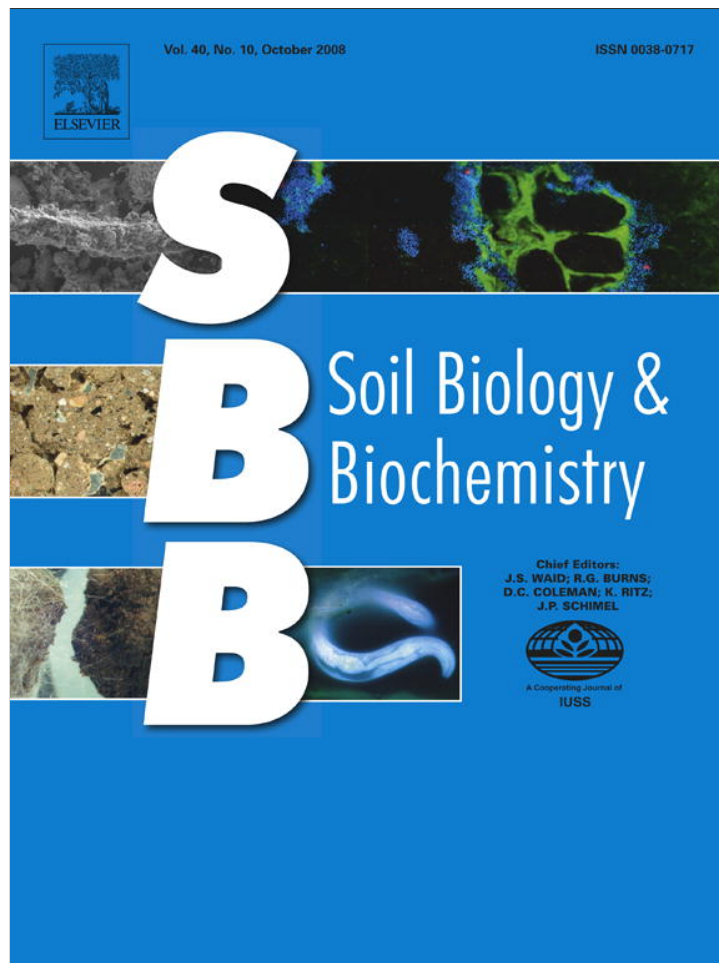


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## Detritivorous earthworms directly modify the structure, thus altering the functioning of a microdecomposer food web

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### ABSTRACT

Epigeic earthworms are key organisms in fresh organic matter mounds and other hotspots of heterotrophic activity. They turn and ingest the substrate intensively interacting with microorganisms and other soil fauna. By ingesting, digesting and assimilating the surrounding substrate, earthworms could directly modify the microdecomposer community, yet little is known of such direct effects. Here we investigate the direct effects of detritivore epigeic earthworms on the structure and function of the decomposer community. We characterized changes in the microfauna, microflora and the biochemical properties of the organic substrate over a short time (72 h) exposure to 4 different densities of the earthworm *Eisenia fetida* using replicate mesocosms (500 ml). We observed a strong and linear density-dependent response of the C and N mineralization to the detritivore earthworm density. Earthworm density also linearly increased CO<sub>2</sub> efflux and pools of labile C and inorganic N. This effect on the function was likely a direct consequence of earthworm activity. Furthermore, earthworms affected the microbial metabolic activity, but this response was not linearly related to the earthworm density, possibly because of indirect effects through the microbial community. Earthworms also had strong effects on the structure of the two trophic levels examined; they enhanced the fungal populations and reduced the numbers of bacterivore nematodes. The effect on the fungi was clearly dependent on the earthworm density, and the reduction of bacterivorous nematodes was also related to the earthworm density, but only marginally. In contrast, earthworms did not have significant effects on microbial biomass carbon, flagellate protozoa or ciliate protozoa. A meaningful part of the short term changes in microflora and microfaunal communities after some hours might be attributable to the earthworm gut associated processes. Hence, detritivore earthworms can directly and quickly modulate the decomposer community altering the decomposition rates of organic matter.

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### 1. Introduction

Earthworms are known to be key species in the soil system, where they modify microbial communities and nutrient dynamics (Lee, 1985; Edwards and Bohlen, 1996). Earthworm casts are usually enriched in nutrients such as C, N or P (Scheu, 1987; Aira et al., 2003). Further, microbial communities of casts and earthworm burrows differ from those of uningested soil (Sampedro and Whalen, 2007; Tiunov and Scheu, 1999, 2000), which would finally result in changes in decomposition rates (Swift et al., 1979; Saetre, 1998). Most of these studies have been focused on long-term or indirect effects of earthworms. Despite there is increasing understanding of the effect of earthworms in mineral nutrient cycling

and fluxes, the relationships that earthworms establish with microorganisms and their proximate effects on microfauna and microbial communities are far to be fully understood.

Studies investigating the direct effect of earthworms on microorganisms are in need particularly for epigeic earthworm species living in organic matter-rich environments, because most such studies focus on soil-dwelling endogeic and anecic species. It is known that epigeic earthworms can modify the fungal composition of forest soils (McLean and Parkinson, 2000), that they possess a diverse digestive enzyme pool (Lavelle and Spain, 2001), and that their feeding habits rely on microorganisms or organic matter, depending on the species (Scheu and Schaefer, 1998). In nature, epigeic earthworms live in fresh organic matter of forest litter, in litter mounds, in herbivore dungs, and in anthropogenic environments such as manure heaps, vegetal debris and vermicomposting beds common in agricultural landscapes. These habitats are hotspots of heterotrophic activity, where epigeic earthworms intensively interact with microorganisms and other soil fauna within

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the decomposer community, strongly affecting decomposition processes (Swift et al., 1979; Monroy et al., 2006; Sampedro and Domínguez, 2008). Microorganisms are the main agents of biochemical decomposition, whereas earthworms are involved in the indirect stimulation of microbial populations through comminution of organic matter, e.g. by increasing the surface area available for attack by microbes. Earthworms also modify the microbial populations through digestion, stimulation and dispersion in casts (Edwards, 2004). Epigeic earthworms closely interact with other biological components of the soil system, and they can affect the structure of microfauna and microflora (Domínguez et al., 2003; Lores et al., 2006).

Detritivorous earthworms modulate the stabilization of organic matter and strongly modify the physical and biochemical properties of the substrates in which they live (e. g. Aira et al., 2007). Broadly, the influence of epigeic earthworms on decomposition might be due to their gut associated processes (GAPs), the proximate effects of ingestion, digestion and assimilation in the gut (hours); or to cast associated processes (CAP), which are more related to ageing processes and to the physical modification of the egested materials (days to weeks; Scheu, 1987; Parthasarathi and Ranganathan, 2000; Aira et al., 2005). Physical modification of the substrate by burrowing habits such as aeration and promotion of leaching (Domínguez et al., 2004), are expected to be more remarkable in soil dwelling than in epigeic earthworms.

Because the direct effects of earthworms should be essentially density-dependent (i.e. proportional to the number of earthworms and to the length of their gut and gut transit time), these effects could be separated from other indirect subsequent effects by exposing during a short time the microdecomposer community to high earthworm densities. In this study we investigate the direct, proximate, influence of detritivore earthworms on the structure and function of the decomposer community. Using mesocosms, we examine how the activity of an epigeic earthworm modifies the microfauna, microflora and biochemical properties of the organic substrate where they live during a very short time period (72 h).

## 2. Materials and methods

We performed a bifactorial experiment in order to study the effect of (i) earthworm density and (ii) time on the structure and function of a microdecomposer foodweb. We used pig manure as substrate, which is known to support a dense decomposer food web (Sampedro and Domínguez, 2008). Pig manure was obtained from a farm near the University of Vigo, NW Spain, homogenized, stored at 5 °C until use, and preincubated before the experiment for 2 d at 20 °C. Some physico-chemical characteristics are summarized in Table 1. The mesocosms consisted of 500 ml mason jars filled with 100 g (fresh weight, fw) of substrate. After filling the jars, we allowed the substrate to stabilize for 72 h before inoculating the

earthworms. We used the epigeic earthworm species *Eisenia fetida* (Savigny, 1826), broadly distributed and easy to manage under lab conditions. We allowed mature individuals ( $303 \pm 7$  mg; mean individual fw  $\pm$  SEM) of our culture bins to empty their guts on moistened tissue paper for 24 h at room temperature before the experiment. We considered four densities of earthworms: control, low, medium and high (0, 25, 50 and 100 earthworms per mesocosm, respectively). Fifteen replicate mesocosms were established for each density, with a mean weight of  $7.3 \pm 0.1$ ,  $14.4 \pm 0.3$  and  $29.8 \pm 0.4$  g fw of earthworms (low, medium and high earthworm density respectively). We covered the jars (containing the substrate and the earthworms) with perforated lids, stored them at random in a dark cabinet, and maintained under laboratory conditions ( $20 \pm 2$  °C) for 72 h. In total we established 72 mesocosms corresponding to 6 levels of time, 4 levels of earthworm density and 3 replicates each. A random subset of mesocosms of each density ( $n = 3$ ) was destructively sampled after 0, 12, 24, 36, 48 and 72 h.

The mineralization rate was immediately determined by measuring the production of CO<sub>2</sub> from the whole mesocosm (earthworms included) after 30 min incubation. The evolved CO<sub>2</sub> was trapped in 0.02 M NaOH and subsequently measured by titration with HCl to a phenolphthalein endpoint, after adding excess BaCl<sub>2</sub> (Anderson, 1982). Earthworms were then removed from each jar, and the substrate was weighed, gently homogenized and immediately processed for chemical, biochemical and faunal analyses.

We determined the moisture content of the manure gravimetrically after drying at 105 °C for 24 h, and the organic matter content after ashing at  $550 \pm 50$  °C for 4 h. Chemical and biological soil characteristics are expressed on the basis of the oven-dry weight (dw). The extractable mineral N (N-NH<sub>4</sub><sup>+</sup> and N-NO<sub>3</sub><sup>-</sup>) content of samples was determined in 0.5 M K<sub>2</sub>SO<sub>4</sub> extracts (1:5, fw:v) using the indophenol blue technique in a Bio-Rad Microplate Reader 550 (Sims et al., 1995). Microbial biomass C (C<sub>mic</sub>) was analyzed by the chloroform fumigation–extraction method using a  $K_{EC} = 2.64$  (Vance et al., 1987). Dehydrogenase enzyme activity was measured colorimetrically at 545 nm by estimation of the rate of reduction of triphenyltetrazolium chloride (TTC) (1.5%) to triphenylformazan (TPF) after incubation at 30 °C for 24 h (Casida et al., 1964). Dissolved organic carbon (DOC) was determined also colorimetrically in microplates after moist digestion (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> and H<sub>2</sub>SO<sub>4</sub>) of aliquots of 0.5 M K<sub>2</sub>SO<sub>4</sub> extracts of the unfumigated samples. Microbial activity within the substrate was determined by measuring the CO<sub>2</sub> evolution from 5 g fw samples during 6 h incubation as above.

Fungal biomass was estimated analyzing the ergosterol content in the samples at the end of the experiment (72 h samples). Ergosterol was extracted by microwave-assisted extraction (MAE) and determined by HPLC analysis (Young, 1995; Aira et al., 2006). Briefly, for the MAE procedure, 500 mg fw samples were digested in a microwave oven into Teflon bottles with 2 ml of methanol and 0.5 ml of 2 M NaOH. The digested samples were extracted with pentane ( $3 \times$  ca. 2 ml), then evaporated to dryness under a stream of N<sub>2</sub> and redissolved with methanol (1 ml). Subsequently, quantitative determination of ergosterol was performed by reverse-phase HPLC analysis using a C18 column of 12.5  $\times$  4 mm 5  $\mu$ m Hypersil with a mobile phase of methanol:water (95:5, v: v) and detection set at 282 nm.

Nematodes were extracted from 10 g fw samples in modified Baermann funnels, counted live at a magnification of 140 $\times$  under a dissecting microscope and sorted into trophic groups (bacterivores, fungivores, omnivores and herbivores) using oesophageal morphology, as described by Parmelee and Alston (1986). Ciliate and flagellate protozoa were grown in NBAS medium (Griffiths, 1989) from 1 g fw samples of substrate in a 1:100 (fw:v) ratio. The estimation of the number of each category was done in serial dilutions of these original cultures in microplates, following a modification of the more probable number technique

**Table 1**  
Physico-chemical characteristics of fresh pig manure used as substrate

Moisture content (%)	86 $\pm$ 10
pH	8.3 $\pm$ 1.0
Electrical conductivity (mS cm <sup>-2</sup> )	0.25 $\pm$ 0.01
Organic matter (mg g <sup>-1</sup> dw) <sup>a</sup>	860 $\pm$ 10
Total carbon (mg g <sup>-1</sup> dw) <sup>b</sup>	455 $\pm$ 60
Dissolved organic carbon ( $\mu$ g g <sup>-1</sup> dw)	4100 $\pm$ 100
Microbial biomass carbon ( $\mu$ g g <sup>-1</sup> dw)	12361 $\pm$ 895
Total nitrogen (mg g <sup>-1</sup> dw) <sup>b</sup>	24 $\pm$ 2
N-NH <sub>4</sub> <sup>+</sup> ( $\mu$ g g <sup>-1</sup> dw)	2400 $\pm$ 100
N-NO <sub>3</sub> <sup>-</sup> ( $\mu$ g g <sup>-1</sup> dw)	70 $\pm$ 10

Mean  $\pm$  SEM;  $n = 3$ .

<sup>a</sup> dw = Dry weight.

<sup>b</sup> Carlo Erba elemental analyzer.

(MNP; Darbyshire et al., 1974). Extraction of microfauna was performed in the 72 h samples.

The effects of incubation time (5 levels) and earthworm density (4 levels) on extractable C and N, total respiration, microbial biomass carbon and microbial activity were analyzed with a two-way ANOVA. The effect of earthworm density after 72 h of incubation (final samples) on these variables and on the ergosterol content and microfauna was analyzed by one-way ANOVA. Post hoc comparisons of means were performed by a Tukey HSD test at  $\alpha = 0.05$ . If a response variable at the end of the experiment (72 h) was significantly affected by earthworm density, we explored the dependence of that variable to the earthworm density by regression analysis. All statistical analyses were carried out using SPSS v. 14 software.

### 3. Results

#### 3.1. Proximate effects of epigeic earthworms on C and N mineralization

The proximate activity of earthworms significantly enhanced the mineralization of both carbon and nitrogen in the substrate. The cumulative C mineralization from mesocosms was significantly affected by earthworm density (Fig. 1; Table 2). The net fluxes of CO<sub>2</sub> after 72 h of incubation were also significantly greater in the high earthworm density, reaching up to 1.3 times over the control (one-way ANOVA  $F_{3,8} = 20.7$ ;  $P < 0.001$ ; Fig. 1). This effect on the function was positively, closely, and linearly related to the earthworm density ( $R^2 = 0.835$ ;  $P < 0.0001$ ).

The labile C pool (DOC) was also significantly affected by earthworm activity throughout the experiment (Table 2). The greatest overall mean values corresponded to the treatment with the high earthworm density, reaching  $2174 \pm 253 \mu\text{g C g}^{-1} \text{ dw}$ , which was significantly different from the control ( $1446 \pm 207 \mu\text{g C g}^{-1} \text{ dw}$ ), while the low and medium earthworm densities showed intermediate values. The significant interaction earthworm  $\times$  time (Table 2) was due to the extremely high values of DOC concentration observed in low and high earthworm densities after 24 and 48 h, respectively (data not shown). The DOC concentration after 72 h was also significantly greater in mesocosms containing earthworms than in control ones (Fig. 2a). This effect was positively and closely related to earthworm density ( $R^2 = 0.617$ ;  $P = 0.002$ ).

Mineral N concentration slightly decreased through time irrespectively to the earthworm density (Table 2); however, the content of inorganic extractable N was significantly greater in the

mesocosms with earthworms after 72 h (Fig. 2b), due to the increase observed in the high density treatment. N mineralization was linearly related to earthworm activity ( $R^2 = 0.749$ ;  $P < 0.001$ ).

#### 3.2. Proximate effects of epigeic earthworms on the decomposer community

Earthworm density significantly affected the variables associated with microbial metabolic activity, i.e. microbial respiration and dehydrogenase activity (i.e. aerobic oxidative activity) throughout the experiment (Table 2). Overall mean values of dehydrogenase activity were the highest in medium earthworm density, being significantly different from that in high density, and showing the control and the lowest earthworm density intermediate values (data not shown). Microbial respiration showed a similar trend, with the overall mean for the low earthworm density being significantly greater and different than the other treatments, and with changing values throughout the experiment (significant interaction earthworm  $\times$  time, Table 2), but the regular trend was the highest values for the treatment with 25 earthworms, and the lowest for any of the other earthworm densities. After 72 h of exposure to the earthworms, microbial respiration was significantly increased at low densities compared to the control without earthworms and markedly decreased at high densities (Fig. 3a). Microbial respiration after 72 h was not linearly related to earthworm density ( $R^2 = 0.216$ ;  $P = 0.128$ ). We did not detect significant differences in the final values of dehydrogenase activity.

Despite the consistent effect on microbial activity, earthworms had no effect on microbial biomass carbon, neither throughout the experiment (Table 2) nor after 72 h (one-way ANOVA;  $F_{3,8} = 1.16$ ;  $P = 0.382$ ). However, earthworms significantly directly enhanced fungi populations after only 72 h of incubation (Fig. 3b). Ergosterol content at high earthworm density was twice that of the control (Fig. 3b), and final values of ergosterol content were positively and linearly related to earthworm density ( $R^2 = 0.673$ ;  $P = 0.004$ ).

We did not detect significant effects of earthworms on flagellated protozoa (one-way ANOVA  $F_{3,8} = 0.944$ ;  $P = 0.464$ ) or ciliated protozoa (one-way ANOVA  $F_{3,8} = 2.39$ ;  $P = 0.144$ ) after 72 h. In contrast, earthworms markedly affected the nematode community, composed only of bacterivorous nematodes. After 72 h, earthworms' presence had significantly reduced the abundance of bacterivorous nematodes (by 30%) in comparison to the control (Fig. 3c). However, the decrease of bacterivore nematodes was only marginally dependent to the density of earthworms ( $R^2 = 0.327$ ;  $P = 0.051$ ).

### 4. Discussion

#### 4.1. Effects on C and N mineralization

The proximate activity of earthworms significantly enhanced the mineralization of both carbon and nitrogen in the substrate. As expected, these effects were proportional to earthworm density. At the highest earthworm density, CO<sub>2</sub> emission and mineral N content were 1.3 and 1.15 times, respectively, that of the control. According to our rationale, this strong and density-dependent effect on the function is due to the direct activity of earthworms on substrate mineralization. That is the proximate direct consequence of gut passage derived from ingestion, digestion and assimilation of the substrate by epigeic earthworms. Thereby it should be proportional to earthworm numbers. This is not new, and several authors have reported similar responses in detritivorous organisms involved in organic matter decomposition (Aira et al., 2002; Vetter et al., 2004).

These effects on substrate mineralization resulted in strong modifications in the pools of labile C and mineral N, with greater

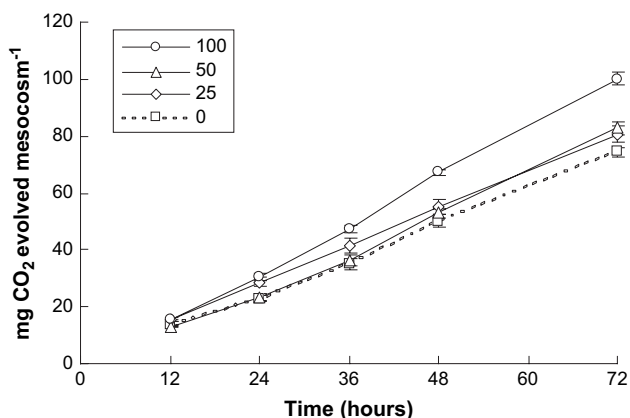


Fig. 1. Proximate effect of epigeic earthworms on organic matter mineralization (cumulative CO<sub>2</sub> evolved) with 0, 25, 50 and 100 earthworms per mesocosm during 72 h incubation. Mean  $\pm$  SEM;  $n = 3$ .

**Table 2**  
Results of the GLM model for the effect of time and earthworm density on the variables analyzed in the destructive sampling

Effect	Effect					
	Earthworm density		Time		Earthworm × time	
	<i>F</i> <sub>(3,40)</sub>	<i>P</i>	<i>F</i> <sub>(4,40)</sub>	<i>P</i>	<i>F</i> <sub>(12,40)</sub>	<i>P</i>
Cumulative respiration (mg CO <sub>2</sub> jar <sup>-1</sup> )	49.7	<0.001	936.7	<0.001	5.3	<0.001
Dissolved organic carbon (μg C g <sup>-1</sup> dw)	3.41	0.026	1.58	0.199	4.11	<0.001
Mineral nitrogen (μg N g <sup>-1</sup> dw)	2.12	0.113	2.71	0.043	0.99	0.469
Microbial respiration (μg CO <sub>2</sub> h <sup>-1</sup> g <sup>-1</sup> dw)	4.1	0.012	4.4	0.005	5.4	<0.001
Dehydrogenase activity (equiv TFF g <sup>-1</sup> dw)	3.2	0.035	4.7	0.003	0.3	0.986
Microbial biomass carbon (μg C g <sup>-1</sup> dw)	1.27	0.298	1.21	0.32	1.72	0.098

*F* values and probabilities are shown for each effect. *P* < 0.05 in italics are significant.

concentrations at higher earthworm densities. The increase in mineral N recorded at high earthworm density can be attributable mainly to mineralization of organic N. Earthworm activity, however, may also have played a role because ammonia is one of their excretion products (Lee, 1985). The significant increase in the DOC with earthworm presence suggests a mobilization of labile C pools likely due to cellulose and hemicellulose breakdown (Saetre, 1998; Aira et al., 2006). Nevertheless, it is also possible that earthworms partly contributed by mucus production (Brown and Doube, 2004). This finding, along with the increase in microbial activity at low earthworm density, suggests that in organic matter-rich environments, activity of epigeic earthworms is not C-limited. It also suggests that earthworms promote the growth and activity of microorganisms perhaps by releasing labile C compounds in the first stages of decomposition.

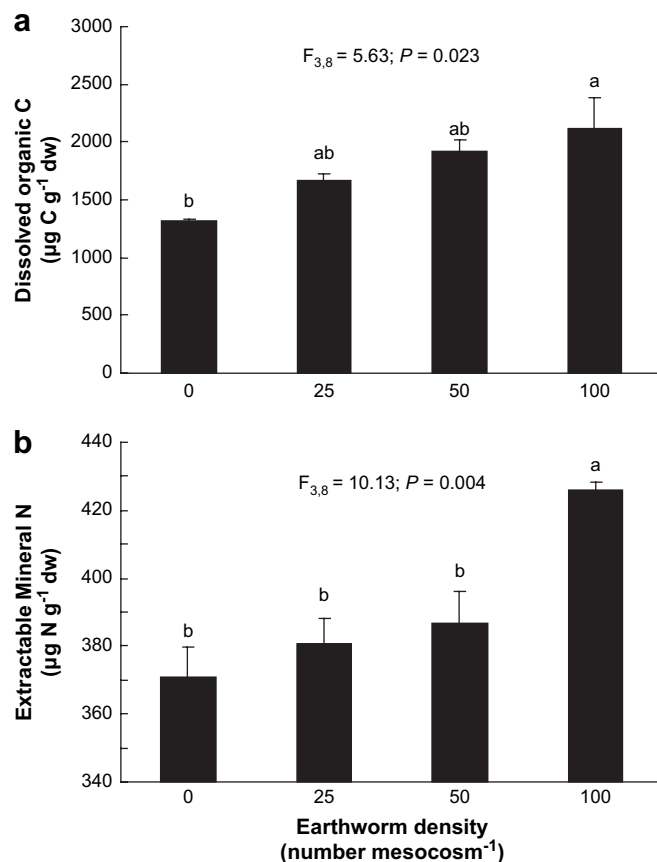
#### 4.2. Effects on decomposer community

Consistent with previous findings (Aira et al., 2002), earthworms seemed to boost microbial metabolism at low densities in the short term. In contrast, at medium and high densities, earthworms exhausted microbial activity. These contrasting, non-linear, results regarding the effect that earthworms exert on microbial activity indicate that contradictory findings among studies may simply reflect differences in substrate quality or earthworm density (Atiyeh et al., 2000; Domínguez et al., 2003).

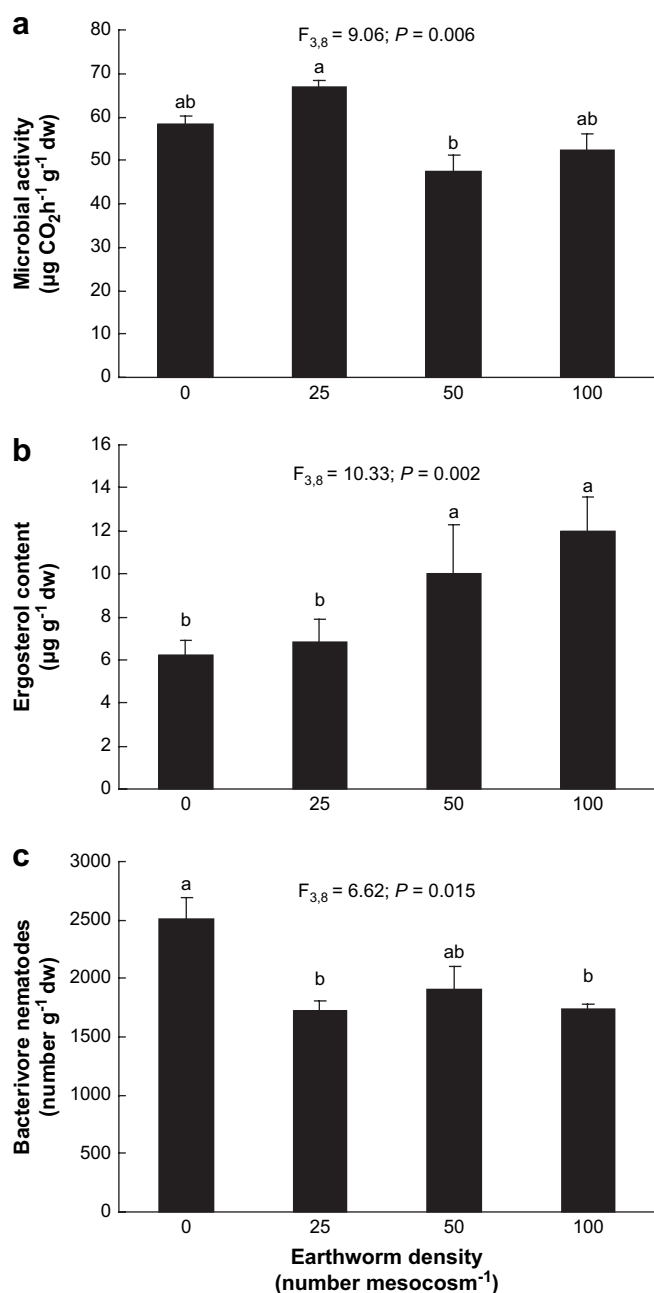
Earthworms significantly affected the structure of the two trophic levels analyzed, i.e. microflora and microfauna. Earthworms enhanced the fungal populations and reduced the numbers of bacterivore nematodes, but they did not affect microbial biomass C. Animal manures are known to be microbial rich environments in which bacteria constitute the largest fraction, with the fungi present mainly as spores (Garrett, 1981). Microorganisms, especially fungi, might be the main constituent of earthworm diet (Edwards, 2004). Moreover, earthworms can graze selectively on fungal species (Cooke, 1983; Moody et al., 1995), which can be digested by earthworms (Schönholzer et al., 1999; Sampedro et al., 2006). Earthworms affected the microbial community structure of pig slurry, and contrary to expectations, they favoured fungal growth in only 72 h. Hence, the significant increase in ergosterol content at intermediate and high earthworm densities suggests that there should be a threshold number of earthworms at which fungal growth is triggered. This is consistent with previous studies (Aira et al., 2006; Lores et al., 2006). Similarly, Pízl and Novakova (2003), found a higher density of microfungi in the substrate worked by *Eisenia andrei* than in the control substrate. Activation of fungi might be attributed to the grazing and dispersal of the spores as well as to the physical changes in substrate structure, favouring fungal growth. In this way, Vetter et al. (2004) found that the accelerating effect of soil fauna on decomposition was associated with substrate homogeneity. In this study, earthworm casting activity

may have likely reversed the homogeneity of pig slurry into a heterogeneous substrate, allowing the rise of new microbial communities, like those formed by fungi.

The reduction in nematode numbers in the earthworm treatments may be due to the direct grazing by earthworms. Nevertheless, under this scenario, it should be expected a stronger negative association between nematode numbers and earthworm density. Our results therefore suggest also an indirect effect of earthworms, possibly due to the physical modifications that they produce in the substrate. These results are important because the decrease in nematodes appeared to be independent of the increase in labile carbon and microbial activity, which usually leads to an increase in the number of bacterivores, because they are closely related to the dynamics of their resources



**Fig. 2.** Proximate effect of epigeic earthworms on carbon and nitrogen mineralization. Concentration of (a) dissolved organic carbon and (b) extractable mineral nitrogen in the substrates after 72 h incubation with 0, 25, 50 and 100 earthworms per mesocosm. *F* and *P* values of the effect of earthworm density analyzed by one-way ANOVA. Mean ± SEM; *n* = 3. Different letters indicate significantly different means (*P* < 0.05, Tukey HSD test).



**Fig. 3.** Proximate effect of epigeic earthworms on the decomposer community. (a) Microbial activity, (b) fungal biomass measured as ergosterol concentration, and (c) bacterivorous nematodes. Conditions and statistical analyses as given in Fig. 2.

(Bongers and Ferris, 1999; Zelenev et al., 2004). Earthworms can ingest and digest nematodes (Dash et al., 1980; Monroy et al., 2008), affecting the fertility, viability and germination of cysts once expelled in casts (Roessner, 1981). Earthworms can also exert indirect effects on nematodes by modifying environmental characteristics, such as moisture content, and nutrient cycling processes (Yeates, 1981). Alternatively, the lack of fungivorous nematodes in our study may be explained by the early stages of decomposition covered by this experiment. In a vermicomposting study fungivorous nematodes only appeared after 6 weeks of incubation, showing higher numbers in the absence than in the presence of *E. andrei* (Domínguez et al., 2003). Furthermore, the life cycle of some fungivorous nematode species is characterized by a slow growth rate (Bongers and Bongers, 1998).

#### 4.3. Conclusions

As expected, we found a significant and clear density-dependent response of the C and N mineralization to the detritivore earthworm numbers. Thus, this effect on the function would be mainly derived from gut associated processes, i.e. the proximate direct consequence of gut passage. Furthermore, earthworms modified the microbial metabolic activity, although such activity was not linearly related to the earthworm density. This may partly reflect indirect effects of earthworms on the microbial community. We also showed that epigeic earthworms directly and strongly affected the structure of the two trophic levels examined, enhancing the fungal populations and reducing the numbers of bacterivore nematodes. The effect of earthworms on fungi was linearly dependent on earthworm density, and the reduction of bacterivorous nematodes was also related to earthworm density, although only marginally. Thus, we propose that a considerable part of these changes in microflora and microfaunal communities after 72 h could be attributable to the gut associated processes, although we cannot discard that other processes also play a role even at that short time scales. Hence, detritivore earthworms could directly modulate decomposition rates and decomposer community composition in the short term. Further research is needed to untangle the complex mechanisms by which detritivore earthworms and the microdecomposer community interact to increase decomposition rates and lignocellulose triggering.

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