Carlo randomization test was used to assess the statistical significance of between-class analyses. Data were analyzed with the R 2.8.1 environment.

3. Results

The discriminant analysis of the 27 identified PLFAs revealed large differences among the microbial communities of the samples, which explained 83% of variance in the data (Fig. 1). Samples were significantly classified into six groups according to type (casts, soil below casts and bulk soil) and age (1 and 2 months) (Monte-Carlo test based on 999 simulations, p-value = 0.001). One- and two-month-old casts were differentiated by an increase in fungal PLFA (see table in Fig. 1). Furthermore, separation between casts, and the two soil samples (soil below and bulk soil) was due to the greater amount of bacterial PLFAs in casts.

The concentration of total PLFAs was significantly higher in casts ($41.40 \pm 1.37\,\mu g\,g^{-1}$ dw) than in the bulk soil ($36.26 \pm 1.32\,\mu g\,g^{-1}$ dw) and soil below casts ($36.64 \pm 0.78\,\mu g\,g^{-1}$ dw) (Table 1), and this effect did not depend on time (Table 1). In the same way, the concentration of bacterial PLFAs was significantly higher in casts ($32.91 \pm 0.99\,\mu g\,g^{-1}$ dw) than in soil below casts ($29.54 \pm 1.01\,\mu g\,g^{-1}$ dw) and bulk soil ($29.42 \pm 0.51\,\mu g\,g^{-1}$ dw) (Table 1); this effect did not depend on time. The concentrations of both Gram-positive and Gram-negative bacterial PLFAs were higher in casts than in bulk soil (Table 1), and were intermediate in soil below casts (Fig. 2a); the effect did not depend on time. The concentration of fungal PLFAs was also higher in casts than in soil (Table 1); this effect was enhanced by ageing, as after 2 months the concentrations of fungal PLFAs in casts were higher than in soil samples (bulk and below casts), producing an interaction between sample and time (Table 1 and Fig. 2b). Microbial activity was significantly higher in casts and soil below casts thanin bulk soil (Table 1) and decreased

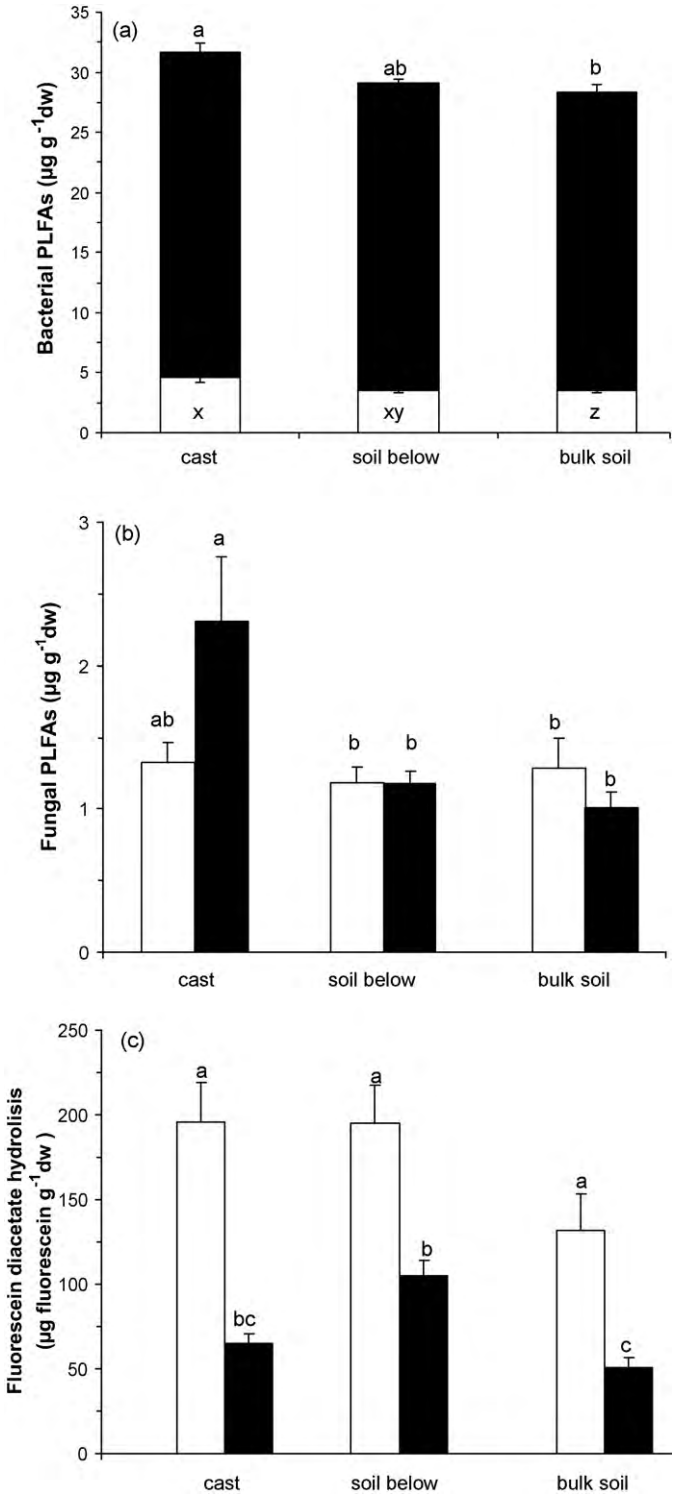


Fig. 2. Changes in (a) Gram-positive and Gram-negative bacterial PLFAs (white and black bars, respectively) in casts, soil below casts and bulk soil samples, (b) fungal PLFAs in casts, soil below casts and bulk soil samples 1 and 2 months after deposition of the casts (white and black bars, respectively) and (c) microbial activity (fluorescein diacetate hydrolysis) in casts, soil below casts and bulk soil samples, 1 and 2 months after deposition of the casts (white and black bars, respectively). Mean ± S.E.M., $n = 20$ (no time effect) for bacterial PLFAs and $n = 10$ for fungal PLFAs and microbial activity. Post hoc comparisons were made with Tukey HSD test ($P < 0.05$).

in all samples significantly from 1 to 2 months (Table 1 and Fig. 2c).

4. Discussion

In the present study we showed that aged casts of the earthworm species *A. caliginosa* hold a different microbial community structure than those of soil (below casts and bulk soil). Thus, we only found minor modifications in the structure of the soil microbial community, when present, in the bulk soil inhabited by the earthworms, with microbial communities of soil below casts being slightly different from those of bulk soil. Although surface casting in this grassland appears to account for a large percentage of released casts, we have to take into account that *A. caliginosa* usually casts onto the soil profile, attached to the gallery walls. However, we found that microbial parameters in the soil below casts and the bulk soil were clearly different from those of casts; thus, if casting in the galleries was high we should have collected some casts at sampling in the galleries included in the soil below and bulk soil samples, which would reduce differences between casts and soil samples. However, discriminant analysis strongly discards this view, with a clear separation of microbial communities in casts from those in soil (below casts and bulk), as previously found in studies with other earthworm species (Egert et al., 2004; Furlong et al., 2002).

We found overall higher values of total microbial biomass and the main microbial groups (bacteria and fungi) as well as in microbial activity in casts relative to parent soil, as previously shown (Aira et al., 2003, 2005). We did not find any differences in the Gram-positive and Gram-negative bacterial PLFAs contents of casts and soil below casts, which suggests some kind of stimulation of these microorganisms from casts to soil. If true, this effect should decrease with ageing since the microbial and nutrient characteristics of casts tend to become impoverished over time (Aira et al., 2005), although such a trend was not observed in the present study. These results may also be due to increased earthworm activity in the gallery that leads to casts, which may have stimulated bacterial microorganisms by mucus addition (Tiunov and Scheu, 1999).

Surprisingly, ageing did not modify microbial biomass, except for fungi, which biomass increased in 2-month-old casts. This contradicts previous findings (Aira et al., 2005) of decreases in fungal populations from fresh to aged casts. Lack of changes in most of the microbiological parameters may be due to the formation of stable aggregates of organic matter in the casts, an effect that is stronger in endogeic earthworm species like *A. caliginosa* (Lavelle and Spain, 2001). This implies the retention and gradual release of nutrients to the microorganisms (Edwards, 2004), which are able to remain active for longer than in the undigested soil, thus creating hotspots of microbial activity. However, we found a reduction of microbial activity after 2 months, which was almost the same for casts and soil samples, suggesting exhaustion of nutrients or adverse weather conditions like temperature reduction (14 °C and 8 °C at the start and end of experiment). There is some controversy regarding succession of bacteria–fungi in aged casts; some authors believe that bacteria dominate in fresh casts, and after depletion of labile C fungi are the major contributors to microbial biomass (Wolters and Joergensen, 1992). However, Devliegher and Verstraete (1995) and Tiunov and Scheu (2000) did not find any changes in the ratio of bacteria–fungi during ageing of casts. The present data support the first hypothesis, although we did not find the expected decrease in bacterial populations.

We have shown that the earthworm *A. caliginosa* had a strong effect on soil microbial communities with casts showing a different microbial community structure and activity from soil. In fact, our data indicates that casts seems to function as isolated hot spots of microbial biomass and activity, with minimal interactions with soil at least after 2 months.

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