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# Changes in density of nematodes, protozoa and total coliforms after transit through the gut of four epigeic earthworms (Oligochaeta)

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ABSTRACT

Epigeic earthworms are detritivorous organisms that live and feed in the soil litter layer. These earthworms exert important effects on the presence of decomposer micro-organisms and their microbial grazers, which lead to changes in the rate of decomposition of the organic matter. To assess the effect of the transit through the gut of epigeic earthworms on the decomposer organisms, we analysed the gut contents of four epigeic species reared under laboratory conditions, with pig slurry as food source. The numbers of nematodes, protozoa (flagellates, testate and naked amoebae) and total coliforms in the hindgut of the earthworm species, *Eisenia fetida*, *Eisenia andrei*, *Lumbricus rubellus* and *Eudrilus eugeniae*, were compared with the numbers of the same organisms in the pig slurry. No nematodes were found after transit through the gut of the earthworms and there was a decrease between 85% and 98% in the numbers of total coliforms of the pig slurry ($723 \pm 125 \times 10^3$ CFU $g^{-1}$ dw). The effect on the numbers of protozoa depended on both groups of protozoa and species of earthworm considered. The numbers of flagellates were greater in the gut samples of *E. fetida* and *E. andrei* ($363 \pm 46 \times 10^3$ MPN $g^{-1}$ dw) than in the ones of *L. rubellus* and *E. eugeniae* ($105 \pm 26 \times 10^3$ MPN $g^{-1}$ dw). The density of testate amoebae was not affected by the transit through the gut of earthworms, and the numbers of naked amoebae were greater in the gut samples of *L. rubellus* ($2500 \pm 780 \times 10^3$ MPN $g^{-1}$ dw) than in the fresh pig slurry ($755 \pm 311 \times 10^3$ MPN $g^{-1}$ dw). The results indicate that short-time effects associated with the digestive activity in the earthworm gut play an important role in the changes that epigeic species exert on the decomposer community.



## 1. Introduction

Although it is well known that soil decomposer organisms interact in a complex way, most studies addressing these interactions focus on the relationships between soil fauna and micro-organisms (fungi and bacteria) (Bardgett, 2005). Micro-organisms are directly responsible for the decomposition process, and show a high degree of specialization and display a large number of enzymes for the breakdown of the organic matter (Lavelle and Spain, 2001). Microbial communities also support a large number of soil invertebrates, which in turn have an important regulatory effect on the microbial populations (Edwards, 2000). The way that soil invertebrates interact with soil micro-organisms largely depends on their size and morphology (Swift et al., 1979). Microfauna (2–100 μm body width) such as nematodes and protozoa, are restricted to films of water and water-filled pores, and feed directly on fungi and bacteria; meso- and macro-fauna (0.1–2 mm and 2–20 mm

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body width, respectively) are detritivores that ingest soil and decaying materials together with micro-organisms (Lavelle and Spain, 2001). Nematodes are the most abundant metazoans in soils (Dindal, 1990), and together with protozoa constitute the main consumers of soil bacteria (Ekelund and Rønn, 1994). These microbial grazers have an important regulatory effect on the decomposition process, since they affect microbial activity and increase nutrient mineralization (Ingham et al., 1985; Bamforth, 1988).

Some larger animals, such as earthworms, can simultaneously affect soil micro-organisms and their microbial grazers through their feeding, burrowing and casting activities (Brown et al., 2000). The relative importance of each of these activities on the soil community depends on the ecological features of the earthworm species under consideration (Lavelle and Spain, 2001). Epigeic earthworms (i.e. those that feed and live in the litter layer) increase decomposition rates and strongly affect populations of other litter inhabiting organisms (Domínguez et al., 2003; McLean and Parkinson, 2000a,b). Nevertheless, little is known about whether and to what extent these changes are due to direct effects of earthworms on the decaying material (i.e. transformations of the ingested material during passage through the gut) or to indirect effects related to their casting and burrowing activities (Brown et al., 2000; Domínguez, 2004).

The first step of the interactions between these animals and the rest of the soil community are the short-term changes associated with digestion of the organic material in earthworms' guts (Domínguez, 2004). In the present study we tested the hypothesis that digestion of organic material by epigeic earthworms has negative effects on the density of both microfauna and micro-organisms, and that those effects are earthworm species-specific. In order to test this hypothesis, the densities of protozoans, nematodes and total coliforms in the gut contents of four epigeic earthworm species were quantified and compared with the densities of the same organisms in the food source supplied to the earthworms.

## 2. Materials and methods

### 2.1. Earthworm species and breeding culture

Four earthworm species belonging to two different families were used in the study. Specimens of *Eisenia fetida* (Savigny, 1826), *Eisenia andrei* Bouché, 1972 and *Lumbricus rubellus* Hoffmeister, 1843 (family Lumbricidae) were obtained by sampling (hand-sorting method) a manure heap and a pig-breeding farm near the University of Vigo, Spain. Specimens of *Eudrilus eugeniae* Kinberg, 1867 (family Eudrilidae) were obtained from a commercial supplier (Minhobox, Brazil). Earthworms of all four species were reared in the laboratory, with pig slurry as the substrate, for 2 months before the start of the experiment. Fresh pig slurry, obtained from a pig-breeding farm near the University of Vigo, NW Spain, was homogenized in a slurry pit, then stored in sealed plastic containers and kept at 5 °C until use. The solid fraction (15% dry weight) of the pig slurry was used to avoid the worms coming into contact with the percolates, which may be harmful to earthworms. The main physicochemical characteristics of the pig slurry are shown in Table 1.

**Table 1 – Physicochemical characteristics of the fresh pig slurry used**

| | |
|---|---|
| Moisture content (%) | 85 ± 2.0 |
| Organic matter content (%) | 86 ± 1.0 |
| pH | 8.3 ± 1.0 |
| Electrical conductivity (mS $cm^{-1}$) | 0.25 ± 0.01 |
| Total nitrogen (mg $g^{-1}$ dw) | 24 ± 2 |
| $N\text{-}NH_4^+$ (μg $g^{-1}$ dw) | 2400 ± 100 |
| $N\text{-}NO_3^-$ (μg $g^{-1}$ dw) | 250 ± 50 |
| Total carbon (mg $g^{-1}$ dw) | 455 ± 60 |
| Dissolved organic carbon (mg $g^{-1}$ dw) | 11.1 ± 0.1 |

dw: the dry weight.

### 2.2. Mesocosms

For each of the four earthworm species, we set up five mesocosms consisting of plastic containers (3 L), which were filled to three-fourth of their capacity with sieved (>2 mm) and moistened (80% moisture content) vermiculite. We chose vermiculite as a substrate because it is chemically inert and does not contain any nutrients, which thus obliges the earthworms to ingest the pig slurry provided. Each of the mesocosms was inoculated with either 50 mature earthworms (*E. fetida*, *E. andrei*) or 20 mature specimens (*L. rubellus*, *E. eugeniae*). A plastic mesh (1 cm pore size) was placed over the surface of the vermiculite and the pig slurry (ca. 200 g, fresh weight) was placed on top of the mesh, to avoid mixing the pig slurry with the vermiculite and to facilitate removal of consumed material. Mesocosms were checked twice weekly, the pig slurry replaced and the vermiculite washed to prevent the earthworms ingesting casts. The mesocosms were maintained at a constant temperature (20 °C) in a scientific incubator.

### 2.3. Sampling method

After 1 week in the mesocosms, several mature earthworms of similar weight were removed from each mesocosm, washed three times with sterile distilled water, and the gut contents released by gently pressing the bodies of intact worms, with tweezers, from the last third to the posterior end (Bonkowski and Schaefer, 1997). In doing this, we assumed that the gut content corresponding to the final section of the intestine (hindgut) was obtained (Horn et al., 2003). Gut contents from earthworms belonging to the same species and mesocosms were pooled to obtain samples that weighed approximately 50 mg (fresh weight). Five specimens of *E. eugeniae* and 15 each of *E. fetida*, *E. andrei* and *L. rubellus* were required to achieve the appropriate weight of sample.

### 2.4. Microfaunal and microbial analysis

Numbers of three different groups of protozoa (flagellates, naked and testate amoebae) were estimated by the MPN method (Darbyshire et al., 1974). For this, 30–60 mg of gut content from each earthworm species and 1 g of pig slurry ($n = 5$) were suspended in NBAS (1:100) (Griffiths, 1989). Twofold dilution series with NBAS were set up in microtitre plates (8 × 12 wells) that were incubated at 15 °C in dark conditions. The plates were checked with an inverted microscope (200×, Olympus CK2) after 4 days for the presence

of naked amoebae and flagellates, and after 10 days for testate amoebae. The presence of protozoa was transformed to MPN by following the tables from Rowe et al. (1977).

The nematodes in the pig slurry were extracted from 10 g of sample (fresh weight, $n = 5$) in modified Baermann funnels and live specimens were counted at a magnification of 140× under a dissecting microscope (Hooper, 1986). The numbers of nematodes in the gut contents of the different earthworm species were counted directly in suspensions of the gut content of each earthworm prepared in 1 mL of saline solution ($n = 20$). The nematodes were sorted in trophic groups (Yeates et al., 1993) using their oesophageal structure as an indicator (Parmelee et al., 1995).

The presence of total coliforms was estimated by the modified membrane filtration technique (Clesceri et al., 1998; EPA, 2000). Samples of 1 g of fresh pig slurry and 30–60 mg of gut content from the different earthworm species ($n = 5$) were suspended at 1:100 in sterile saline. Aliquots of 100 μL from each sample were diluted in 100 mL of sterile saline and then filtered through membrane filters. The filters were incubated in Petri dishes with Chromocult® Coliform Agar (Merk KGAA, Darmstad, Germany) for 24 h in an incubator chamber at 37 °C. The presence of total coliforms was then estimated by counting the number of colony forming units (CFU), at 140× under a dissecting microscope.

### 2.5. *Statistical analyses*

The data were analysed by the GLM procedure of the statistical package Statistica (vers. 7). Data (except for flagellates) were log transformed to fulfil the requirement of homogeneity of variances. Because some of the protozoan cultures were lost during the incubation process, post hoc comparisons were performed by Unequal N HSD test for flagellates and naked amoebae, and by Tukey HSD test for testate amoebae and total coliforms. Values given throughout the text are means ± S.E.

## 3. Results

All the nematodes extracted from the pig slurry (1450 ± 110 individuals $g^{-1}$ dw) were bacterivores. Passage of the pig slurry through the gut of all the earthworm species studied had a noteworthy effect on the presence of nematodes, since no live specimens were found in any of the gut content samples analysed ($n = 20$). The presence of nematodes was reduced to the appearance of a residual number of fragmented nematode cuticles in some of the samples.

The effect of the gut passage on the presence of protozoa was less evident. In general there was no great difference between the numbers of flagellates, testate and naked amoebae in the pig slurry and in the gut contents of the four earthworm species studied (Fig. 1). Nevertheless, the density of flagellates in the gut samples differed in the different earthworm species, and was three times higher in *E. fetida* and *E. andrei* than in *E. eugeniae* and *L. rubellus* (Fig. 1a). The testate amoebae were the least abundant group of protozoa in both the fresh pig slurry and the gut content samples (52.7 ± 10.5 and 50.9 ± 9.7 × $10^3$ MPN $g^{-1}$ dw, respectively), and there were no significant differences in the densities of these organisms

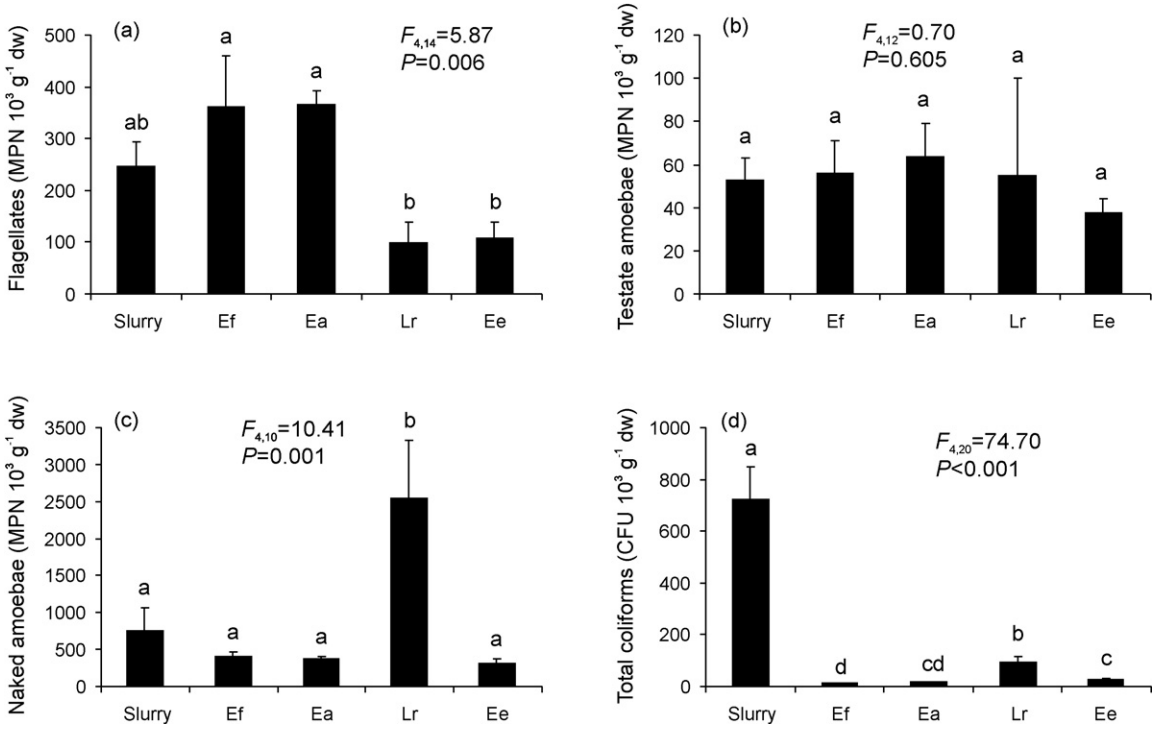


**Fig. 1 – Differences in the densities of protozoa and total coliforms in samples of pig slurry and gut contents from four species of epigeic earthworms (GLM test). Ef, *Eisenia fetida*; Ea, *Eisenia andrei*; Lr, *Lumbricus rubellus*; Ee, *Eudrilus eugeniae*. Means ± S.E. Different letters indicate significant differences (graphs (a) and (c): unequal N HSD test; graphs (b) and (d): Tukey HSD test).**

after the passage through the gut of the earthworms (Fig. 1b). Naked amoebae were the most abundant protozoa in the pig slurry ($1.4 \pm 0.5 \times 10^6$ MPN $g^{-1}$ dw). Passage through the gut of *E. fetida*, *E. andrei* and *E. eugeniae* did not affect the density of naked amoebae; however the number of amoebae in the gut samples of *L. rubellus* was 3.4 times higher than in the pig slurry (Fig. 1c).

Significantly lower numbers of total coliforms were observed in the earthworm gut contents than in the fresh pig slurry (Fig. 1d). Passage through the gut of earthworms decreased total coliforms by as much as 85% and this effect was species-specific (Fig. 1d).

## 4. Discussion

Passage through the guts of epigeic earthworms affected the composition of the microfaunal community of pig slurry. The observed reduction in numbers of nematodes is consistent with the results of Dash et al. (1980), who reported a large decrease in numbers of nematodes in a soil after passage through the gut of the tropical earthworm *Lampito mauritii*. In our study, the presence of nematode cuticles in the gut samples indicated that the nematodes were ingested by the earthworms; because of the high density of nematodes present in the pig slurry, these organisms may constitute an important part of the diet of the earthworm species under study. The earthworms probably digest nematodes by the proteolytic activity of the enzymes present throughout their gut (Edwards and Fletcher, 1988), which would allow the earthworms to assimilate amino acids and other compounds from the nematodes (Pokarzhevskii et al., 1997). There is no direct evidence that earthworms can feed on nematodes, but application of nematicides has a negative effect on earthworm populations (McColl, 1984), which may suggest a trophic relationship between these organisms. Such a relationship would also explain the observed reduction in nematode numbers in the presence of earthworms (Yeates, 1981; Hyvonen et al., 1994; Räty and Huhta, 2003; Domínguez et al., 2003).

In the present study, the density of protozoa in the pig slurry was not reduced after transit through the earthworm guts. Although there is some evidence of a negative effect of earthworms on the density of protozoans in the materials that they ingest (Bonkowski and Schaefer, 1997; Cai et al., 2002), such reductions occurred only when the active forms of the protozoa were considered and did not affect estimates of the total population (Bonkowski and Schaefer, 1997). Soil protozoan populations are formed by active and resistant forms (Ekelund and Rønn, 1994); dilution culture techniques favour the presence of excysting individuals (Berthold and Palzenberger, 1995; Stevik et al., 1998), and most-probable-number calculations probably underestimate the effect of the passage through the gut of the earthworms. The secretion of substances in the midgut of *Lumbricus terrestris* is associated with a decrease in the presence of protozoa (Piearce and Phillips, 1980); lysis of protozoan cells entails the release of organic compounds that can be assimilated by the earthworms and that appear to be essential for their development (Miles, 1963). In fact, *E. fetida* can grow with protozoa as the only food source (Neuhauser et al., 1980), which supports the idea that these organisms constitute an important part of the diet of earthworms (Bonkowski and Schaefer, 1997).

The remarkable increase in the density of naked amoebae after the transit through the gut of *L. rubellus* indicated that earthworms could also have positive effects on protozoan populations. In organic matter-rich substrates like the pig slurry, earthworms could feed preferentially on organic C compounds than on micro-organisms (Aira et al., 2006); in some cases, this selective use of the food resources could favour the populations of protozoa, which remain undigested in the earthworm gut. The observed differences among earthworms regarding the density of flagellates and naked amoebae in the gut samples seem to be associated with the genus of earthworm considered. There were no differences between *E. fetida* and *E. andrei*, and the effects of these species on flagellates were in direct contrast to the effects exerted by *E. eugeniae* and *L. rubellus*. These differences may be due to the morphological and physiological characteristics of the digestive system of these earthworms. The oesophagus is longer in lumbricids (*E. fetida*, *E. andrei* and *L. rubellus*) than in other families (Edwards and Bohlen, 1996); the gut typhlosole and the gut enzymatic array vary with the species considered (Edwards and Fletcher, 1988; Makeschin, 1997), and the gut transit time is also species-specific (Dash et al., 1986; Hendriksen, 1991). Moreover, *E. fetida* and *E. andrei* are closely related species that differ morphologically only in their body pigmentation (Domínguez et al., 2005), and thus close similarities in their digestive abilities should be expected.

As well as the effects on the microfauna, epigeic earthworms also reduced the presence of total coliforms, which indicates that gut transit has an important effect on the microbiological composition of the pig slurry. Total coliforms are characteristic of pig manures (Zhu, 2000), and Joergensen et al. (1998) showed that the presence of these bacteria was related to the total microbial biomass in soil; nevertheless, the density of total coliforms was up to 60 times lower in the gut samples than in the fresh pig slurry, which suggests that the effect on coliforms was stronger than on the rest of micro-organisms. Although Aira et al. (2006) reported a significant decrease of 1.74-fold in the microbial biomass of the pig slurry after passage through the gut of *E. eugeniae*, several authors found that the densities of bacteria, actinomicetes and yeasts in earthworm faeces were equal or greater than in parent materials (Shaw and Pawluk, 1986; Schönholzer et al., 1999; Wolter and Scheu, 1999). Earthworm digestion can increase the availability of nutrients for micro-organisms, rising microbial numbers in casts (Parthasarathi and Ranganathan, 1999) and changing the microbial composition (Brown, 1995). There is increasing evidence that earthworms have a specific gut microflora (Karsten and Drake, 1995; Horn et al., 2005), and the decrease in total coliforms might be related to competitive interactions between coliforms and micro-organisms that are specific to the earthworm gut (Brown and Mitchell, 1981). Moreover, the negative effect of the passage through the earthworm gut observed in enterobacteria such as *Serratia marcescens*, *Escherichia coli* and *Salmonella enteridis* (Day, 1950; Brüsewitz, 1959; Brown and Mitchell, 1981) suggests the occurrence of selective effects on the ingested micro-organisms.

Overall, epigeic earthworms had a significant direct effect on the density of microfauna and micro-organisms in the pig slurry. As a result of this and the casting activities of the worms, a different decomposer community is introduced into the organic material where the earthworms simultaneously feed and live. Furthermore, the earthworm species under study did not affect all groups of decomposer organisms in the same way, which resulted in significant differences in the decomposer community present in the digested pig slurry, and possible differences in the properties of the casts produced.

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