Microbial Ecology

Eisenia fetida (Oligochaeta: Lumbricidae) Modifies the Structure and Physiological Capabilities of Microbial Communities Improving Carbon Mineralization During Vermicomposting of Pig Manure

Manuel Aira, Fernando Monroy and Jorge Domínguez

Departamento de Ecoloxía e Bioloxía Animal, Facultade de Ciencias, Universidad de Vigo, Vigo, E-36310, Spain

Received: 25 January 2007 / Accepted: 27 January 2007 / Online publication: 25 February 2007

Abstract

Although microorganisms are largely responsible for organic matter decomposition, earthworms may also affect the rates of decomposition directly by feeding on and digesting organic matter and microorganisms, or indirectly affect them through their interactions with the microorganisms, basically involving stimulation or depression of the microbial populations. We tested the general hypothesis that microbial populations, and especially fungi, are enhanced by earthworm activity, and also whether earthworms are able to modify the biodiversity of microbial populations, and its relation to the function of the system. In addition, we examined the metabolic quotient and the effect of labile organic C to assess the relationships between earthworm and microbes. We found that decomposition of pig manure has two stages characterized by the presence or absence of earthworms. Thus, the presence of earthworms was related with increases in overall microbial biomass and activity, which decreased when earthworms left the substrate; the same pattern was observed for fungi. Furthermore, earthworms modified the physiological profiles of microbial communities of pig manure, increasing the diversity of substrates utilized. In addition, earthworms promoted a more efficient use of energy of microbial communities, as the metabolic quotient showed. The rate of carbon loss was almost twice where earthworms were present, revealing faster decomposition. Our data match with the recent findings that to maintain essential processes the functional properties of present species are at least as important as the number of species *per se*. This is in accordance with the "insurance hypothesis," which states that a large number of species is probably essential for maintaining stable processes in changing environments, as the presence of earthworms would have promoted in pig manure.

Introduction

In soil organic matter dynamics and nutrient cycling, soil faunaespecially the so-called ecosystem engineers play an important role in directly or indirectly regulating resource availability to other species, thereby causing physical changes in biotic or abiotic materials [21]. Earthworms are considered ecosystem engineers [22] because they affect the physicochemical and biological properties of the soils that they inhabit through their activities such as casting and burrowing [13, 23]. In this way, it is known that microbial biomass and activity are usually enhanced in the drilosphere, with greater numbers of microbial colony forming units (CFUs) in the burrow walls [25, 38] and earthworm casts [31, 40] than in the parent soil. This appears contradictory to the fact that earthworms can digest bacteria and fungi [34] and that fungi are supposed to be a major component of the diet of earthworms (see review in [40]). Doube and Brown [12] concluded that the capability of earthworms to digest organic residues is minimal and that they obtain their nutrients from the microorganisms associated with the ingested substrate. In addition, it has been demonstrated that the earthworm *Lumbricus terrestris* is able to alter the composition of the ingested microbiota, modifying both the bacterial and fungal communities of soils via casting [14, 40].

It is well known that carbon availability controls the rate of decomposition processes, and it has been

Correspondence to: Manuel Aira; E-mail: aira@uvigo.es

DOI: 10.1007/s00248-007-9223-4 • Volume 54, 662–671 (2007) •

established that it also limits the growth of earthworms, especially endogeic and anecic ones, suggesting the existence of some degree of competition between earthworms and microbes for C pools [32, 41]. It is therefore essential to establish what the relationships between earthworms and microorganisms are because whether the earthworms stimulate or depress microbiota has an important effect on the decomposition of organic matter, as microorganisms are main agents responsible for the decomposition process [8]. Most of the current knowledge regarding interactions between earthworms and microorganisms has been obtained from studies with endogeic and anecic earthworms, maybe because of their significance in earthworm communities of studied areas. Nevertheless, some studies with epigeic earthworms demonstrate that these earthworms also are able to modify the microbial communities of soils, at least in medium and large time scales (6 months and 2 years, respectively) [27, 28]. This lack of research regarding epigeic earthworms is quite surprising because these earthworm species, also called litter feeding species, has a capital role in organic matter decomposition [22].

Vermicomposting, where the substrate is transformed within a short time, has been found to be a suitable system for studying the relationships between epigeic earthworms and microorganisms and their effects on the decomposition of organic matter and allows understanding the chemical and biological consequences of earthworm activities [1]. Vermicomposting is the biooxidation and stabilization of organic matter involving the joint action of earthworms and microorganisms. Although the biochemical degradation of organic matter is carried out by microbes, earthworms are important drivers of the process, as their actions help to condition the substrate, increase the surface area for microbiological activity, and alter its biological activity [11]; indeed, earthworms are able to modify the substrate as a result of their own digestive enzymes [23].

The aims of the present study were first to test whether earthworms are able to change quantitatively the microbial community (i.e., stimulate or depress microbial biomass and activity and the contribution of fungal populations); second to establish if these modifications are also qualitative, altering the functional diversity of microbial communities (i.e., the community level physiological profiles); and third, to study if the dynamics of changing microbial communities affect function in the system (i.e., rate of carbon loss). To do this, we designed a reactor that allowed us to monitor the age of the substrate and sample it without interfering with either newer or older substrates within the reactor. At the end of the experiment we obtained a profile of layers in which we were able to observe the different phases of interaction among earthworms and microorganisms in the process of organic matter decomposition. We analyzed microbial biomass (microbial biomass-C) and activity (basal respiration), and calculated the metabolic quotient to assess the metabolic state of microorganisms. Then we carried out a study on microbial communities by analyzing a biomarker for fungi (ergosterol), the active microorganisms (substrate-induced respiration), and analyzed the functional microbial diversity on the basis of their physiological profiles, using Biolog® Ecoplate analysis. In addition, we analyzed the total carbon and dissolved organic carbon contents to understand how changes in microbial parameters affect the rates of decomposition and to establish the dynamics of labile organic C pools during vermicomposting.

Materials and Methods

Pig Manure. Fresh pig manure was obtained from a pig breeding farm near the University of Vigo, NW Spain. Pig manure was homogenized in a manure pit, then stored in sealed plastic containers and kept at 5°C until use. We chose pig manure as substrate because it is a microbial-rich substrate mainly composed of anaerobic or facultative anaerobic bacteria [46, 49] in which fungi are present mainly as spores [16].

Reactor Setup and Functioning. The reactors were comprised of modules that were added sequentially to the system. The modules, which resembled sieves, were made of PVC. The external diameter of each was 30 cm, with a height of 2 cm, giving a volume of 1413 cm^3. The bottom of the modules was a mesh size 5 mm, which allowed earthworms to move between modules. Each reactor was initially composed of one module containing vermicompost, in which earthworms were placed, and another module containing a layer of 1.5 kg of fresh pig manure (300 g of dry mass, moisture content $80 \pm 10\%$). Vermicompost was obtained from laboratory cultures of *E. fetida* fed with pig manure; its main physicochemical characteristics together with those of fresh pig manure are given in Table 1. New modules containing the same amount of fresh pig manure were added when the earthworms feeding activity required (i.e., changes in the appearance of pig slurry, making the coarse fraction, such as seeds and straw, more evident); this procedure allowed us to date the age of each module within the reactor [1]. A detailed graph explaining the procedure and the construction of modules is given in Fig. 1.

Experimental Design and Sampling Method. We set up a batch of six reactors, three without earthworms (control) and three containing 500 mature earthworms (ca. 90 ± 10 g, fresh weight; *Eisenia fetida*) each. At the end of the experiment (i.e., after 36 weeks), the reactors comprised 12 modules with an increasing gradient of age, resembling a soil profile, from upper to lower layers,

Table 1. Physicochemical characteristics of the fresh pig slurry used

	Pig manure	*Vermicompost*
Moisture content (%)	86 ± 10	78 ± 2
Organic matter content (%)	86 ± 10	65 ± 1
pH	8.3 ± 1.0	6.2 ± 0.1
Electrical conductivity (mS cm^{-2})	0.25 ± 0.01	1.3 ± 0.4
Total nitrogen (mg g^{-1} dw[a])	24 ± 2	27 ± 1
$N-NH_4^+$ (μg g^{-1} dw)	2400 ± 100	30 ± 10
$N-NO_3^-$ (μg g^{-1} dw)	250 ± 50	730 ± 80
Total carbon (mg g^{-1} dw)	455 ± 60	360 ± 2
Dissolved organic carbon (μg g^{-1} dw)	11,100 ± 100	1200 ± 100

[a]dw = dry weight

as follows: 2, 4, 7, 8, 11, 18, 21, 25, 27, 29, 33, and 36 weeks. For sampling, the reactors were dismantled and the modules isolated to avoid the earthworms escaping. Five samples of substrate per module were taken at random and gently mixed for biochemical analyses, i.e., microbial biomass-C (C_{mic}), ergosterol content, basal and substrate-induced respiration, and Biolog® Ecoplate analysis.

Analytical Procedures. Total carbon content was measured on oven-dried (60°C) and ball-milled samples using a Carlo Erba NA 1500 C/N analyzer. Dissolved organic carbon (DOC) of pig manure 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 [20, 42]. Microbial biomass-C (C_{mic}) was determined by the chloroform fumigation–extraction method [43] with field-moist samples (5 g fresh weight). The filtered soil extracts of both fumigated and unfumigated samples were analyzed for soluble organic C using a Microplate Reader (BioRad Microplate Reader 550, 590 nm). C_{mic} was estimated as the difference between the organic C extracted from the fumigated and that from the nonfumigated sample, multiplied by the K_2SO_4 extract efficiency factor for microbial C ($k_c = 2.64$) [43].

Ergosterol was determined after microwave-assisted extraction [47]. Samples (500 mg of substrate, fresh weight) were placed into a 10-mL vial, to which 2 mL of methanol and 0.5 mL of 2 M NaOH were added, and the vial was tightly sealed with a Teflon-lined screw cap. Three such culture vials were placed within Teflon PFA® vessels and tightly sealed. The vessels were then placed on the turntable drive stub of a scientific microwave oven (CEM Corporation MDS-2000, operating at 2450 MHz and 630 W maximum output) and irradiated three times at medium power (60% of maximum output power, manufacturer's setting) for 20 s, with 1 min of cooling between each time. After cooling for approximately 30 min, the vials were removed from the Teflon PSA® vessels. The contents were neutralized with 1 M HCl and then extracted with pentane (3 × ca. 2 ml), all within the 10 mL vial. The combined pentane extracts were evaporated to dryness under a stream of N_2 gas, and then redissolved with 1 mL of methanol and filtered through a 0.2-μm syringe filter (MFS) before high-performance

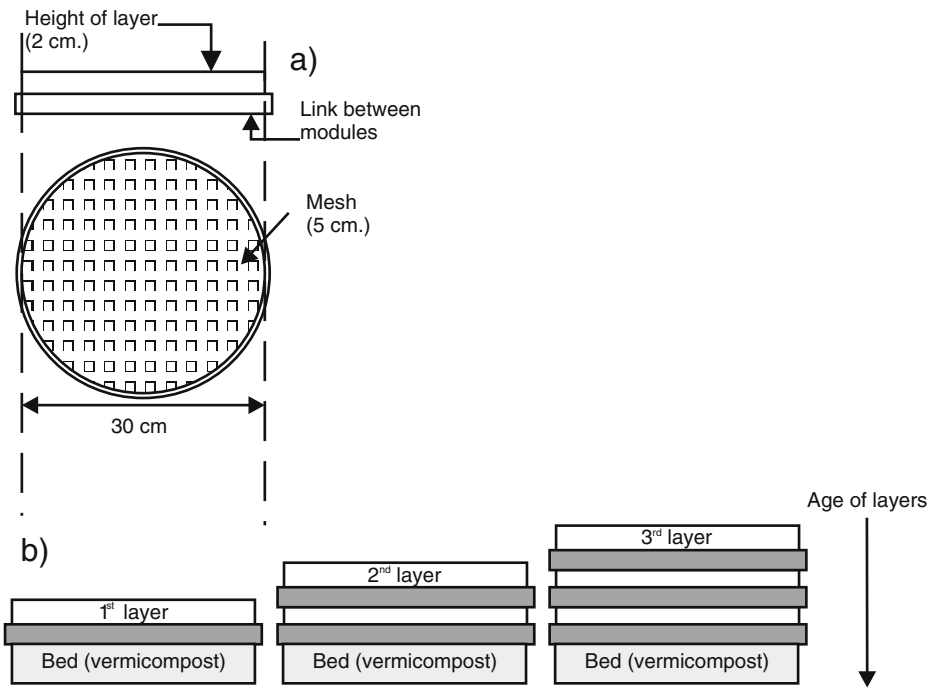

Figure 1. Scheme of module construction and set up (a) and the procedure used to add new modules during vermicomposting process (b).

liquid chromatography (HPLC) analysis. Ergosterol from sample extracts was also separated on a 12.5 × 4 mm Hypersil 5 C18 (366349) reverse-phase column packed with ODS 4 mm and eluted with methanol/water (95:5 *v*/*v*) at a flow rate of 2 mL min^{-1}. Ergosterol was detected with a Jasco UV-1570 variable wavelength detector (Jasco) set at 282 nm.

Basal respiration was determined by measuring CO_2 evolution. Field-moist samples (5 g fresh weight) were placed in 100-mL airtight glass vessels and incubated at 22°C for 6 h. The CO_2 produced from the sample was trapped in NaOH 0.02 M and subsequently measured by titration with 0.01 M HCl to a phenolphthalein endpoint, after adding excess $BaCl_2$ [3]. For substrate-induced respiration, 0.75 mL glucose solution (equal to 100 mg g^{-1} pig manure) was added to samples (5 g fresh weight). After 30 min, the 100-mL vessels were closed and incubated at 22°C for 12 h. The CO_2 produced from the sample was trapped in 0.06 M NaOH and subsequently measured by titration with 0.03 M HCl to a phenolphthalein endpoint, after adding excess $BaCl_2$. Metabolic quotient (qCO_2) for each layer was calculated by dividing the CO_2 released from the samples in 1 h by the microbial biomass-C content [4].

Community-level physiological profile of microbial communities (CLPP) was assessed using the Biolog® Ecoplate microplate identification system (BIOLOG Inc., Hayward, CA, USA). We sampled fresh pig manure and layers of 2, 4, 7, 8, and 11 weeks of age because these contained the highest densities of earthworms and the biochemical analyses revealed that changes in microbial biomass and activity had taken place. We also analyzed the two oldest layers (25 and 36 weeks old), which functioned as a contrast because they presented lower values of microbial biomass and activity. Thoroughly mixed substrate samples, i.e., of pig manure 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 maintained at 20°C for 5 days. The absorbance of plates was recorded after 24, 48, 72, 96, and 120 h, with a BioRad Microplate Reader 550 at 595 nm. For the BIOLOG® data the average well color development (AWCD) of all 31 carbon sources for each sample were calculated before any statistical analysis to eliminate variation in well color development caused by different cell densities [15]. The 48 h absorbance data were used for the analysis as this was the time necessary for microbial growth and color development, and at this time 75% or more of the wells showed a positive response for microorganisms [19]. 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 [48]).

Statistical Analysis. Data were analyzed using a split plot repeated measures ANOVA (ANOVAR) where single reactors were subjects, earthworm treatment was fixed as between subject factor and the incubation time (i.e., each single module) was fixed as the within subject factor. This model assumes correlation between treatment levels within a block, i.e., the modules of each vermireactor [45]. The same ANOVA design was performed to analyze indices of substrate diversity, richness, and evenness obtained from the Biolog Ecoplate absorbance data [36], and a Tukey HSD test was carried out when ANOVA was significant at $P < 0.05$. All variables analyzed fulfilled sphericity assumptions (Mauchly's test). Cluster analysis was used to estimate relationships between earthworms and functional diversity of microorganisms on the basis of similarities between carbon substrate oxidation patterns [35]. The Euclidean distance

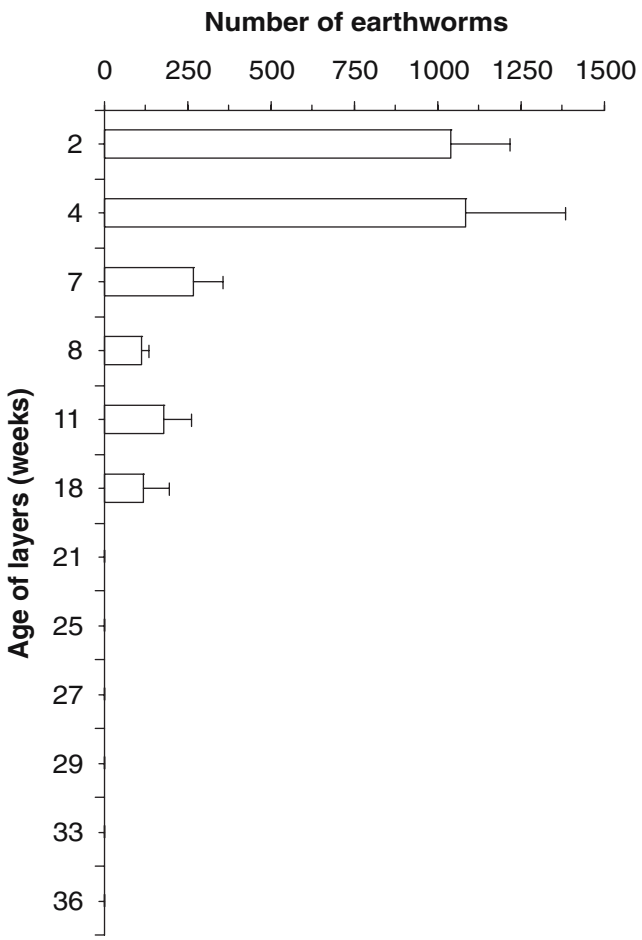

Figure 2. Earthworm population in vermireactors at different sampling times. Number of earthworms (mean ± S.E.) in each layer, from 2 to 36 weeks of age, are shown.

method was used to determine the distances in space, and the Ward method was used to add layers to clusters [24]. All statistical analyses were performed using SPSS 11.5 software.

Results

The mean number of earthworms per reactor at the end of the experiment (after 36 weeks) was 2800 ± 200, with a mean biomass of 700 ± 30 g, more than a fivefold and eightfold increase over the initial population of 500 mature earthworms, which had a mean biomass of 90 ± 10 g. Earthworms were located mainly in the younger layers (Fig. 2) and two groups of layers were distinguished based on their earthworm density. The first group was formed by 2- and 4-week-old layers with more than 1040 ± 180 and 1080 ± 300 earthworms in each layer, respectively; the mean biomass of earthworms of these two layers were 240 ± 61 and 280 ± 64 g (2- and 4-week-old layers, respectively). The second group was formed by 7-, 8-, 11-, and 18-week-old layers, with 267 ± 90, 113 ± 20, 180 ± 80, and 120 ± 70 earthworms, with corresponding weights of 70 ± 20, 30 ± 10, 50 ± 25, and 30 ± 20 g, respectively. No earthworms were found in the remaining layers.

The process of vermicomposting was characterized by a continuous and significant loss of C through the increasing age of layers of reactors until 21 weeks (Fig. 3a; ANOVA, $F_{11,44} = 17.90$, $P < 0.0001$). Moreover, earthworms produced a significant intense decrease in total C content of pig manure (Fig. 3a; ANOVA, $F_{1,4} = 351.00$, $P < 0.0001$), doubling the rate of C loss with respect to reactors without earthworms (440 and 200 μg C day^{-1}, with and without earthworms, respectively). Further, the loss of C in the reactors with earthworms was 1.5 times higher in layers where earthworms were present (762 and 492 μg C per day with and without earthworms, respectively), and remained constant once earthworms left the substrate in the oldest layers, resulting in a significant interaction between earthworms and age (Fig. 3a; ANOVA, $F_{11,44} = 5.57$, $P < 0.0001$).

A slightly higher DOC content was found in layers in which earthworms were present (2-, 4-, 7-, 8-, and 11-week-old layers; Fig. 3b), with values ranging between 2100 and 2800 μg g^{-1} dry weight. However, the main effect of earthworms during vermicomposting of pig manure was significantly reduced DOC pools, which became more evident in 18- to 36-week-old layers, with values of DOC below 1500 μg g^{-1} dw (Fig. 3b; ANOVA, $F_{1,4} = 12.28$, $P < 0.05$). These opposing effects of earthworms on DOC contents, increase and decrease through age of layers, resulted in a significant interaction between earthworms and age of layers (Fig. 3b; ANOVA, $F_{11,44} = 5.29$, $P < 0.0001$).

Time (i.e., age of layers) produced a significant decrease of C_{mic} in both with and without earthworm treatments (Fig. 4a; ANOVA, $F_{11,44} = 5.45$, $P < 0.0001$). Although the overall effect of earthworm on C_{mic} was not significant, this was higher in layers with earthworms (2, 4, 8, and 11 weeks old) than in layers without earthworms (Fig. 4a). This effect was more intense in 2- and 4-week-old layers with values of 22,500 ± 2300 and 26,300 ± 1700 μg g^{-1} dw, respectively, which were higher

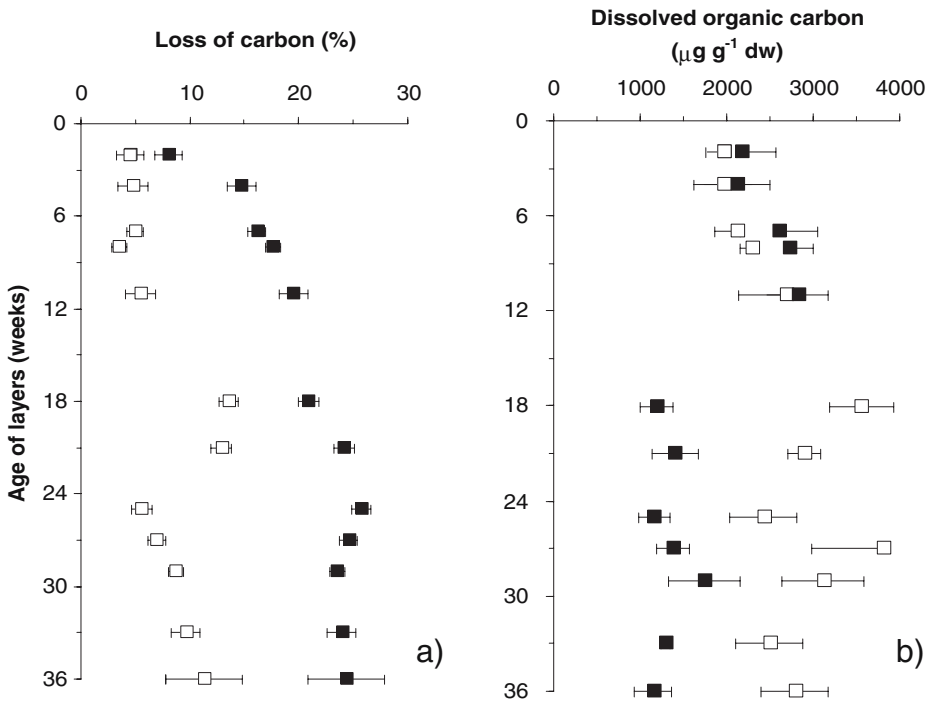

Figure 3. Development with time of (a) loss of carbon (mean ± S.E.) and (b) dissolved organic carbon in layers of vermireactors with (black squares) and without *Eisenia fetida* (white squares). The vertical distributions of variables values corresponding to age of layers of pig manure, from 2 to 36 weeks, are shown on the *y* axis.

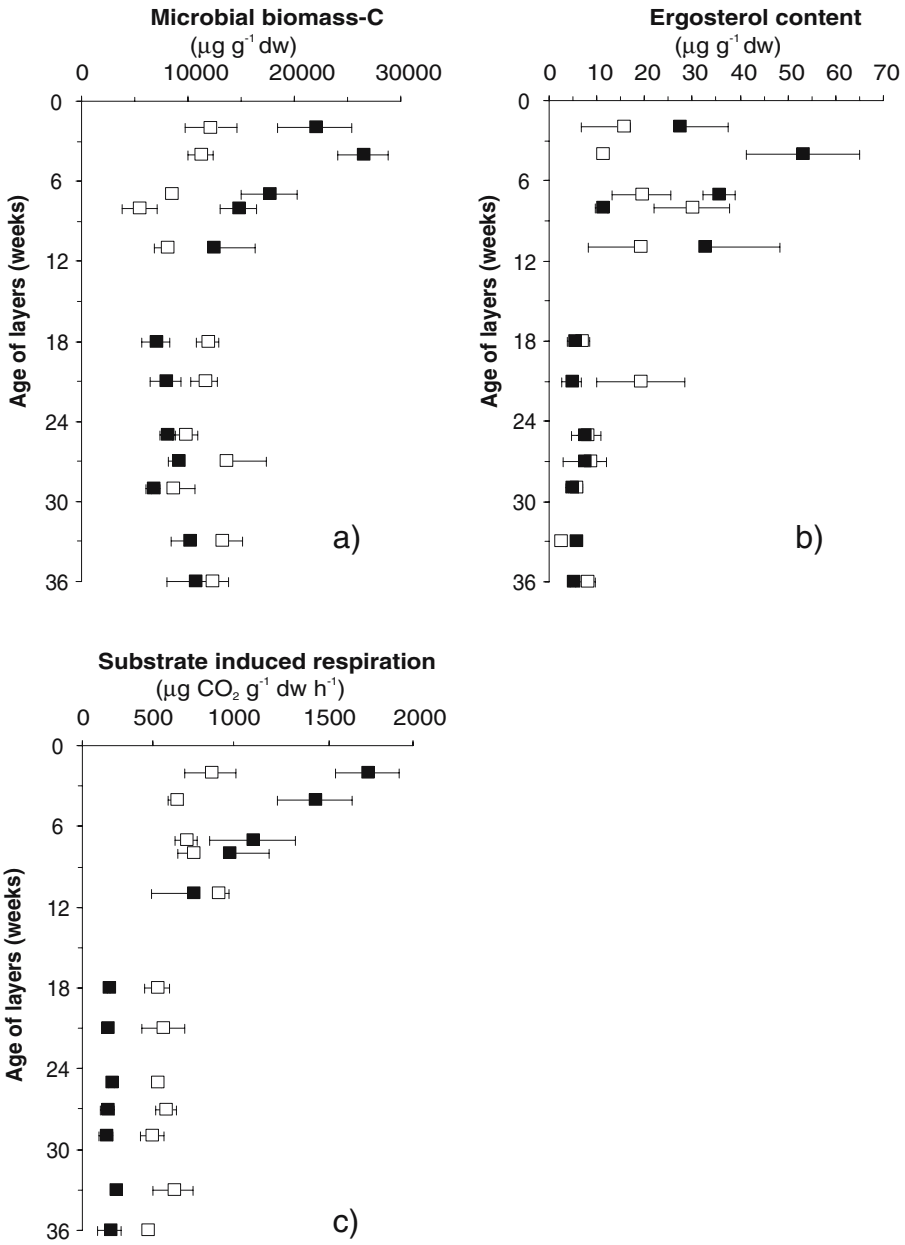

Figure 4. Development with time of (a) microbial biomass-C (mean ± S.E.), (b) ergosterol content, and (c) substrate-induced respiration in layers of reactors with (black squares) and without *Eisenia fetida* (white squares). The distributions of variable values corresponding to age of layers of pig manure, from 2 to 36 weeks, are shown on the *y* axis.

than C_{mic} of the initial pig manure (19,000 ± 1200 μg g^{-1} dw). In the layers where earthworms were absent, 18- to 36-week-old layers, C_{mic} fell to below 10,000 μg g^{-1} dw, whereas it was higher than 10,000 μg g^{-1} dw in the vermireactors without earthworms (Fig. 4a), leading to a significant interaction between earthworm and age of layers (Fig. 4a; ANOVA, $F_{11,44} = 6.09$, $P < 0.0001$).

Ergosterol concentration increased in layers of reactors where earthworms were present, 2-, 4-, 7-, and 11-week-old layers, with a peak of 53 ± 12 μg g^{-1} dw in the 4-week-old layer (Fig. 4b); these values were higher than those corresponding to the initial fresh pig manure (10 ± 1 μg g^{-1} dw). In the oldest layers (18 to 36 weeks old) the ergosterol concentrations decreased to below 8 μg g^{-1} dw in reactors once earthworms have left the layers, leading to a significant interaction between earthworms and age of layers (Fig. 4b; ANOVA, $F_{11,44} = 3.41$, $P < 0.01$). Furthermore, age of layers produced a decrease in ergosterol content in reactors with and without earthworms (Fig. 4b; ANOVA, $F_{11,44} = 5.52$, $P < 0.0001$).

Earthworm presence in the younger layers of the reactors (2- to 8-week-old layers) clearly increased SIR with respect to those without earthworms (Fig. 4c). Thus, SIR values in these layers ranged between 1735 and 993 μg CO_2 g^{-1} dw h^{-1}, respectively, with a peak of 1735 ± 229 μg CO_2 g^{-1} dw h^{-1} in the 2-week-old layer. This trend was opposed to the significant decrease produced by age of layers in the reactors (Fig. 4c;

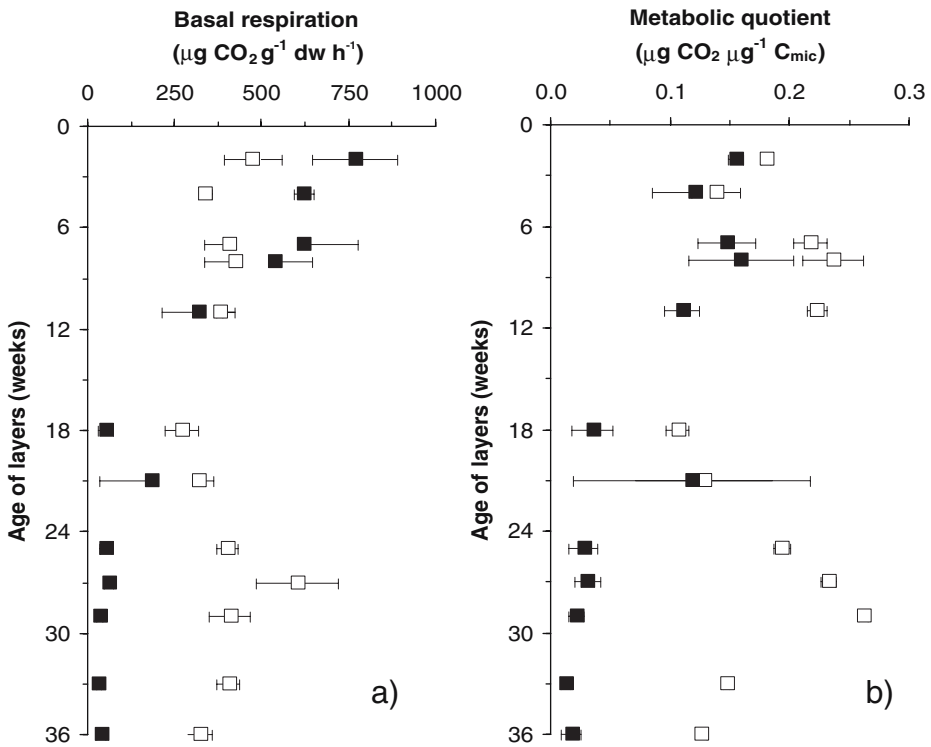

Figure 5. Development with time of (a) basal respiration (mean ± S.E.) and (b) metabolic quotient in layers of reactors with (black squares) and without *Eisenia fetida* (white squares). The distributions of variable values corresponding to age of layers of pig manure, from 2 to 36 weeks, are shown on the *y* axis.

ANOVA, $F_{11,44} = 14.92$, $P < 0.0001$). This was much more marked in reactors with earthworms with values below 250 μg CO_2 g^{-1} dw h^{-1} in 11- to 36-week-old layers, which resulted in a significant interaction between earthworms and age of layers (Fig. 4c; ANOVA, $F_{11,44} = 7.45$, $P < 0.0001$).

Decomposition of pig manure was characterized by a significant loss of basal respiration with the increasing age in reactors (Fig. 5a; ANOVA, $F_{11,44} = 8.53$, $P < 0.0001$). Although the main effect of earthworm presence was to intensify this decrease (Fig. 5a; ANOVA, $F_{1,4} = 13.82$, $P < 0.05$), basal respiration was higher in 2-, 4-, 7-, and 8-week-old layers in the reactors with earthworms than in those without earthworms, with a maximum of 745 ± 107 μg CO_2 g^{-1} dw h^{-1} in the 2-week-old layer (Fig. 5a). In the lower layers, those older than 18 weeks, basal respiration in reactors with earthworms dropped below 60 μg CO_2 g^{-1} dw h^{-1} resulting in a significant interaction between earthworms and age of layers (Fig. 5a; ANOVA, $F_{11,44} = 7.49$, $P < 0.0001$).

Despite of stimulation of C_{mic} and basal respiration in fresh layers of reactors with earthworms, the qCO_2 revealed that respiration of microorganisms was significantly lower through the entire profile of vermireactors with earthworms (0.07 ± 0.02 μg CO_2 $\mu g^{-1} C_{mic}$) than in vermireactors without earthworms (0.18 ± 0.02 μg CO_2 $\mu g^{-1} C_{mic}$; Fig. 5b; ANOVA, $F_{1,4} = 10.93$, $P < 0.05$). Although the qCO_2 showed a continuous decrease with age of layers (Fig. 5b; ANOVA, $F_{11,44} = 3.09$, $P < 0.01$), the difference of values between fresh and old layers in

Table 2. Values (mean ± SE) of different ecological indices (substrate richness, diversity, and evenness) calculated from Biolog data in layers of vermireactors with and without earthworms (EW)

	Substrate richness		*Substrate diversity*		*Substrate evenness*	
Age of layers (weeks)	*With EW*	*Without EW*	*With EW*	*Without EW*	*With EW*	*Without EW*
0	28.67 ± 0.44	28.67 ± 0.44	2.53 ± 0.38	2.53 ± 0.38	1.74 ± 0.30	1.74 ± 0.30
2	29.00 ± 0.55	29.00 ± 0.44	3.84 ± 0.22	3.72 ± 0.38	2.63 ± .037	2.54 ± 0.30
4	29.56 ± 0.56	29.78 ± 0.30	3.93 ± 0.11	3.64 ± 0.22	2.67 ± 0.38	2.47 ± 0.20
7	29.33 ± 0.50	29.56 ± 0.24	3.80 ± 0.10	3.61 ± 0.11*	2.59 ± 0.34	2.46 ± 0.17*
8	29.11 ± 0.44	29.11 ± 0.33	3.76 ± 0.10	3.68 ± 0.33	2.57 ± 0.30	2.51 ± 0.22
11	28.78 ± 0.42	29.00 ± 0.23	3.66 ± 0.22	3.51 ± 0.19	2.51 ± 0.29	2.40 ± 0.15
25	28.89 ± 0.31	29.00 ± 0.34	3.56 ± 0.44	3.68 ± 0.33*	2.43 ± 0.22	2.51 ± 0.23*
36	28.56 ± 0.44	29.11 ± 0.64*	3.70 ± 0.22	4.01 ± 0.11*	2.54 ± 0.30	2.74 ± 0.44*

Values marked with asterisk are significantly different at $p < 0.05$ (post hoc Tukey HSD test).

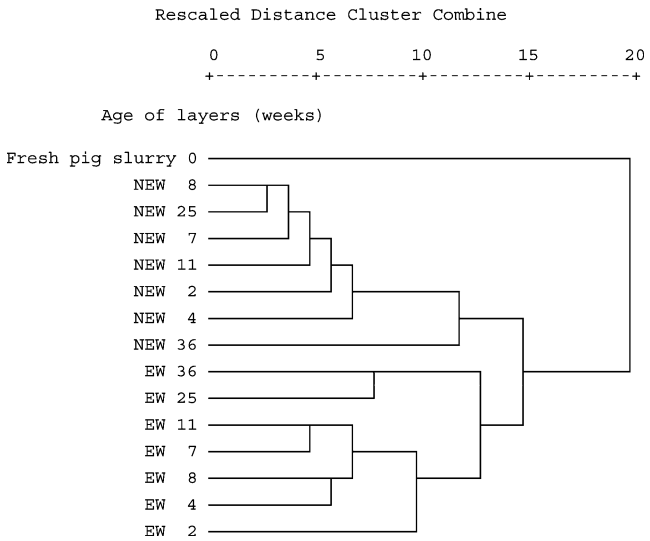

Figure 6. Cluster analysis of the Biolog Ecoplate physiological profiles from layers of 0, 2, 4, 7, 8, 11, 25, and 36 weeks of age in vermireactors without earthworms (NEW) and with earthworms (EW). Clusters were determined by the Ward method and by Euclidean distance.

vermireactors with earthworms resulted in a significant interaction between earthworms and age of layers (Fig. 5b; ANOVA, $F_{11,\ 44} = 2.26$, $P < 0.05$).

Earthworms significantly decreased the number of different carbon compounds (substrate richness) used by microorganisms, especially in 36-week-old layers (Table 2; ANOVA, $F_{1,4} = 10.51$, $P < 0.05$); this decrease was intensified with age of layers (Table 2; ANOVA, $F_{6,24} = 3.28$, $P < 0.05$). The presence of earthworms in 7-week-old layer produced an increase of substrate diversity, but in the oldest layers (25 and 36 weeks old) substrate diversity was significantly lower than in the reactor without earthworms (Table 2); thus, earthworm presence resulted in a significant decrease in substrate diversity (Table 1; ANOVA, $F_{1,4} = 17.49$, $P < 0.05$). Earthworms promoted a significant decrease in substrate evenness in oldest layers, although substrate evenness was significantly higher in the 7-week-old layer (Table 2; ANOVA, $F_{1,4} = 13.80$, $P < 0.05$).

Cluster analysis of the data from Biolog Ecoplate (Fig. 6) grouped the layers of the different treatments (earthworm presence and age of layers) into two principal clusters, one comprising the initial fresh pig manure and the other the aged layers of reactors with and without earthworms. Layers in the reactors with earthworms were grouped into two clusters formed by young layers (2, 4, 7, 8, and 11 weeks old) and old layers (25 and 36 weeks old). Finally, layers of reactors without earthworms were grouped into two clusters formed by the oldest layer (36 weeks old) and other layers (2, 4, 7, 8, 11, and 25 weeks old).

Discussion

Our results showed that earthworms played an important role in modifying the functional diversity (i.e., community-level physiological profiles) of pig manure during vermicomposting. Moreover, the above-mentioned changes resulted in alterations on the system functioning, herein measured as C loss, which was clearly accelerated by the joint action of *E. fetida* and microorganisms. We found that the effect of earthworms on organic matter decomposition was clearly separated into two stages. In the first stage, corresponding to the 2- to 18-week-old layers, in which earthworms were still present, we recorded the highest values of microbial biomass and activity, up to two to four times higher than corresponding layers of reactors without earthworms. The second stage, corresponding to the 21- to 36-week-old layers, characterized by the absence of earthworms and the stabilization of organic matter, showed an intense decrease in microbial biomass and activity.

Earthworms enhanced both components of the microbial community (bacteria vs fungi,) [1], although previous studies showed that fungi could be one of the main components of the earthworms' diet [9], and that earthworms can digest and graze selectively on determinate species of fungi [29, 34]. We recorded a high stimulation of fungal growth in layers with earthworms: a two- and fivefold increase in 2- and 4-week-old layers with respect to corresponding layers in vermireactors without earthworms. Furthermore, SIR measures revealed that earthworms increased the biomass of active microorganisms in young layers, being up to 2.5 times higher than in vermireactors without earthworms. The enhancement in microbial biomass may be explained partly by the production of mucus by the earthworms, because mucus is known to have a stimulating effect on microorganisms [12]. Furthermore, it is known that microbial biomass is stimulated in earthworm burrows [39] and by breakdown of organic matter, thereby increasing the surface available for microorganisms, a process that would be increased by the high earthworm densities. These two processes would have increased with the high earthworm densities observed in these layers. The effects that earthworms exert on soil fungi may be caused by either physicochemical modification of substrate [22] or dispersion of propagules [44]. The observed physical alteration of the substrate and the concentration of straw fractions of pig manure may favor suitable conditions for fungi. Dispersion of spores, the main form of fungi in manure [16], with earthworm casts may have played an important role in the observed fungal growth.

The consequence of the intense microbial activity was a quick mineralization of C, and although DOC contents increased slightly in young layers, these pools of

easily assimilable carbon were rapidly exhausted. However, it has been reported that earthworms could mobilize labile C pools in early stages of decomposition [30]. Our data suggest that during the first stages of organic matter decomposition, the relationships established between earthworms and microorganisms are close to some kind of mutualism [8], although in this case it would take place outside earthworm gut. This mechanism could be similar to nutrient enrichment process as described by Devliegher and Verstraete [10], but in this case *E. fetida* modified the structure of substrate and released new nutrient pools as a result of its feeding and casting activities, which stimulated microbial metabolism. In fact, Scheu *et al.* [33] found that the presence of epigeic earthworms (*E. fetida*, *Lumbricus rubellus*, *Dendrodrilus rubidus*) was able to counteract the reduction in microbial biomass produced by endogeic earthworms. The exhaustion of labile C pools and maturation of casts are largely responsible for the decrease in microbial biomass and activity of later stages, as several studies on cast aging indicated [2, 31]. These processes together with the stabilization of C loss may be related with protection and sequestration of C in casts, which increase with aging [reviewed in 23].

Earthworms not only produced quantitative changes in the biomass and activity of microbial populations of pig manure, but also modified the functional diversity of microbial community, in this article measured as community-level physiological profile (i.e., physiological diversity). Hill *et al.* [18] reported that if profiles segregate in a cluster, communities would be considered functionally different, so cluster analysis clearly separated microbial communities of reactors with and without earthworms. Despite the fact that Biolog® selects for only a portion of the microbial community [37], results showed that earthworms promoted a change in microbial communities increasing their capabilities to use more diverse carbon pools. This result suggests that earthworms could have been optimized the way in which microorganism use resources, favoring the existence of a more specialized microbial community, as suggested by the high rates of C loss.

The metabolic quotient evaluates the efficiency of microorganisms in utilizing organic C compounds with larger values indicating an inefficient use of energy [4]. Despite the overall stimulation of microbial biomass and activity, the low values of metabolic quotient found in reactors with earthworms suggest that microbial communities of these reactors may have been under a lesser stress that the corresponding ones in reactors without earthworms. Further, microorganisms of reactors with earthworms would be using more efficiently the energy available than microorganisms of reactors without earthworms. As a consequence, the functionality of the system was better, as shown by the strong C loss, which in these young layers (2–18 weeks old) was 1.5 times higher in reactors with earthworms. Our data match with the recent findings that for maintaining essential processes the functional properties of present species are at least as important as the number of species *per se* [5, 6]. This is in accordance with the "insurance hypothesis" proposed by Loreau *et al.* [26], which states that a large number of species is probably essential for maintaining stable processes in changing environments as the presence of earthworms would create in pig slurry. It has been stated that changes in community-level physiological profiles should not be equated to changes in microbial community structure because it only records the fastest growing portion of community [7]. However, our study suggests that these modifications could be caused by the increase of fungal populations, as ergosterol content revealed, favoring the proliferation of new species and physicochemical alteration of the substrate finally homogenized by the earthworms. The homogenization of the substrate by earthworms could have led to a "community conditioning" [17], where microorganisms increase that are specialized in metabolizing compounds produced or released by earthworms, improving the rate of mineralization and decomposition of the substrate, as it was previously reported with cellulose decomposition [1].

Acknowledgments

This research was supported by CICYT (AGL2003-01570) and Xunta de Galicia (PGIDIT03PXIB30102PR) grants. Manuel Aira was financially supported by a postdoctoral fellowship from Xunta de Galicia. Manuel Aira also acknowledges Paul Fraiz for his highly valuable help in language editing.

References

1. Aira, M, Monroy, F, Domínguez, J (2006) *Eisenia fetida* (Oligochaeta, Lumbricidae) activates fungal growth, triggering cellulose decomposition during vermicomposting. Microb Ecol (in press, doi:10.1007/s00248-006-9109-x)
2. Aira, M, Monroy, F, Domínguez, J (2005) Ageing effects on nitrogen dynamics and enzyme activities in casts of *Aporrectodea caliginosa* (Lumbricidae). Pedobiologia 49: 467–473
3. Anderson, JPE (1982) Soil respiration. In: Page AL (Ed.) Methods of soil analysis, Part 2. Chemical and microbiological properties. American Society of Agronomy, Madison, Wisconsin, pp 831–871
4. Anderson, JPE, Domsch, KH (1993) The metabolic quotient for CO_2 (qCO_2) as a specific activity parameter to assess the effects of environmental conditions, such as pH, on the microbial biomass of forest soils. Soil Biol Biochem 25: 393–395
5. Andren, O, Balandreau, J (1999) Biodiversity and soil functioning from black box to can of worms? Appl Soil Ecol 13: 105–108
6. Bardgett, RD, Shine A (1999) Linkages between plant litter diversity, soil microbial biomass and ecosystem function in temperate grasslands. Soil Biol Biochem 31: 317–321

7. Bossio, DA, Scow, KM (1998) Impacts of carbon and flooding on soil microbial communities: phospholipid fatty acid profiles and substrate utilization patterns. Microb Ecol 35: 265–278
8. Brown, GG, Barois, I, Lavelle, P (2000) Regulation of soil organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains. Eur J Soil Biol 36: 177–198
9. Dash, HK, Beura, BN, Dash, MC (1986) Gut load, transit time, gut microflora, and turnover of soil, plant, and fungal material by some tropical earthworms. Pedobiologia 29: 13–20
10. Devliegher, W, Verstraete, W (1995) *Lumbricus terrestris* in a soil core experimentnutrient-enrichment processes (NEP) and gut-associated processes (GAP) and their effect on microbial biomass and microbial activity. Soil Biol Biochem 27: 1573–1580
11. Domínguez, J (2004) State of the art and new perspectives on vermicomposting research. In: Edwards CA (Ed.) Earthworm ecology, 2nd edition, CRC Press, Boca Raton, pp 401–424
12. Doube, BM, Brown, GG (1998) Life in a complex community: functional interactions between earthworms, organic matter, microorganisms, and plant growth. In: Edwards CA (Ed.) Earthworm ecology, St. Lucie Press, Boca Raton, pp 179–211
13. Edwards, CA (1998) Earthworm Ecology. St. Lucie Press, Boca Raton
14. Egert, M, Marhan, S, Wagner, B, Scheu, S, Friedrich, MW (2004) Molecular profiling of 16S rRNA genes revealed diet-related differences of microbial communities in soil, gut and casts of *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae). FEMS Microbiol Ecol 48: 187–197
15. Garland, JL (1997) Analysis and interpretation of community-level physiological profiles in microbial ecology. FEMS Microbiol Ecol 24: 289–300
16. Garrett, SD (1981) Soil fungi and soil fertility. Pergamon Press, Oxford
17. Griffiths, BS, Bonkowski, M, Roy, J, Ritz K (2001) Functional stability, substrate utilization and biological indicators of soils following environmental impacts. Appl Soil Ecol 16: 49–61
18. Hill, GT, Mitkowski, NA, Aldrich-Wolfe, L, Emele, LR, Jurkonie, DD, Ficke A, Maldonado-Ramirez S, Lynch ST, Nelson EB (2000) Methods for assessing the composition and diversity of soil microbial communities. Appl Soil Ecol 15: 25–36
19. Ibekwe, AM, Kennedy, AC (1998) Phospholipid fatty acid profiles and carbon utilization patterns for analysis of microbial community structure under field and greenhouse conditions. FEMS Microbiol Ecol 26: 151–163
20. Jackson, ML (1958) Soil chemical analysis. Constable & Co. Ltd, London
21. Jones, CG, Lawton, JH, Shachak, M (1994) Organisms as ecosystem engineers. Oikos 69: 373–386
22. Lavelle, P, Bignell, D, Lepage, M, Wolters, V, Roger, P, Ineson, P, Heal, OW, Ghillion, S (1997) Soil function in a changing world: The role of invertebrate ecosystem engineers. Eur J Soil Biol 33: 159–193
23. Lavelle, P, Spain, AV (2001) Soil ecology. Kluwer Academic Press, London
24. Logan, NA (1994) Bacterial systematics. Blackwell Scientific, London
25. Loquet, M, Bhatnagar, T, Bouché, MB, Rouelle, J (1977) Essai d'estimation de l'influence écologique des lombriciens sur les microorganismes. Pedobiologia 17: 400–417
26. Loreau, M, Naeem, S, Inchausti, P, Bengtsson, J, Grime, JP, Hector, A, Hooper, DU, Huston, MA, Raffaelli, D, Schmid, B, Tilman, D, Wardle, DA (2001) Biodiversity and ecosystem functioning: current knowledge and future challenges. Science 294: 804–808
27. McLean, MA, Parkinson, D (1998) Impacts of the epigeic earthworm *Dendrobaena octaedra* on microfungal community structure in pine forest floor: a mesocosm study. Appl Soil Ecol 8: 61–75
28. McLean, MA, Parkinson, D (2000) Field evidence of the effects of the epigeic earthworm *Dendrobaena octaedra* on the microfungal community in pine forest floor. Soil Biol Biochem 32: 351–360
29. Moody, SA, Briones, MJI, Pierce, TG, Dighton, J (1995) Selective consumption of decomposing wheat straw by earthworms. Soil Biol Biochem 28: 533–537
30. Saetre, S (1998) Decomposition, microbial community structure, and earthworm effects along a birch–spruce soil gradient. Ecology 79: 834–846
31. Scheu S (1987) Microbial activity and nutrient dynamics in earthworm cast (Lumbricidae). Biol Fertil Soils 5: 230–234
32. Scheu S, Schaefer M (1998) Bottom-up control of the soil macrofauna community in a beechwood on limestone: manipulation of food resources. Ecology 79: 1573–1585
33. Scheu, S, Schlitt, N, Tiunov, AV, Newington, JE, Jones, TF (2002) Effects of the presence and community composition of earthworms on microbial community functioning. Oecologia 133: 254–260
34. Schönholzer, F, Hahn, D, Zeyer, J (1999) Origins and fate of fungi and bacteria in the gut of *Lumbricus terrestris* L. studied by image analysis. FEMS Microbiol Ecol 28: 235–248
35. Scott, JS, Knudsen, GR (1999) Soil amendment effects of rape (*Brassica napus*) residues on pea rhizosphere bacteria. Soil Biol Biochem 31: 1435–1441
36. Siciliano, SD, Germida, JJ (1998) Biolog analysis and fatty acid methyl esther profiles indicate that pseudomonad inoculants that promote phytoremediation alter the root-associated microbial community of *Bromus biebersteinii*. Soil Biol Biochem 30: 1717–1723
37. Smalla, K, Wachtendorf, U, Heuer, H, Liu, WT, Forney L (1998) Analysis of Biolog BG substrate utilization patterns by microbiological communities. Appl Environ Microbiol 64: 1220–1225
38. Tiunov, AV, Dobrovolskaya, TG, Polyanskaya, LM (1997) Microbial community of *Lumbricus terrestris* burrow walls. Microbiology 66: 349–353
39. Tiunov, AV, Scheu, S (1999) Microbial respiration, biomass, biovolume and nutrient status in burrow walls of *Lumbricus terrestris* L. (Lumbricidae). Soil Biol Biochem 31: 2039–2048
40. Tiunov, AV, Scheu, S (2000) Microfungal communities in soil, litter and cast of *Lumbricus terrestris* L. (Lumbricidae): a laboratory experiment. Appl Soil Ecol 14: 17–26
41. Tiunov, AV, Scheu, S (2004) Carbon availability controls the growth of detritivores (Lumbricidae) and their effect on nitrogen mineralization. Oecologia 138: 83–90
42. Tyurin, IV (1931) A new modification of the volumetric method of determining soil organic matter by means of chromic acid. Pochvovedenie 26: 36–47
43. Vance, ED, Brookes, PC, Jenkinson, DS (1987) An extraction method for measuring soil microbial biomass C. Soil Biol Biochem 19: 703–707
44. Visser, S (1985) Role of soil invertebrates in determining the composition of soil microbial communities. In: Fitter AH, Atkinson D, Read DJ, Usher MB (Eds.) Ecological interactions in soil, Blackwell, Oxford, pp. 297–317
45. von Ende, CN (2001) Repeated-measures analysis. In: Scheiner SM, Gurevitch J (Eds.) Design and analysis of ecological experiments, Oxford University Press, Oxford, pp 134–157
46. Whitehead, TR, Cotta, MA (2001) Characterisation and comparison of microbial populations in swine faeces and manure storage pits by 16S rDNA gene sequence analyses. Anaerobe 7: 181–187
47. Young, JC (1995) Microwave-assisted extraction of the fungal metabolite ergosterol and total fatty acids. J Agric Food Chem 43: 2904–2910
48. Zak, JC, Willig, MR, Mooread, DL, Wildman, HG (1994) Functional diversity of microbial communities: a quantitative approach. Soil Biol Biochem 26: 1101–1108
49. Zhu, J (2000) A review of microbiology in swine manure odor control. Agr Ecosyst Environ 78: 93–106