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Earthworms strongly modify microbial biomass and activity triggering enzymatic activities during vermicomposting independently of the application rates of pig slurry

Manuel Aira *, Fernando Monroy, Jorge Domínguez

Departamento de Ecoloxia e Bioloxía Animal, Facultade de Ciencias, Universidad de Vigo, Vigo E-36200, Spain

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Abstract

We studied the relationships between earthworm activity, microbial biomass and the activation and dynamics of several enzyme activities. We carried out an experiment in which low and high rates (1.5 and 3 kg respectively) of pig slurry were applied to small scale reactors with and without earthworms. We found that extracellular enzyme activity increased with rate of pig slurry. In both rates of pig slurry applied, the presence of earthworms in young layers stimulated microbial growth which decreased once earthworms left the slurry and the layers aged. This increase was related to the initial activation of the microbial enzymes studied as correlations between microbial biomass and enzymes showed, which indicated an increase of intracellular enzyme activity. In the aged slurry, the pattern of activity of the four enzymes assayed depended on the rate of pig slurry applied. Thus, in low rate reactors, enzymatic activity through layers appeared to be related to microbial biomass, but in high rate reactors the activity of enzymes was more or less continuous. Further, these differences in overall enzyme activity agree with the variation found in extracellular enzyme activity suggesting certain dependence on substrate availability.

Keywords: Decomposition; Pig slurry; Microbial biomass; β-glucosidase; Cellulase; Phosphatase; Protease; Vermicomposting

1. Introduction

Enzyme activities have been used widely as an index of soil fertility or ecosystem status because they are involved in the biological transformations of native and foreign compounds in soils (Tate, 2000). Several enzymatic activities have been measured to describe organic matter decomposition in two microbial-driven processes, composting and vermicomposting (Garcia et al., 1994, 1995; Benıtez et al., 2002, 2005). Vermicomposting involves the bio-oxidation and stabilization of organic matter through the joint action of earthworms and microorganisms. The transformations in physicochemical and biochemical properties (Domınguez, 2004) and the short time in which they can occur (Aira et al., 2002) make vermicomposting a suitable system for studying microbe–earthworm interactions (Aira et al., 2006c, in press).

Usually there is not any correlation between the enzyme activities and microbial biomass and respiration (Tate, 2000), and this may depend on the fact that enzymatic activity is due to enzymes which may be in a living or dead cell, cell debris, free enzymes and/or enzymes adsorbed by clay or immobilized in humic complexes (Ceccanti and Garcia, 1994; Alef and Nannipieri, 1995a; Nannipieri et al., 2002). Thus, it is

* Corresponding author. Tel.: +34 986812593; fax: +34 986812556.
E-mail address: aira@uvigo.es (M. Aira).

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necessary to determine the relationships between microbial populations and enzymatic activity during organic matter decomposition; further, it is also important to quantify the amount of extracellular enzyme activity. Such knowledge would lead to better understanding of how earthworms and microorganisms interact during organic matter decomposition. In our study we focused on how earthworms affected the patterns of microbial enzymatic activities during the vermicomposting of pig slurry, and hence modified the rate of organic matter decomposition. Thus, we analyzed enzymes of the C cycle, such as β-glucosidase and cellulase, because they have been associated with litter mass loss and therefore with the turnover of carbon in a wide range of ecosystems (Sinsabaugh and Linkins, 1993; Sinsabaugh, 1994; Sinsabaugh and Moorhead, 1994). Alkaline phosphatase is involved with the P cycle, hydrolysing organic P esters (Alef et al., 1995) to inorganic phosphorus, which then is available to plants. Protease activity arising from by depolymerization of dissolved organic nitrogen from N-containing compounds (Paul and Clark, 1996), is assumed to be a critical point in the N cycle (Schimel and Bennet, 2004) as polymers are not accessible to microorganisms (Chapin et al., 2002). Therefore, since these enzymes metabolize large organic polymers into smaller ones, analyzing them we can study decomposition rates of organic matter.

Decomposition systems, like vermicomposting, are characterized by having a control donor dynamics, that is, decomposer and detritivores do not control the rate of regeneration of their resources (Pimm, 1982). Thus, we also studied how the amount of manure affects earthworm populations and hence microbial biomass and activity and enzyme activities.

2. Material and methods

2.1. Slurries

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

2.2. Experimental design and sampling method

We set up twelve reactors, six each for low (1.5 kg) and high rates (3 kg) of pig slurry; three for each rate of pig slurry without earthworms (control) and three for each rate, containing 500 mature individuals of the earthworm *Eisenia fetida* with a mean biomass of 85± 5 g (fresh weight). At the end of the experiment (i.e. after 36 weeks), each reactor comprised 12 layers with an increasing gradient of age, resembling a soil profile, from upper to lower layers of: 2, 4, 7, 8, 11, 18, 21,25, 27, 29, 33 and 36 weeks (Aira et al., 2006a,c, in press).

At sampling time the reactors were dismantled and the layers isolated to avoid escape of earthworms. The earthworms were then picked by the hand-sorted method from the substrate, counted and weighted. Five samples of substrate per layer were taken at random and mixed gently for biochemical analyses, i.e. microbial biomass C (C_{mic}), microbial activity (basal respiration), β-glucosidase, cellulose, alkaline phosphatase and protease activities. The number of analytical replicates per treatment was three giving a total number of 144 samples (12 layers × 2 earthworm (with and without) and 2 rates of pig slurry application (low and high)).

2.3. Microbial biomass and enzyme activities

Microbial biomass C (C_{mic}) was determined by the chloroform fumigation–extraction method (Vance et al., 1987) with field-moist samples (5 g fresh weight(fw)). The filtered extracts (1:50 wt:v with 0.5 M K_2SO_4) of both fumigated and unfumigated samples were analyzed for soluble organic C at 590 nm using a Microplate Reader (Bio-Rad Microplate Reader 550). The C_{mic} was estimated as the difference between the organic C extracted from the fumigated and that from the non-fumigated sample, multiplied by the K_2SO_4 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 h incubation. The evolved CO_2 was trapped in 0.02 M NaOH and then measured by titration with HCl to a phenolphthalein endpoint, after adding excess $BaCl_2$ (Anderson, 1982).

β-glucosidase activity was assessed by incubating samples (1 g fw) with *p*-nitrophenyl glucoside (0.025 M) for 1 h at 37 °C, and measuring the *p*-nitrophenol (PNP) released in a Bio-Rad Microplate Reader at 400 nm (Eivazi and Tabatabai, 1988). Cellulase activity was estimated by incubating soil samples (5 g fw) with carboxymethylcellulose (CMC) sodium salt (0.7%) for 24 h at 50 °C, and measuring released reducing sugars in a Bio-Rad Microplate Reader at 690 nm (Schinner and von Mersi, 1990). Alkaline phosphatase (alkaline phosphomonesterase) activity was assessed by incubating soil samples (1 g fw) with *p*-nitrophenyl glucoside (0.015 M) for 1 h at 37 °C, and measuring the released *p*-nitrophenol in a Bio-Rad Microplate Reader at 400 nm (Eivazi and

Tabatabai, 1977). Protease activity was measured by incubating soil samples (1 g fw) with sodium caseinate (2%) for 2 h at 50 °C using Folin-Ciocalteu reagent, and quantifying the released amino acids in a Bio-Rad Microplate Reader at 700 nm (Ladd and Butler, 1972).

To quantify the amount of extracellular enzyme activities we applied the approach of McLaren and Pukite (1973) as in Nannipieri et al. (1996). It consists in plotting the enzyme activity against the microbial biomass and, if the correlation between them is significant, the extrapolation to zero biomass will give the extracellular enzyme activity (Nannipieri et al., 1996; Dilly and Nannipieri, 2001).

2.4. Statistical analysis

Data were analyzed using a split plot repeated measures ANOVA (ANOVAR) where single reactors were subjects, rate of pig slurry and earthworm treatment were fixed as between subject factor and week (i.e. each single layer) was fixed as the within subject factor. This model assumes correlation between treatment levels within a subject, i.e. the layers of each reactor (Von Ende, 2001). When sphericity assumptions for ANOVAR (Mauchly's test) could not be met we used Huynh–Feldt correction of P whenever values of ε were close to 1 (Potvin et al., 1990). Regression analyses were performed to examine the relationships between microbial biomass and activity and the four enzyme activities. All results are mean±S.E. All statistical analysis were performed with SPSS 11.5 software.

3. Results

After 36 weeks we found a mean population of 2800± 200 earthworms per reactor (in reactors with earthworms), with a mean biomass of 700±30 g, more than a 5-fold and 8-fold increase respectively over the initial numbers and biomass of inoculated population of earthworms. There were not differences between low and high rate of pig slurry applied in earthworm populations. Earthworms were mainly located in the 2 and 4 week-old layers with over 1000 earthworms in each layer, whereas the 7, 8, 11 and 18 week-old layers showed no more than 200 earthworms per layer. No earthworms were found in the remaining layers (21, 25, 27, 29, 33 and 36 weeks old).

Earthworm activity enhanced microbial biomass C (C_{mic}), since it was 1.3 times greater in reactors with earthworms than in reactors without earthworms (ANOVAR, $F_{1,8}=7.85$, $P<0.05$; Fig. 1a, b); rate of application of pig slurry did not affect C_{mic}. Moreover, C_{mic} showed a clear pattern of distribution depending on earthworm presence, with higher values in 2 to 18 week-old layers in both the low and high rate reactors, whereas in the remaining layers (21 to 36 weeks-old) C_{mic} decreased to values similar to those showed by reactors without earthworms. This increase–decrease pattern across layers promoted by earthworms resulted in a

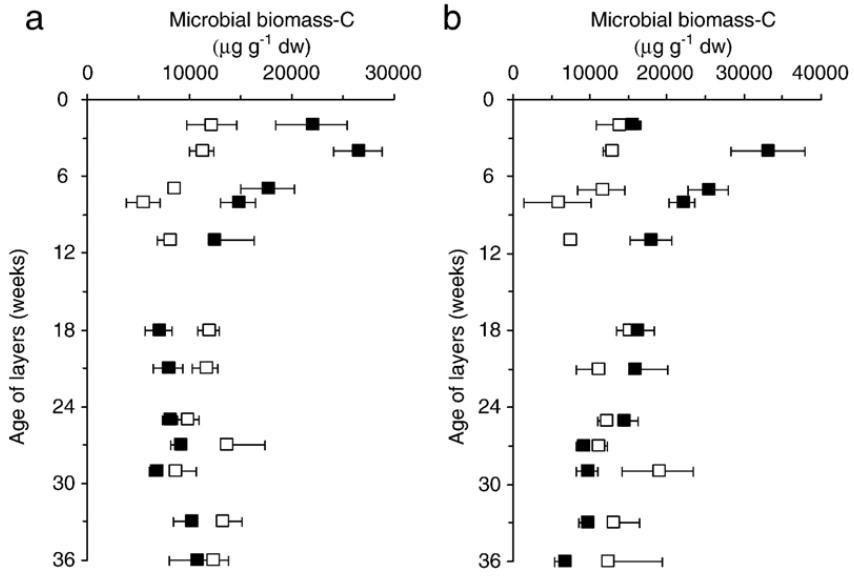

Fig. 1. Microbial biomass C in layers of vermireactors fed with a) 1.5 kg of pig slurry or b) 3 kg of pig slurry with *E. fetida* (closed squares) and with no *E. fetida* (open squares). The vertical distributions of variable values (mean±SE) corresponding to the age of layers of pig slurry between 2 and 18 weeks, are on the *y* axis.

significant interaction between age of layers and earthworms (ANOVAR, $F_{11,88}=8.77$, $P<0.001$). C_{mic} was highly related to earthworm populations ($r=0.60$, $P<0.001$), and this relationship was higher for low ($r=0.67$) than high rate reactors ($r=0.52$, $P<0.001$ for both reactors).

Earthworms significantly decreased basal respiration (1.4 times) through layers of reactors (ANOVAR, $F_{1,8}=48.39$, $P<0.01$). However, basal respiration was higher (1.7 times) in high than in low rate reactors (ANOVAR, $F_{1,8}=7.85$, $P<0.001$; Fig. 2a, b). Earthworms produced a marked pattern of basal respiration in reactors, fostering microbial activity in young layers and decreasing it in the older layers, producing a significant interaction between age of layers and earthworm presence (ANOVAR, $F_{11,88}=13.43$, $P<0.0001$; Fig. 2a, b). Overall microbial activity (i.e. including two rates of application and reactors with and without earthworms) showed a significant relationship with C_{mic} ($r=0.44$, $P<0.0001$). This relationship increased in low rate reactors ($r=0.56$, $P<0.0001$), and decreased in high rate reactors ($r=0.29$, $P<0.05$).

Earthworms increased significantly β-glucosidase activity (up to 1.2 times), increase which was greater in low than in high rate reactors producing a significant interaction between rate of application and earthworms (ANOVAR, $F_{1,8}=13.12$, $P<0.01$; Fig. 3a, b). β-glucosidase activity was between 2.7 and 1.3 greater in low rate than in high rate reactors with earthworms. However, the pattern reversed with activities being between 1.3 and 1.9 higher in the 18 to 36 week-old layers in high rate reactors than in low rate reactors (Fig. 3a, b), producing significant interactions between age of layers and earthworms (ANOVAR, $F_{11,88}=6.23$, $P<0.01$), and age of layers and rate of application (ANOVAR, $F_{11,88}=4.36$, $P<0.05$). Overall activity of β-glucosidase highly correlated with C_{mic} ($r=0.45$, $P<0.0001$), this correlation increased in high ($r=0.49$) and low rate reactors ($r=0.66$, $P<0.0001$ for both rates of manure). These correlations gave extracellular β-glucosidase activities of 760 and 1080 μg PNP g^{-1} dw h^{-1} for low and high rate reactors. Microbial activity also correlated with β-glucosidase activity ($r=0.25$, $P<0.01$), but disappeared in high rate reactors and it was highly significant for low rate reactors ($r=0.77$, $P<0.0001$).

Cellulase activity was greater in high rate reactors (between 3 and 8 times) than in low rate reactors in the layers where earthworms were present (2 to 18 week-old layers), although effects of earthworms on cellulase activity were more evident in the low rate reactors, resulting in a significant interaction between rate of application and earthworms (ANOVAR, $F_{1,8}=6.99$, $P<0.05$; Fig. 4a, b). Furthermore, in 21 to 36 week-old layers this ratio even increased, with cellulase activity being 10 to 21 times higher in the high rate than in low rate reactors with earthworms, resulting in a significant interaction between age of layers and rate of application (ANOVAR, $F_{1,8}=2.31$, $P<0.05$). C_{mic} and enzyme activity correlated ($r=0.35$, $P<0.0001$), incresing the correlation in the low ($r=0.61$) and high rate reactors ($r=0.48$, $P<0.0001$ for both rates of manure). These correlations gave extracellular cellulase activities of 3250 and 44900 μg meq glucose g^{-1} dw 24 h^{-1} for

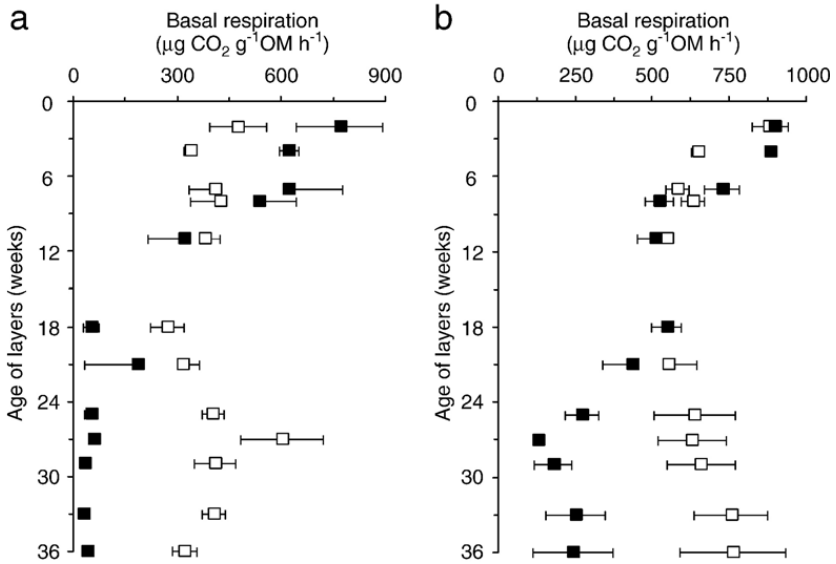

Fig. 2. Basal respiration in layers of vermireactors fed with a) 1.5 kg of pig slurry or b) 3 kg of pig slurry with *E. fetida* (closed squares) and with no *E. fetida* (open squares). The vertical distributions of variable values (mean±SE) corresponding to the age of layers of pig slurry between 2 and 18 weeks, are on the *y* axis.

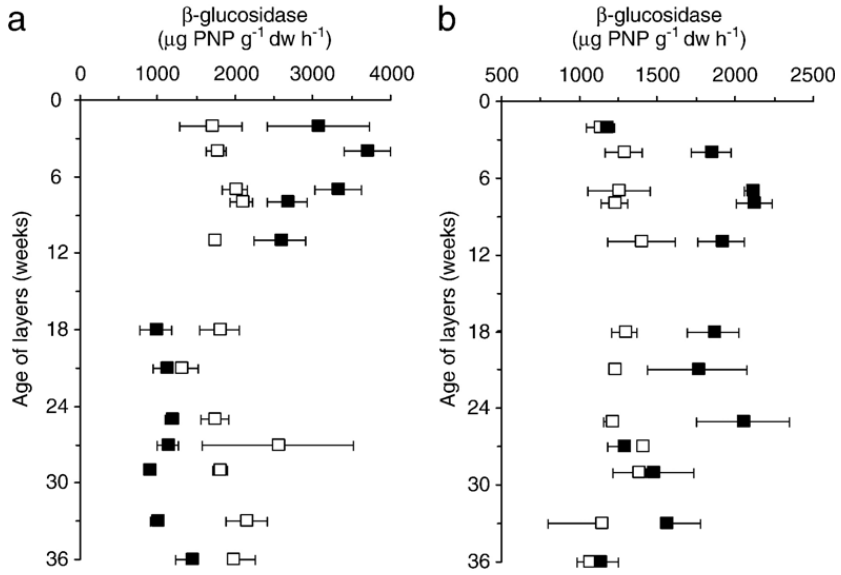

Fig. 3. β-glucosidase activity in layers of vermireactors fed with a) 1.5 kg of pig slurry or b) 3 kg of pig slurry with *E. fetida* (closed squares) and with no *E. fetida* (open squares). The vertical distributions of variable values (mean±SE) corresponding to the age of layers of pig slurry between 2 and 18 weeks, are on the *y* axis.

low and high rate reactors. Microbial activity correlated with cellulase activity ($r=0.36$, $P<0.0001$), correlation which it was greater in low rate reactors ($r=0.46$, $P<0.0001$).

Alkaline phosphatase significantly increased in the low (up to 2.7 times) than in high rate reactors (ANOVAR, $F_{1,8}=270.29$, $P<0.0001$; Fig. 5a, b); moreover alkaline phosphatase showed reverse trends across the layers in the low (decrease) and the high rate reactors (increase) resulting in a significant interaction between age of layers and rate of pig slurry (ANOVAR, $F_{11,88}=2.33$, $P<0.05$). Earthworms produced an increase in 2 to 18 week-old layers, which decrease in the older ones, resulting in a significant interaction between the age of layers and earthworms (ANOVAR, $F_{11,88}=4.62$, $P<0.001$; Fig. 5a, b). Overall alkaline phosphatase did not correlate with C_{mic}; however, it increased in low ($r=0.53$) and high rate reactors

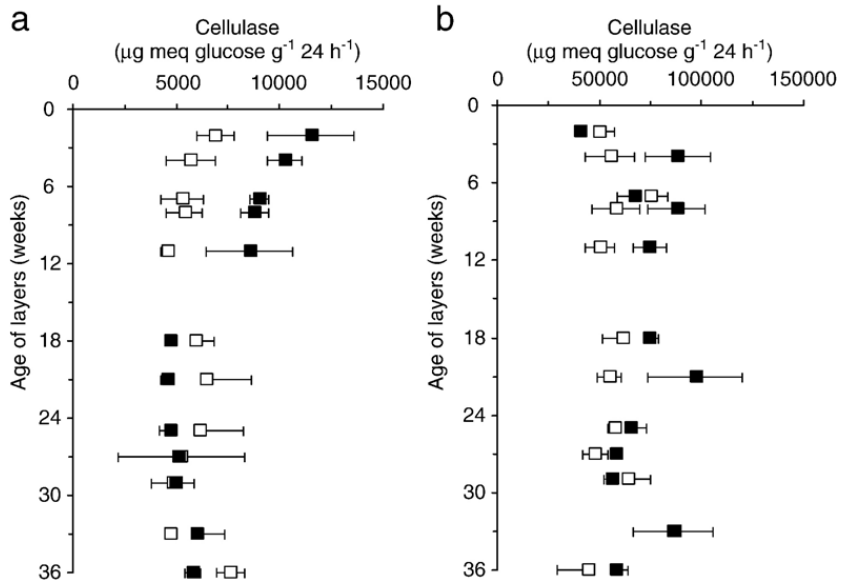

Fig. 4. Cellulase activity in layers of vermireactors fed with a) 1.5 kg of pig slurry or b) 3 kg of pig slurry with *E. fetida* (closed squares) and with no *E. fetida* (open squares). The vertical distributions of variable values (mean±SE) corresponding to the age of layers of pig slurry between 2 and 18 weeks, are on the *y* axis.

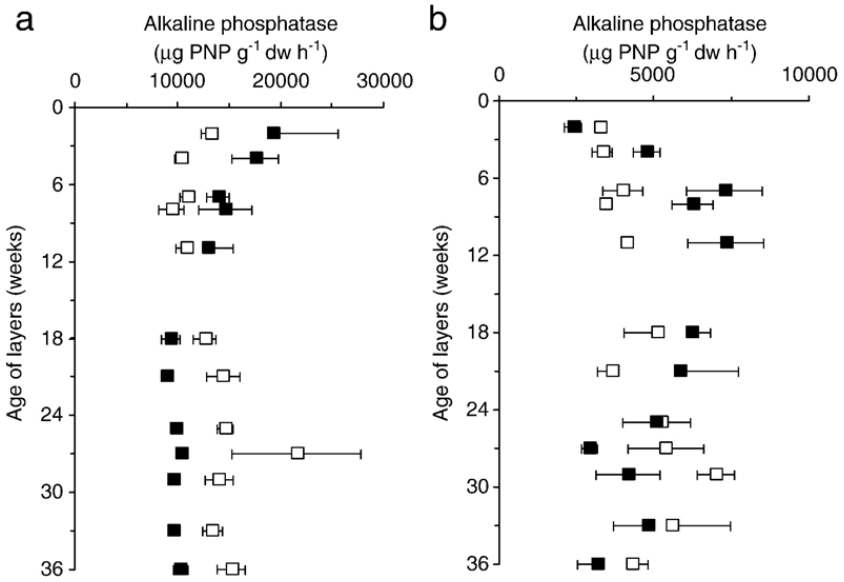

Fig. 5. Alkaline phosphatase activity of layers in vermireactors fed with a) 1.5 kg of pig slurry or b) 3 kg of pig slurry with *E. fetida* (closed squares) and with no *E. fetida* (open squares). The vertical distributions of variable values (mean ± SE) corresponding to the age of layers of pig slurry between 2 and 18 weeks, are on the *y* axis.

($r=0.49$; $P<0.0001$ for both rates of manure). These correlations gave extracellular alkaline phosphatase activities of 8070 and 2950 µg PNP g^{-1} dw h^{-1} for low and high rate reactors. Overall alkaline phosphatase did not correlate with basal respiration, although did it in low rate reactors ($r=0.62$, $P<0.0001$), but did not appear in high rate reactors.

Protease activity was significantly greater in high rate reactors (1.4 times) than in low rate reactors (ANOVAR, $F_{1,8}=52.64$, $P<0.001$; Fig. 6a, b). Furthermore, protease activity in the low rate reactors trended to decrease through layers, whereas in the high rate reactors remained more or less stable, producing a significant interaction between age and rate of application (ANOVAR, $F_{11,88}=3.06$, $P<0.01$). Earthworms significantly decreased (1.3 times) protease activity (ANOVAR, $F_{1,8}=23.39$, $P<0.01$; Fig. 6a, b). The contrasted patterns of protease activity from earthworms through the layers of reactors produced significant interactions between age and earthworms (ANOVAR, $F_{11,88}=2.88$,

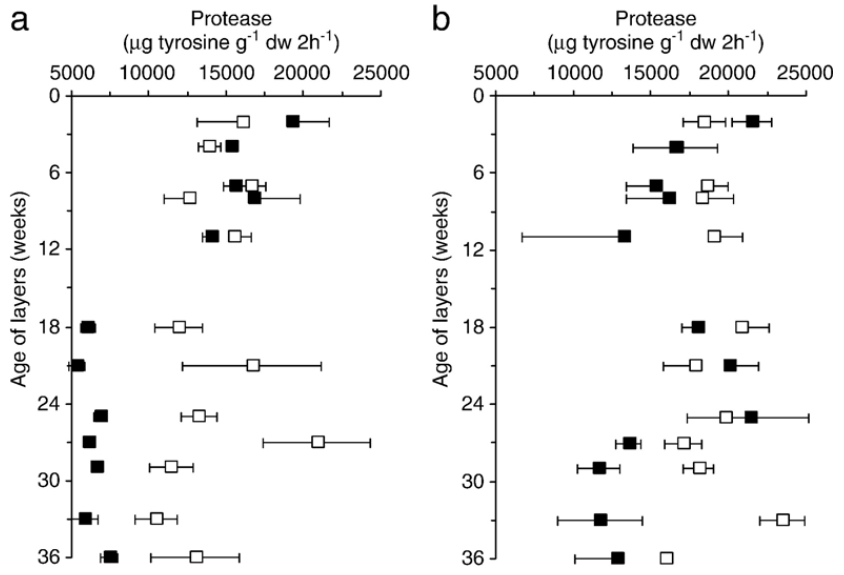

Fig. 6. Protease activity in layers of vermireactors fed with a) 1.5 kg of pig slurry or b) 3 kg of pig slurry with *E. fetida* (closed squares, $n=3$) and with no *E. fetida* (open squares, $n=3$). The vertical distributions of variable values (mean ± SE) corresponding to the age of layers of pig slurry between 2 and 18 weeks, are on the *y* axis.

$P<0.01$). Overall protease activity correlated with C_{mic} ($r=0.33$, $P<0.0001$), and increased in low rate reactors ($r=0.45$, $P<0.0001$). These correlations gave extracellular protease activities of 7710 and 16160 μg tyrosine g^{-1} dw 2 h^{-1} for low and high rate reactors. Moreover, overall protease activity correlated with basal respiration ($r=0.63$), which increased in low rate reactors ($r=0.63$, $P<0.0001$) and decreased in high rate reactors ($r=0.31$, $P<0.01$).

4. Discussion

Earthworm activity had a strong effect on microbial biomass during vermicomposting of pig slurry. Our data showed that the vermicomposting process can be separated in two stages which were related to the presence of the earthworms in the layers, as the high correlation found between earthworm density and microbial biomass showed. Thus, the first stage comprised the youngest layers in which earthworm were located and was characterized by a transient increase in microbial biomass that occurred independently of the rate of slurry applied. By the contrary, older layers were characterized by low microbial biomass contents, due to exhaustion of nutrient pools (Domínguez, 2004), despite of in previous stages (i.e. when they were added to reactors and were "young") probably followed the same dynamic of increases in microbial biomass. Although pig slurry should not be nutrient limited, this stimulatory effect of earthworms on microbial biomass could be explained by mucus and casts production; mucus is a source of easy assimilable carbon for microorganisms (Doube and Brown, 1998) and casts are structures enriched in available forms of C, N and *P* (Scheu, 1987; Aira et al., 2003). The highest levels of microbial biomass were observed in layers with highest numbers of earthworms (2 and 4 week-old layers) where these processes were more intense. 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 (Brown et al., 2000), although in this case would take place outside earthworm gut. This mechanism could be similar to nutrient enrichment process as described by Devliegher and Verstraete (1995, 1997), but in this case *E. fetida* modified the structure of substrate and released new nutrient pools due to its feeding and casting activities which stimulated microbial metabolism.

The interpretation of data from enzyme assays is complicated since enzyme activity depends on many factors and different location of enzymes contributing to the measured enzyme activity in the studied system (Nannipieri et al., 2002). Probably, enzyme assays on moist samples measure most of extracellular enzyme activity because polar esterified compounds cannot diffuse into cells (Breewuer et al., 1995). However, these assays do not separate the contribution of activity due to free enzymes from the activity of enzymes linked to humic substances (Burns, 1982). The strong correlation between C_{mic} and enzymes, especially in the young layers suggest that there was synthesis of new enzymes, and part of the measured enzyme activity was from intracellular origin (Nannipieri et al., 1996, 2002; Dilly and Nannipieri, 2001), which would have triggered following enzyme activity. Moreover, the ratio between extracellular enzyme activity between high and low rate reactors (1.4, 13.8 and 2.1 for β-glucosidase, cellulase and protease) match with the ratio between overall enzyme activities in layers where earthworm left suggesting a baseline of extracellular enzyme activity during organic matter degradation. Measuring extracellular activity as proposed by McLaren and Pukite (1973) implies the assumption of the non-existence of substances which repress or induce the enzyme synthesis. This may complicate the results in nutrient rich environments like pig slurry, and conclusions have to be taken with care. However, this method has been tested and probed its suitability through a huge amount of soils, including those treated with sewage sludges (Nannipieri et al., 1996).

β-glucosidases may catalyze the hydrolysis of glucosides and their activity depends on substrate availability since it can be induced by the substrate (Nannipieri et al., 1990; Alef and Nannipieri, 1995b), which it is assumed to be double in high rate reactors. Earthworms played an important role in the initial activation of β-glucosidase and cellulase activities in reactors by two possible actions: i) by increasing the availability of substrate and ii) by the activation of microbial metabolism. Microorganisms appeared to be more closely related to the β-glucosidase and cellulase activities in low rate reactors (higher correlations); however, this was not the case in high rate reactors, in which β-glucosidase and cellulase activities peaked through all layers. These results also explain the maintenance of β-glucosidase activity in high rate reactors since cellulase activity catalyses the release of substrate for β-glucosidase. In addition, casting may have also caused the initial increase in cellulase activity in layers with earthworms since it has been reported that casts from the tropical earthworm species *Martiodrilus* sp. had higher cellulolytic activity than the uningested

material (Mora et al., 2005), and similar results have been reported for *E. fetida* (Zhang et al., 2000) and *Eudrilus eugeniae* (Aira et al., 2006b) which have similar digestive capabilities. However, enzyme activity decreases with ageing of casts (Parthasarathi and Ranganathan, 2000; Aira et al., 2005), which suggests that other processes are involved to maintain enzyme activities through layers. The accumulation and protection of extracellular enzymes in humic complexes, compounds that are known to increase during vermicomposting (Domınguez, 2004), must be also taken into consideration in explaining the maintenance of a high β-glucosidase and cellulase activities found in the older layers of the high rate reactors (Benıtez et al., 2000, 2005; Ceccanti and Garcia, 1994), since in these complexes enzyme are protected from proteolysis (Nannipieri et al., 2002). The β-glucosidase activity curve in layers in low rate reactors was similar to those reported by Benıtez et al. (1999) for vermicomposting of sewage sludge, whereas the pattern of enzyme activity in layers in high rate reactors was more similar to the continuous increase in β-glucosidase activity during vermicomposting of lignocellulosic olive wastes reported by Benıtez et al. (2005); these patterns support the hypothesis of substrate availability.

Phosphomonoesterases catalyse the release of inorganic phosphorus (orthophosphate) from organic phosphomonesters (Alef et al., 1995), and it is known that orthophosphate is an inhibitor of soil phosphatases (Appiah et al., 1985; López-Hernández et al., 1989). The decrease in phosphatase activity might be due to the increase in available P caused by the decrease in microbial biomass. This hypothesis is supported by significant correlations found between microbial biomass and phosphatase activity in both high and low rate reactors. Similar patterns of phosphatase activity, with an initial increase and subsequently decrease was reported by Benıtez et al. (2005) during vermicomposting of a lignocellulosic olive waste with the earthworm *Eisenia andrei*. Phosphatase activity has been shown to be greater in the casts of several earthworm species with different feeding habits, such as *Lumbricus rubellus*, *Aporrectodea caliginosa* and *A. molleri* (Sharpley and Syers, 1976; Aira et al., 2003) but was also lesser in casts of other earthworm species such as *Metaphire guillemi* (Zhang et al., 2000) and *Martiodrilus camariguensis* (Jiménez et al., 2003) and *E. eugeniae* in pig slurry (Aira et al., 2006b).

Protease activity is linked to the N cycle, because it catalyses the hydrolysis of protein N (Paul and Clark, 1996), which can increases the pool of available dissolved organic N (Schimel and Bennet, 2004). This enzyme activity also depended on increased available substrate being high in high rate reactors; the initial increase in reactors containing earthworms may be due to casting, since protease activity was greater in casts of the epigeic earthworms *E. eugeniae* and *Lampito mauritii* than in uningested material (Parthasarathi and Ranganathan, 2000). This pattern of protease activity appears to be characteristic of the vermicomposting process, since is similar to those described by Benıtez et al. (2002) for vermicomposting of sewage sludge with *E. fetida*. Microorganisms seemed to play an important role in shaping patterns of protease enzyme as significant and high correlations between protease activity and microbial biomass revealed. However, enzyme activity in the older layers of the high rate reactors was greater than those of low rate reactors (up to 1.3 times) suggesting some dependence of substrate availability for protease activity.

Earthworms increased microbial biomass during vermicomposting of pig slurry independently of the rate of application of pig slurry. This increase was related with the initial activation of the four enzymes assayed, mainly via intracellular enzyme activity. Substrate availability regulated the activity of enzymes, thus β-glucosidase and alkaline phosphatase showed greater activities in the low rate reactors, whereas cellulase and protease did it in high rate reactors. Moreover, we found strong extracellular enzyme activities which remained active through decomposition process; this extracellular activity increased with rate of pig slurry application indicating substrate availability for extracellular enzyme activity.

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