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E. Benítez · R. Nogales · G. Masciandaro
B. Ceccanti

Isolation by isoelectric focusing of humic-urease complexes from earthworm (*Eisenia fetida*)-processed sewage sludges

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Abstract Vermicomposting is an eco-biotechnological process that transforms energy-rich and complex organic substances into a stabilized humus-like product. In a laboratory experiment, *Eisenia fetida* (Sav.) earthworms were employed to process putrescible sewage sludges into a high-value biofertilizer, very rich in urease activity and humic-urease complexes (stabilized extracellular enzymes). Extracellular humic-urease complexes were extracted by a single 24-h extraction at 37 °C using neutral pyrophosphate (0.1 M); then, the extracts were dialysed and characterized by means of an analytical isoelectric focusing technique. This technique gave a multiplicity of humic bands enzymatically active, with isoelectric points ranging from 4.8 to 5.6. The results demonstrated that, after an 18-week incubation period, sewage sludge had undergone a biochemical evolution, which caused a doubling of absolute urease activity and a six-fold increase in specific activity (activity with reference to the humic C fraction). The biochemical evolution of the vermicompost was evaluated also from the sharp decrease in pyrophosphate-extractable C and water-soluble C. Stabilization of organic C during vermicomposting and the activity of humic-urease complexes expressed at low pH values are of extreme importance when organic wastes are used in acid soils for biochemical restoration purposes.

Key words Sewage sludge · Vermicomposting · Extracellular urease · Humic substance · Isoelectric focusing

E. Benítez (✉) · R. Nogales
Estación Experimental de Zaidín,
Consejo Superior de Investigaciones Científicas (CSIC),
Profesor Albareda 1, P. O. Box 419, 18080 Granada, Spain
e-mail: Emilio-Benitez§eez.csic.es

G. Masciandaro · B. Ceccanti
Istituto per la Chimica del Terreno, Consiglio Nazionale delle Ricerche (CNR), via F. Corridoni 78, 56125 Pisa, Italy

Introduction

Vermicomposting is considered a feasible method for enhancing the value and detoxification of organic wastes, before their use as soil conditioners (Edwards and Neuhauser 1988; Nogales et al. 1999). Products with a relatively high content of humic-like compounds, active microorganisms and enzymes, greatly contribute to the enhancement of the biochemical fertility of soils degraded by intensive-cultivation, pollution or other natural causes (Perucci 1992; Dick 1992; Nannipieri 1994; García et al. 1994). Enzymatically active humic fractions, extracted from vermicompost, have been found to be particularly efficient in mitigating soil salinity and in improving its physical structure when added in fertirrigation solutions, both in laboratory and field experiments (Garcia et al. 1995; Masciandaro et al. 1997). It is known that many extracellular enzymes can become bound to humic matter during a composting or a vermicomposting process, regardless of the type of organic matter used (Ceccanti and Garcia 1994; Ceccanti et al. 1997), but knowledge of the chemical and biochemical properties of such extracellular enzymes is very scanty. The study of these humic-enzyme complexes and the separation of enzymatically active from non-active humic matter can be done through the isoelectric focusing (IEF) technique (De Nobili et al. 1983; Ceccanti et al. 1986, 1989; Garcia et al. 1995). IEF electrophoretically separates amphoteric molecules according to their net surface charge density or isoelectric points (pI); pI is the pH value at which the net surface electrical charge is "zero". Compounds with a comparable molecular weight and electrophoretic mobility, such as those in humic or fulvic fractions, but differing slightly in their electrical surface charge, may be sharply separated by IEF. The surface electrical charge of a humic-enzyme complex is of particular importance when predicting its stability and movement (solubility) in a given physical environment characterized by an amount of electrical charges similar to that existing in many soils. The purpose of this work was the extraction and quan-

tification of humic-urease complexes from vermicomposted sewage sludge and their purification through an analytical IEF technique.

Materials and methods

Sewage sludge

A 50% mixture of two different kinds of sewage sludges, an anaerobically digested sewage sludge produced from a wastewater treatment plant of a paper mill plant and an aerobically digested municipal sludge, was used as a substrate for the vermicomposting process. For the determination of the heavy metals content, the mixture was air dried and crushed to pass through a 2-mm sieve. In accordance with the United States Environmental Protection Agency (1983), HNO_3–$HClO_4$ and diethylenetriaminepentaaceticacid procedures were used for determining total and available heavy metals, respectively. Concentrations of metals are given in Table 1.

Vermicomposting

The mixture of sewage sludges was vermicomposted during an 18-week period. All characteristics of the process have already been described by Benítez et al. (1999). Samples were collected weekly during vermicomposting and stored in plastic vials at 4 °C until both chemical analyses and urease tests were carried out.

Organic C extraction

Water-soluble C (WSC) was extracted from vermicompost with distilled water in a 1:10 solid:liquid ratio by mechanical shaking at 60 °C for 1 h. WSC was analyzed in the supernatant after centrifugation. Pyrophosphate-extractable C (PEC) was extracted at 37 °C for 24 h under shaking, using 100 g organic product suspended in 1 l $Na_2P_4O_7$ (0.1 M, pH 7.1) as extractant. Then, the suspension was centrifuged at 18,000 rpm and filtered through a 0.22-μm Millipore membrane. The extract was dialysed against distilled water and, once dialysed, concentrated to the initial volume by a molecular sieving Amicon PM-10 diaflomembrane (molecular cut-off 10,000) under a N atmosphere (Ceccanti et al. 1989). The extract was used for testing total C and urease activity. A concentration factor of 20 was necessary to load sufficient C and urease on the IEF tubes.

C analysis

The C contents of WSC and PEC were determined by acid digestion with $K_2Cr_2O_7$ and H_2SO_4 at 160 °C for 30 min. A spectrophotometric method was used to quantify the Cr^{3+} produced by the reduction of Cr^{6+} ($\lambda = 590$ nm) (Garcia et al. 1994).

Urease activity

Urease activity in PEC was determined using 0.5 ml dialysed extract, 2 ml of 6.4% urea as substrate and 2 ml phosphate buffer (0.1 M pH 7.0). Controls were run as an enzyme test, but organic extract was added at the end of incubation, before the determination of NH_3. Enzyme tests and controls were incubated at 37 °C for 2 h, then kept at 2 °C for 15 min to stop the reaction. The NH_4^+ released into the solution from the hydrolytic reaction of urea was measured as gaseous NH_3 through a selective electrode (model 95–12; Orion, Cambridge, Mass.).

Table 1 Concentration (mg kg^{-1}) of heavy metals in sewage sludge. *DTPA* Diethylenetriaminepentaaceticacid extractable, *nd* not detected

	Mn	Cu	Zn	Ni	Pb	Cd	Co
Total	1314	417	750	50	139	3.5	4.1
DTPA	306	96	180	13	49	nd	1.8

Isoelectric focusing

IEF was carried out in cylindrical tubes (0.5 × 8 cm) containing polyacrylamide gel (5% w/v) and carrier ampholines at pH 4–6 (BioRad, Richmond, Calif.) at a final concentration of 2% (Ceccanti et al. 1986, 1989). Gel tubes were preferred to slab gel as they permitted the loading of more sample and, at the same time, facilitated the collection of the humic bands for testing urease activity. A densitometric scanning of native humic bands was carried out directly on gel tubes at 460 nm with a Nasatron 821 densitometer (Milan), without any pre-treatment or staining since the focused humic bands were naturally brown. Gel pH was measured at 0.5-cm intervals by using a microelectrode (Gel Pro-pHiler; BioRad). These measurements were recorded by an Orion Ionalyzer 901 microcomputer (Orion).

Urease activity on humic bands

To analyse urease activity of the humic bands obtained by IEF, the gel was gently removed from the inside of the glass tubes and cut into thin sections, each corresponding to a band for analysis. Polyacrylamide sections that contained the humic band were separately incubated to test for enzyme activity as follows:

Two millilitres of phosphate buffer (0.1 M, pH 7.1) was added to the sectioned band. Shaking the band for 2 h at 37 °C was necessary for the removal of ampholines. After shaking, the buffer was decanted and replaced by 2 ml fresh buffer, then 0.5 ml of 6.4% urea was added and the mixture incubated at 37 °C for 2 h. After incubation, the samples were kept at 2 °C for 15 min to stop the reaction. The NH_4^+ released by the hydrolytic reaction was measured by means of an NH_4^+-selective electrode (model 95-12, Orion). Specific urease activity in a humic band was expressed as the ratio between urease activity and organic C present in the band. For each band, C was estimated by relating the recorded area given by densitometric scanning at 460 nm to the total area occupied by all the bands, i.e. the signal intensity of the recorder proportional to the C loaded on the gel.

Statistical analysis

All the results reported are means of three replicates. To test the effect of time on chemical and biochemical parameters, ANOVA was conducted using Statgraphics Plus statistical software (Statistical Graphics, Princeton, N.J.).

Results and discussion

Evolution of extractable organic C

Figure 1 shows the decrease with time in the content of PEC and WSC of sludge. Both PEC and WSC decreased sharply during the first 2 weeks, reaching nearly 30% and 50% of their initial contents, respectively; thereafter, a further 18 weeks was required to further halve their concentration. WSC is considered as an index of mineralization of organic C mainly composed of labile and easily degradable compounds (Garcia et al.

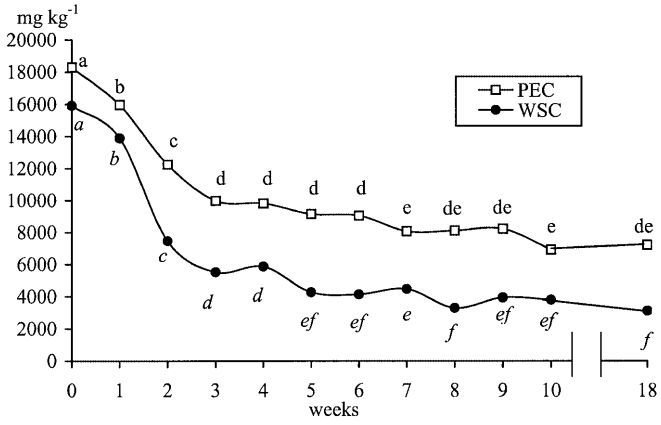

Fig. 1 Pyrophosphate extractable C (*PEC*) and water-soluble C (*WSC*) during sewage sludge vermicomposting. On the *same line*, data with the *same letter* are not significantly different ($P<0.05$)

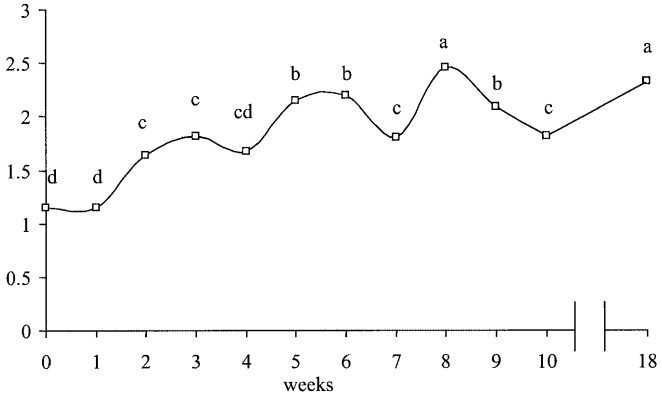

Fig. 2 PEC:WSC ratio during sewage sludge vermicomposting. Data with the *same letter* are not significantly different ($P<0.05$). For abbreviations, see Fig. 1

1994), while PEC was supposed to contain humic-like compounds, synthesized during vermicomposting, and more recalcitrant compounds (structural proteins, fats, polysaccharides, etc.). The evolution of PEC and WSC indicated the extent by which sludges changed their biological composition. The biological changes were better indicated by the fluctuation of the ratio PEC:WSC (Fig. 2). The continuous increase in the ratio during the first 6 weeks of the incubation was due to a faster disappearance of WSC than PEC, while the fluctuation may have been correlated with the innumerable chemical and microbiological processes taking place during the organic matter decomposition. In a previous paper, Benítez et al. (1999) reported that two phases, mineralization and maturation, characterize the sewage sludge vermicomposting process. In the maturation phase the compost is generally characterized by a low level of total (microbial and extracellular) enzymatic activity, reflecting a lack of mineralizable organic compounds, which does not stimulate microbial processes. Although total enzymatic activity is low, extracellular activity may increase during the maturation phase

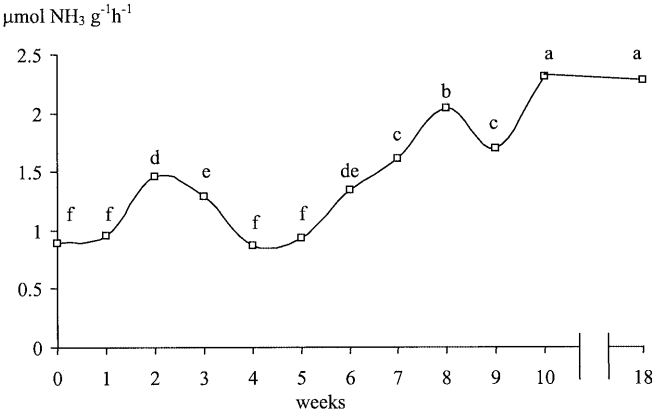

Fig. 3 Extracellular urease activity during sewage sludge vermicomposting. Data with the *same letter* are not significantly different ($P<0.05$)

of composting due to a continuous accumulation of cell-released (extracellular) enzymes onto humic matter, which become stabilized and resistant to physical and/or microbial degradation (Ceccanti and Garcia 1994).

Extracellular humic-urease complexes

The activity of extracellular urease, which was the enzyme measured in the pyrophosphate extract of the vermicompost, increased after 4 weeks (Fig. 3), suggesting its continuous production and accumulation onto humic molecules. Specific urease activity, the enzyme activity with reference to the humic-C fraction, increased as well (Fig. 4), thus confirming that urease accumulated. These data do not agree with those of Ceccanti and Garcia (1994), who, while studying the biochemistry of composting, found that extracellular enzymes in a pyrophosphate extract decreased with time due to the depletion of metabolizable enzyme-inducing substrates, while extracellular specific activity in-

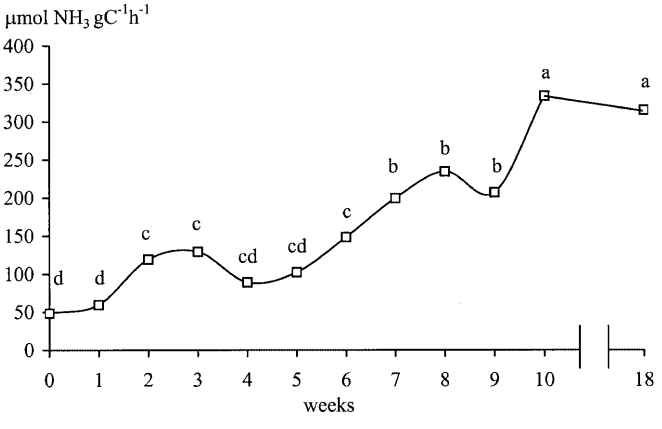

Fig. 4 Specific urease activity during sewage sludge vermicomposting. Data with the *same letter* are not significantly different ($P<0.05$)

creased. The fact that both total and specific urease activity in PEC increased during vermicomposting gave rise to several assumptions:

1. The association of urease with humic substances did not affect the active site of the enzyme.
2. The humic-urease complexes resisted microbial or earthworm attack.
3. The humic-urease complexes were resistant to any inhibitory or degrading effect resulting from either heavy metals or extracellular proteases.
4. Extracellular urease in the vermicompost was more related to the type of humic compound than to the quantity of C in the extract (Garcia et al. 1993)

Fractionation by IEF of humic-urease complexes

The physical and biochemical properties of humic-urease complexes were determined through analytical IEF. The distribution of the humic substances after IEF is reported in Figs. 5 and 6. The humic bands (Fig. 5a) focused between pH or pI 4–5.5; these values were very similar to those found by Garcia et al. (1995) for humic substances and enzymes extracted from composted manure. The poor resolution of the bands (overlapped peaks) was probably due to the nature of sewage sludge, which was rich in pseudo-humic substances and fats, proteins, polysaccharides, etc. (Boyd et al. 1980; Ceccanti et al. 1997).

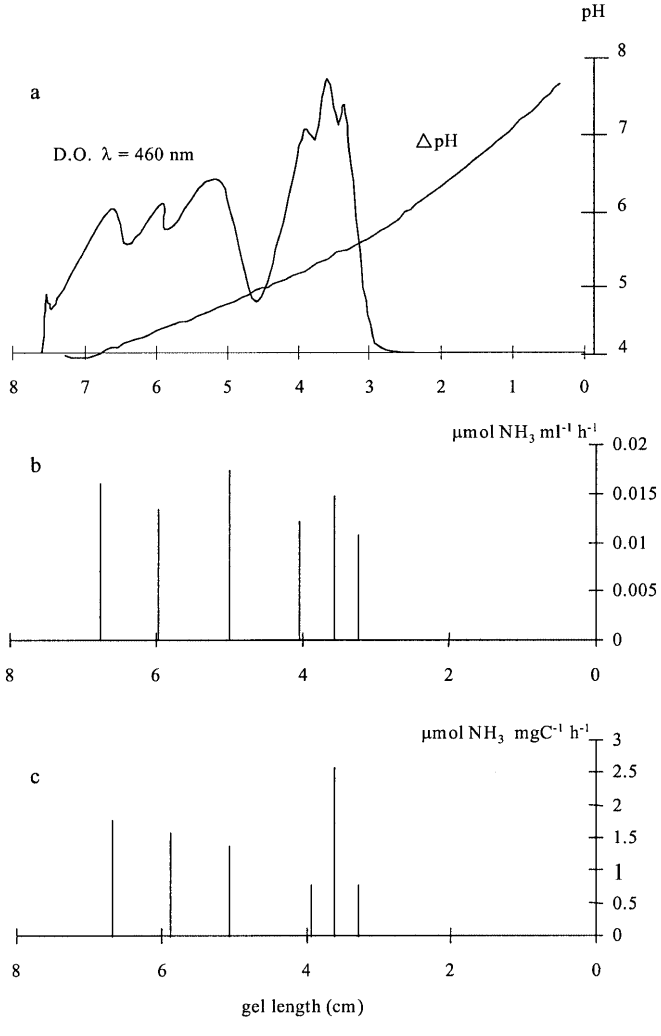

Fig. 5 Isoelectric focusing profile (**a**), absolute urease activity (**b**) and specific urease activity (**c**) of humic substances extracted from sewage sludge

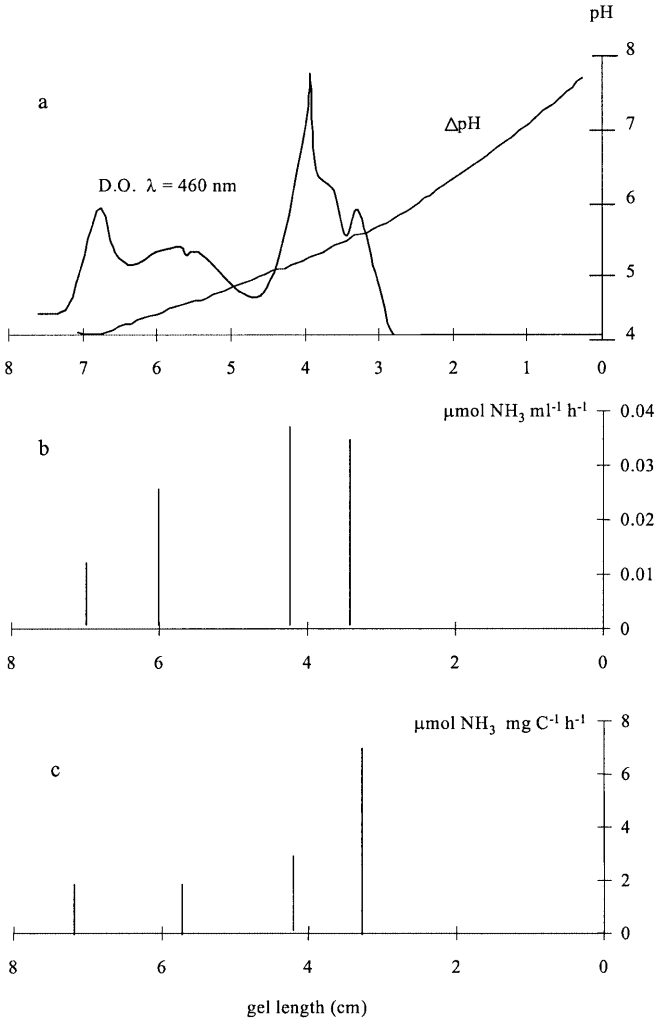

Fig. 6 Isoelectric focusing profile (**a**), absolute urease activity (**b**) and specific urease activity (**c**) of humic substances extracted from vermicomposted sewage sludge

Urease showed heterogeneity of active bands in the IEF profile (Fig. 5b), and coincided with the main peaks of organic matter.

After 18 weeks, the IEF profile showed a redistribution of the humic substances in three well-defined groups of bands that focused at acid pH values (pI < 6; Fig. 6a). This profile seems to be very similar to those reported for humic substances extracted from a variety of soils and composts (Ceccanti et al. 1989; Garcia et al. 1995). The number of humic bands that focused at pI 4–5.5 decreased after 18 weeks of vermicomposting and, consequently, two peaks of urease activity were

lost. The bands that focused at pI 5–5.5 showed an increase by a factor of 3 in absolute activity (Fig. 6b) when compared to those of similar pI obtained before vermicomposting (Fig. 5b). This could mean that the humic-urease complexes had a higher degree of purity (Ceccanti et al. 1989). There was, in fact, an increase in specific enzyme activity in all the bands (activity with reference to humic C) after vermicomposting, except in those that focused at very acid pI (4–4.5), which remained almost unchanged (Fig. 6c).

In conclusion, vermicomposting increased urease activity in the organic extract that appeared to be very stable, and complexed to humic substances possessing very low pIs. The acidic character of these complexes is interesting from both biochemical and ecological points of view, since they are supposed to remain active and chemically stable in soil and water environments. The strong electronegativity shown by the humic-urease complexes under IEF (low pIs) may also decrease the solubility of heavy metals in the environment, functioning like chelating polymers.

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References

Benítez E, Nogales R, Elvira C, Masciandaro G, Ceccanti B (1999) Enzyme activities as indicators of the stabilization of sewage sludges composting by *Eisenia fetida*. Biores Technol 67:297–303

Boyd SA, Sommers LE, Nelson DW (1980) Changes in the humic acid fraction of soil resulting from sludge application. Soil Sci Soc Am J 44:1179–1186

Ceccanti B, Garcia C (1994) Coupled chemical and biochemical methodologies to characterize a composting process and the humic substances. In: Senesi N, Miano TM (eds) Humic substances in the global environment and implications on human health. Elsevier, Amsterdam, pp 1279–1284

Ceccanti B, Alcañiz-Baldellou JM, Gispert-Negrell M, Gassiot-Matas M (1986) Characterization of organic matter from two different soils by pyrolysis-gas chromatography and isoelectric focusing. Soil Sci 142:83–90

Ceccanti B, Bonmati-Pont M, Nannipieri P (1989) Microdetermination of protease activity in humic bands of different sizes after analytical isoelectric focusing. Biol Fertil Soils 7:202–206

Ceccanti B, Garcia C, Nogales R, Benítez E, Masciandaro G (1997) Attività e ruolo delle sostance umiche nell'ambiente: aspetti chimico-structurali e biochimici. In: De Nobili M, Leita L (eds) Dal suolo alla pianta dalla pianta al suolo. Le sostanze umiche come base della sostenibilità. Proc Convegno Nazionale del Capitolo Italiano dell'IHSS, Udine, 13 December 1996, pp 1–9

De Nobili M, Petrussi F, Ceccanti B, Sequi P (1983) Use of the isoelectric focusing technique (IEF) for the characterization of organic substances of different origin and as means of following humification processes. In: Novák B (ed) Transactions of the International Symposium on Humus and Plants, VIII UISGP, Prague,29 August-3 September 1983, vol II. Research Institute of Crop Production, Praque, pp 77–79

Dick RP (1992) A review: long-term effects of agricultural systems on soil biochemical and microbial parameters. Agric Ecosyst Environ 40:25–36

Edwards CA, Neuhaser EF (eds) (1988) Earthworms in waste and environmental management. SPB, The Hague

Garcia C, Hernandez T, Costa F, Ceccanti C, Ganni A (1993) Hydrolases in the organic matter fractions of sewage sludge: changes with composting. Biores Technol 45:47–52

Garcia C, Hernandez T, Costa F, Ceccanti C (1994) Biochemical parameters in soils regenerated by the addition of organic wastes. Waste Manage Res 12:457–466

Garcia C, Ceccanti B, Masciandaro G, Hernandez T (1995) Phosphatase and glucosidase activities in humic substances from animal wastes. Biores Technol 53:79–87

Masciandaro G, Ceccanti B, Garcia C (1997) Soil agroecological management: fertirrigation and vermicompost treatment. Biores Technol 59:199–206

Nannipieri P (1994) The potential use of soil enzymes as indicators of productivity, sustainability and pollution. In: Pankhurst CE. In: Pankhurst CE, Doube BM, Gupta VVSR, Grace PR (eds) Soil biota. Management in sustainable farming systems. CSIRO, Melbourne, pp 238–244

Nogales R, Elvira C, Benítez E, Thompson R, Gómez M (1999) Feasibility of vermicomposting dairy biosolids using a modified system to avoid earthworm mortality. J Environ Sci Health B34:151–169

Perucci P (1992) Enzyme activity and microbial biomass in a field soil amended with municipal refuse. Biol Fertil Soils 14:54–60

United States Environmental Protection Agency (1983) Methods for chemical analysis of water and wastes. EPA-60014-79-020. Cincinnati, Ohio