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SHORT COMMUNICATION

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Changes in microbial biomass and microbial activity of pig slurry after the transit through the gut of the earthworm *Eudrilus eugeniae* (Kinberg, 1867)

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Abstract Here we studied the effects of gut transit through the earthworm *Eudrilus eugeniae*, on the physicochemical, biochemical, and microbial characteristics of pig slurry, by analyzing fresh casts. The reduction in the dissolved organic C contents in casts we recorded suggests that during digestion, earthworms assimilated labile organic C preferentially, which is a limiting growth factor for them. Furthermore, both microbial biomass and activity in pig slurry were significantly decreased by earthworm gut transit. It appears that *E. eugeniae* is able to digest microorganisms, although the addition of glucose to the food increased respiration, indicating that growth of microorganisms in casts could be limited by depletion of labile C. Despite reduced microbial biomass and activity, the metabolic diversity of microbial communities was greater in casts than in original pig slurry. Community level physiological profiles obtained from Biolog Ecoplate data revealed that, after earthworm gut transit, the microbial communities in casts and pig slurry were clearly differentiated by their physiological profiles. The results indicate that first stage in vermicomposting of pig slurry by *E. eugeniae*, i.e., casting, produced changes that will influence the dynamics of the organic matter degradation by reducing forms of N and C available to microorganisms, hence restricting their growth and multiplication. Nevertheless, the reduced microflora of casts was characterized by an increased catabolic potential that might lead to thorough degradation of pig slurry.

Keywords Earthworm feeding · Enzymatic activity · Microbial physiological profile · Microbial biomass · Microbial respiration

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Introduction

Earthworms are major components of the soil fauna in a wide variety of soils and climates and are involved directly or indirectly in organic matter decomposition and stabilization, nutrient turnover, and modification of soil physical properties (Edwards and Bohlen 1996; Lavelle and Spain 2001). Many of these effects are associated with the relationships between earthworms and microorganisms, which mainly occur in earthworm gut, casts, burrows, and middens. Casting has a major importance considering the high rate at which casts are produced, which range between 36 and 108 Mg ha^{-1} $year^{-1}$ in temperate zones (Lavelle and Spain 2001) and strong modifications in biochemical properties with respect to uningested material (Scheu 1987; Aira et al. 2003). Therefore, due to its significance in soil ecosystem processes, changes in nutrient status and microbial composition and activity of casts with respect to the parent soil have been studied widely. It is generally accepted that microbial biomass and respiration are greater in earthworm casts than in the parent soil (Zhang and Hendrix 1995; Tiunov and Scheu 2000a; Aira et al. 2003). However, microorganisms may constitute an important part of the diet of earthworms, which can feed on them selectively (Edwards 2004; Moody et al. 1995). On the other hand, an increase in culturable aerobic microorganisms in the gut contents of earthworms, such as *Lumbricus terrestris* and *Lumbricus rubellus*, has been reported (Kristufek et al. 1992; Fisher et al. 1995; Schönholzer et al. 1999).

Vermicomposting involves biooxidation and stabilization of organic material through the interactions between earthworms and microorganisms. Although microorganisms are mainly responsible for the biochemical degradation of organic matter, earthworms play an important role in the process by fragmenting and conditioning the substrate, increasing surface area for growth of microorganisms, and altering its biological activity (Domínguez 2004; Domínguez and Edwards 2004). High population densities of earthworms in vermicomposting systems result in a rapid turnover of fresh organic matter into earthworm casts

or vermicomposts. These casts can be deposited both inside and outside of fresh organic matrix, thereby affecting the decomposition rates in their proximity because of their different microbial composition. During the vermicomposting process, earthworms can modify the diversity and abundance of the microflora directly, by selective feeding, or by stimulation of particular taxa of microorganisms (Pedersen and Hendriksen 1993; Devliegher and Verstraete 1995; Wolter and Scheu 1999; Tiunov and Scheu 2000a); moreover, earthworms exert other indirect effects on microbial communities, such as microbial dispersion and the release of additional food resources in their casts. For all these reasons, better knowledge of the changes in the chemical and biochemical properties of the organic wastes during the vermicomposting process is required to understand the effect of the earthworms' activities on the processes of biodegradation better. We have chosen the pig slurry as organic waste due to the fact that number of pig breeding farms in Spain is increasing (Plaza et al. 2004), and because most of the produced pig slurry is applied without any treatment to the soil.

The main objective of our study was to monitor the short-time changes (fresh manure to casts) of pig manure, after passing through the gut of the epigeic earthworm *Eudrilus eugeniae* under controlled environmental conditions. We monitored the changes in available pools of C and N of pig slurry; moreover, we analyzed microbial biomass and activity (respiration and substrate utilization patterns (Biolog Ecoplate) and enzyme activities since they have been shown to be reliable indicators of the response of microbial communities to variations in their environmental conditions (Carreiro et al. 2000), and microbial activities are very important in regulating soil properties (Nannipieri et al. 1990; Dick 1992).

Materials and methods

Fresh pig slurry was obtained from a pig breeding farm near the University of Vigo, NW Spain. Pig slurry was homogenized in a slurry pit and then stored in sealed plastic containers and kept at 5°C until use.

Specimens of *E. eugenieae* were originally obtained from a Brazilian commercial supplier (Minhobox, Brazil) and are now reared as laboratory stock in the University of Vigo. Batches of ten mature earthworms (mean 15 g fresh weight) were each placed in plastic boxes (n=10), which were filled with a layer of vermicompost (to ensure the survival of earthworms) and 1 kg of fresh pig slurry. The boxes, which were maintained at 20°C, were reviewed daily, and fresh casts taken from the surface and the consumed pig slurry were replaced when required.

The moisture content of the pig slurry and earthworm casts was determined after drying at 105°C for 24 h, and the organic matter content was determined after heating at 550°C for 4 h. The pH and electric conductivity were recorded from a suspension of the samples in distilled water 1:20 (weight/volume) sample to extractant. Inorganic N ($N\text{-}NH_4^+$ and $N\text{-}NO_3^-$) was determined in 0.5 M K_2SO_4 extracts (1:5 weight/volume) using a modified indophenol blue technique (Sims et al. 1995) with a Bio-Rad Microplate Reader 550. Dissolved organic C (DOC) of pig slurry and earthworm casts was determined colorimetrically after moist digestion ($K_2Cr_2O_7$ and H_2SO_4) of aliquots of 0.5 M K_2SO_4 extracts of the samples.

Microbial biomass C (C_{mic}) was analyzed by the chloroform fumigation–extraction method (Vance et al. 1987) with an extract efficiency factor for microbial C (k_c=2.64, Vance et al. 1987). Microbial activity was assessed by measuring the rates of CO_2 evolution from samples after 6- and 12-h incubation [basal and substrate-induced incubation (SIR), respectively]. Prior to incubation, 0.75 ml of glucose solution (equivalent to 100 mg glucose g^{-1} dw pig slurry) was added to samples for the SIR assay. The evolved CO_2 was trapped in 0.02 and 0.06 M NaOH (basal and SIR, respectively) and then measured by titration with HCl to a phenolphthalein endpoint, after adding excess $BaCl_2$ (Anderson 1982).

Dehydrogenase enzyme activity was measured by estimation of the rate of reduction of triphenyltetrazolium chloride (TTC) (1.5%) to triphenylformazan (TPF), after incubation at 30°C for 24 h, in a Bio-Rad Microplate Reader 550 at 545 nm (Casida et al. 1964). Protease activity was measured by determining amino acids released, after incubation of samples with sodium caseinate (2%) for 2 h at 50°C using Folin-Ciocalteu reagent, in a Bio-Rad Microplate Reader 550 at 700 nm (Ladd and Butler 1972). Alkaline phosphatase activity was estimated by determination of *p*-nitrophenol (PNP) released, after incubation of samples with *p*-nitrophenyl phosphate (0.025 M) for 1 h at 37°C, in a Bio-Rad Microplate Reader 550 at 400 nm (Eivazi and Tabatabai 1977). β-glucosidase activity was assessed by determination of the released *p*-nitrophenol, after the incubation of samples with *p*-nitrophenyl glucoside (0.025 M) for 1 h at 37°C, in a Bio-Rad Microplate Reader at 400 nm (Eivazi and Tabatabai 1988). Cellulase activity was estimated by determination of reducing sugars released, after incubation of soil samples with carboxymethyl cellulose sodium salt (0.7%) for 24 h at 50°C, in a Bio-Rad Microplate Reader at 690 nm (Schinner and Von Mersi 1990).

Microbial functional diversity was assessed using Biolog Ecoplate microplate identification system (BIOLOG Inc., Hayward, CA, USA). This method tests for the utilization of 31 different C compounds, classified into six different groups: carbohydrates, carboxylic acid, polymers, amines/amides, amino acid, and miscellaneous (Insam 1997). Functional diversity is defined as the numbers and types of substrate utilization. Thoroughly mixed substrate, i.e., separated samples of pig slurry and casts (n=3) (1 g fresh weight) were suspended in 100 ml of sterile saline (0.85 M NaCl). The solution was then allowed to settle for 15 min before adding 150 μl aliquots of this solution to each well of Biolog Ecoplates. The inoculated plates were kept at 20°C for 5 days. The absorbance of plates was registered at 24, 48, 72, 96, and 120 h with a Bio-Rad Microplate Reader 550 at 595 nm. For the BIOLOG data, the average well color development (AWCD) of all 31 C sources for each sample were calculated prior to any

Table 1 Physicochemical properties of pig slurry and casts of *E. eugeniae* (n=10)

Physicochemical characteristics	Pig slurry	Casts
Moisture content (%)	79.8±0.4	79.3±0.2
Organic matter content (%)	82±16	77±9
pH	8.26±0.03	8.02±0.05
Electrical conductivity (mS cm^{-2})	0.25±0.02	0.23±0.01
NH_4^+-N (μg g^{-1} dw[a])	2,020±70	600±20[a]
NO_3^--N (μg g^{-1} dw)	400±100	620±120
Dissolved organic carbon (μg g^{-1} dw)	1,200±60	500±30[a]

dw Dry weight
[a]Means were compared by Student's t test (p<0.001).

statistical analysis to eliminate variation in well color development caused by different cell densities (Garland and Mills 1991; Garland 1997). The 48-h absorbance data were used for the analysis because this was the time necessary for microbial growth and color development, and at this time, 75% or more of wells showed a positive response for microorganisms (Ibekwe and Kennedy 1998). The metabolic diversity of microbial communities was estimated as substrate richness (the number of substrates utilized), substrate evenness (the equitability of activities across all utilized substrates), and substrate diversity (using Shannon's diversity index; Zak et al. 1994).

Statistical analysis

Student's t test was used to compare means of different physicochemical, biochemical and microbiological analyses performed on pig slurry and cast samples. The same procedure was used to analyze the indices of substrate diversity, richness, and evenness obtained from absorbance data of Biolog Ecoplate. Cluster analysis was used to estimate relationships among physiological profiles of microflora on the basis of similarities of C substrate oxidation patterns (Scott and Knudsen 1999). The Euclidean distance method was used to determine the distances in space, and the Ward method was used to add samples to clusters (Logan 1994). All statistical analyses were performed using SPSS 11.5 software.

Results

The passage of pig slurry through the gut of *E. eugeniae* did not have any significant effects on its moisture or organic matter content, pH, or electrical conductivity (Table 1). However, transit through earthworm gut did affect available N and C pools; thus, N-NH_4^+ and DOC contents were significantly lower in casts, whereas N-NO_3^- contents were slightly higher than in the pig slurry (Table 1).

Transit through the earthworm gut changed the microbial biomass, enzymatic activities, and function of pig slurry. The C_{mic} and basal respiration in the casts of *E. eugeniae* were significantly lesser than in the fresh pig slurry; in contrast, SIR values were significantly greater in the casts than in the pig slurry (Table 2). Moreover, enzymatic activity was significantly lower in casts than in pig slurry, except for cellulase activity. Dehydrogenase and protease activities were much lower in the casts than in the pig slurry, as were to a lesser extent, β-glucosidase and phosphatase activities (Table 2).

The functional diversity of microorganisms distinguished clearly the microbial communities in pig slurry and in casts. The microflora in the casts was able to catabolize more compounds than the microflora in pig slurry, as

Table 2 Biochemical and microbial parameters of pig slurry and casts of *E. eugeniae* (n=10)

Biochemical and microbial characteristics	Pig slurry	Casts
Microbial biomass-C (μg g^{-1} dw)	8,900±700	5,100±200[a]
SIR respiration (μg CO_2 g^1 om[a] h^{-1})	860±30	1,280±30[a]
Basal respiration (μg CO_2 g^1 om h^{-1})	980±50	350±20[a]
Dehydrogenase activity (μg TPF g^{-1} dw)	1,200±40	50±10[a]
β-glucosidase activity (μg PNP g^{-1} dw)	1,200±10	1,000±10[a]
Alkaline phosphatase activity (μg PNP g^{-1} dw)	4,900±200	3,800±100[a]
Cellulase activity (μg glucose equivalent g^{-1} dw)	10,300±300	10,900±200
Protease activity (μg tyrosine g^{-1} dw)	26,100±1,100	17,300±800[a]

om Ash-free dry weight
[a]Means were compared by Student's t test (p<0.001).

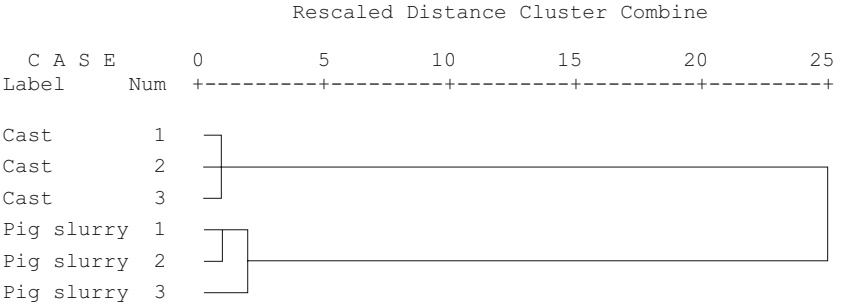

Fig. 1 Cluster analysis of the Biolog Ecoplate physiological profiles from pig slurry and casts from *E. eugeniae*. Clusters were determined by the Ward method and by Euclidean distance.

revealed by a significantly higher values of both substrate richness (28.89±0.11 and 29.44±0.11, pig slurry and casts, respectively) and diversity (3.65±0.07 and 3.86±0.02, pig slurry and casts, respectively); substrate evenness did not indicate preferences for substrates utilized by microorganisms (2.50±0.04 and 2.62±0.02, pig slurry and casts, respectively). Cluster analysis of the data from Biolog Ecoplate grouped the samples analyzed into two principal clusters composed by those from fresh pig slurry and those from casts of *E. eugeniae*. The clusters were separated by Euclidean distances of 1 and 2, respectively (Fig. 1).

Discussion

The transit of pig slurry through the gut of the earthworm *E. eugeniae* produced a reduction in both available N and C forms ($N\text{-}NH_4^+$ and DOC) in the casts. The smaller amounts of $N\text{-}NH_4^+$ observed in the casts may be attributed to losses by volatilization before intake of pig slurry by the earthworms, because the slight increase in $N\text{-}NO_3^-$ content was not enough to account for all of the $N\text{-}NH_4^+$ lost; moreover, soil animals cannot exploit mineral nutrient pools directly. The large reduction in the DOC levels of casts may have been resulted from direct assimilation of DOC by *E. eugeniae* during digestion of pig slurry. This is consistent with the general assumption that labile C compounds are an important part of the diet of earthworms, at least in soils and for endogeic earthworms, and that C availability is a limiting factor for growth of earthworms in soils (Seastedt et al. 1988; Scheu and Schaefer 1998; Tiunov and Scheu 2004).

Microbial biomass and activity of pig slurry were affected strongly by digestion of *E. eugeniae*, although we found some unexpected results, like the lack of fit between SIR and C_{mic} measures in both pig slurry and cast samples, or the higher values of basal respiration than SIR ones in the pig slurry samples. It appears that *E. eugeniae* may be able to feed on the microorganisms present in pig slurry because there was a large decrease in C_{mic}; however, the increase in SIR in casts suggests that the nutrient status in casts was altered by depletion of both labile C and N pools, which may affect microbial growth. The reduction factor for C_{mic} was 1.74, whereas those for DOC levels and basal respiration were 2.8 and 2.4, respectively, providing further evidence that *E. eugeniae* fed preferentially on organic C compounds and, to a lesser extent, on microorganisms. The lower microbial activity observed in casts may have been caused by decreases in DOC due to C limitations for microorganisms similar to those in soil systems (Scheu 1993) rather than due to direct feeding by *E. eugeniae*. Similar reductions in microbial biomass were reported by Devliegher and Verstraete (1995), Bohlen and Edwards (1995) and Zhang et al. (2000) in soil with different earthworm species; Scheu (1987) and Aira et al. (2003) reported increases in microbial biomass in casts of *Allolobophora caliginosa*, whereas there were not any changes in casts of *L. rubellus* (Daniel and Anderson 1992). However, Parthasarathi and Ranganathan (1999) reported a fourfold increase in microbial populations [colony-forming unit (CFU] in casts of individuals of *E. eugeniae* fed with pressmud, indicating that effects of earthworms on microorganisms are clearly dependent on kind of food source and availability and the species of earthworm involved (Tiunov and Scheu 2000b; Flegel and Schrader 2000).

There was also a significant reduction in the enzyme activities analyzed. The low dehydrogenase activity found in casts suggests that *E. eugeniae* intensively fed on aerobic microorganisms. Regarding dehydrogenase assay, it is necessary to note that this analysis only accounts for a limited percentage of respiration since oxygen is a better electron acceptor than the TTC used in our assay (Nannipieri et al. 1990). Thus, the reduction in aerobic microbial metabolism was ten times higher than in overall microbial metabolism, with reduction factors of 24 and 2.4 for enzyme dehydrogenase and basal respiration, respectively. This dramatic decrease is consistent with the recent description of the lumbricid gut lumen as a fermentative anaerobic environment (Horn et al. 2003), in which the ratios of anaerobic/aerobic microorganisms are greater than in ingested soils (Karsten and Drake 1995). Similar decreases in dehydrogenase activity were reported during vermicomposting sewage sludge (Benitez et al. 1999) and pig slurry (Aira et al. 2002), with *Eisenia andrei* and *Eisenia fetida*, respectively. However, our results were not consistent with increased respiration rates and dehydrogenase activity in casts of *E. eugeniae* reported by Parthasarathi and Ranganathan (1999), which were clearly related to higher CFU counts, or to the intense dehydrogenase activities observed during vermicomposting of cattle dung (Bansal and Kapoor 2000) and cow dung (Kaushik and Garg 2003) with *E. fetida*. This contradiction may be explained partly because pressmud is a richer (in nutrients and microflora) substrate than pig slurry, and digestion of *E. eugeniae* appears to increase the availability of these nutrients, thereby increasing the microbial populations. We analyzed the enzyme activities (β-glucosidase, cellulase, phosphatase and protease) to monitor directly the functional responses of the microbial community of pig slurry to changes induced by *E. eugeniae* gut transit. This earthworm species is classified as epigeic (litter feeding) and therefore is characterized by diverse enzyme activities (Lavelle and Spain 2001). The decrease in enzyme activities could depend on the decrease of the relative substrates concentration during transit of pig slurry through the gut. According to this hypothesis, the greatest decrease was in protease activity, showing that during gut transit organic N pools, as it succeed with DOC, were also digested by *E. eugeniae*, diminishing substrate availability to the enzyme. The small decreases in β-glucosidase, cellulase, and phosphatase activities observed may indicate that passage through the earthworm intestine did not affect cellulolytic and organic P compounds to the same extent. Nevertheless, this hypothesis needs to be verified by analyzing how the concentration of enzyme substrates varies during earthworm gut transit.

Similar to microbial biomass and activity, community level physiological profiles were also affected. In this way, the substrate utilization potential of microorganisms of

casts and those from the pig slurry samples were separated into different clusters. Gut transit clearly increased the number of potential substrates used by microflora. However, this result cannot be attributed to differences in microbial species composition because of limitations of the method employed, which shows only the physiological profile of culturable microorganisms present in the wells (Widmer et al. 2001; Nannipieri et al. 2003).

The present results are important because the microbial community in casts will initiate the vermicomposting process, and the metabolic potential will determine the degree of organic matter degradation and stabilization.

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