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Short-term variations in labile organic C, microbial biomass activity and structure after organic amendment of arable soils

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Abstract

Although the application of organic amendments to arable soils is considered to be a suitable tool for improving soil fertility and enhancing carbon (C) stocks, more research is required on the influence of input of organic matter on the activity and structure of the soil's microbial community. The aim of this work was therefore to make a comparative study of the effects of organic materials with different degrees of stabilization and source (an untreated pig slurry, the solid fraction of the digestate from the anaerobic fermentation of pig wastes, a livestock-derived organic matter compost and an urban waste compost) on the size, activity and structure of the microbial community in two arable soils. These effects, studied through a laboratory incubation experiment, were related to the quantity and quality of organic matter added, as well as to the rapid changes in the more labile water-soluble organic matter fraction. Particular attention was devoted to the short-term variations following organic amendment, during which changes in CO_2 emissions, microbial biomass C and water-extractable organic C pools were most pronounced. Phospholipid fatty acid profiles and 16S rDNA sequence analyses evidenced changes in the microbial community structure of amended soils. Modifications of the structure of bacterial communities after amendment, generally involving declining proportions of Gram-positive bacteria (Actinobacteria and Firmicutes) and an increase in abundance of Gram-negative bacteria (Acidobacteria, Bacteriodetes and Proteobacteria), were both quality and quantity dependent, with effects being proportional to the mineralizable organic C content of the added materials.

Keywords: organic amendment, organic C pool, microbial activity, CO_2 emissions.

Introduction

The application of organic materials to soils has gained importance as a consequence of worldwide environmental concerns about contribution of agricultural ecosystem to atmospheric concentrations of greenhouse gases (GHGs), as well as the potential of arable soils to sequester C (Lal, 2004). Land use change together with modern farming practices, intensive cropping, tillage and removal of crop residues generally contribute to enhancing the turnover of soil organic matter (SOM), leading to a decrease in soil C stocks and higher CO_2 emissions (Hutchinson et al., 2007), thus contributing to global warming (Paustian et al., 2000). Moreover, the use of fossil fuels in the production of the large amounts of mineral fertilizers applied to agricultural soils also contributes to GHG emissions (Lal, 2004).

The Intergovernmental Panel on Climate Change provides scientific support on the causes of global warming and identified soil C sequestration as a possible solution for mitigating GHG emissions (Lal, 2004). In this respect, it has been shown that soil amendment with organic-matter-rich materials commonly adopted in agriculture, such as pig slurry, sewage sludge and compost, may increase C stocks (Hutchinson et al., 2007). Such materials are used to increase soil fertility both in terms of a direct supply of plant nutrients thus contributing to the reduction in mineral fertilizers applied, as well as to improve the chemical, physical and biochemical soil properties (Giusquiani et al., 1995). The addition of organic materials to soil strongly influences the size, structure and activity of the microbial biomass as well as its resilience (i.e. the property of a soil system to recover its equilibrium after disturbance), particularly in the short-term immediately after amendment. These processes strongly govern organic C turnover and SOM stabilization, which in turn determine nutrient cycling (Nannipieri et al., 2003; Guerrero et al., 2007). Soil microbial communities utilize different SOM pools as a source of energy and C in a dynamic process (Calbrix et al., 2007), and their activity and growth is generally stimulated by the application of organic materials to the soil (Jedidi et al., 2004). Although the influence of soil amendment on short-term changes in the size and activity of microbial biomass, and turnover of SOM is expected to be determined by the amounts and availability of organic materials added (Blagodatskaya and Kuzyakov, 2008), our knowledge on the importance of these processes is still lacking.

The most labile organic C source supplied with amendment is the water-extractable organic C (WEOC) fraction which is present in variable amounts depending on the degree of stabilization of the organic materials added (Said-Pullicino and Gigliotti, 2007). Moreover, Said-Pullicino et al. (2007a,b) have shown that not only the quantity but also the quality of WEOC changes as a function of organic matter stability with important implications on the labile SOM pool of amended soils. Thus, the effect of soil amendment on microbial activity and related soil processes may strongly depend on the type of stabilization technology the applied materials have been subjected to (e.g. composting, anaerobic digestion). Various authors have shown that the maximum rates of soil respiration resulting from enhanced microbial activity occur in the days immediately following amendment, resulting in strong fluxes of CO_2 to the atmosphere (Bernal et al., 1998; Fangueiro et al., 2007; Bustamante et al., 2010). These changes in microbial activity have been shown to be related to the degree of stabilization (De Neve et al., 2003; Guerrero et al., 2007) and particle size distribution (Fangueiro et al., 2007) of the added materials, as well as the clay content of the receiving soil (Bustamante et al., 2010). However, the extent to which the quantity and composition of added organic matter and its labile fractions affect the microbial community structure is largely unknown. As microbial activity has a strong impact on soil C cycling and nutrient availability, more

knowledge on changes in microbial community structure as a function of organic matter addition is needed for a better understanding of SOM dynamics in amended soils.
Based on these considerations, this work aims at evaluating short-term variations in the quality and quantity of the labile organic C pool, soil microbial biomass and activity, as a consequence of soil amendment. This was achieved by means of a laboratory microcosm involving the amendment of two soils having different properties, with organic materials subjected to different stabilization treatments. Moreover, the influence of exogenous organic matter on the changes in the overall microbial community structure was studied by phospholipid fatty acids (PLFA) and DNA analyses. Phospholipids constitute an important part of all cell membranes, and the PLFA composition can be taken to provide a fingerprint of the microbial community, and also allows for the estimation of fungal and bacterial biomass (Frostegård and Bååth, 1996). Similarly, 16S rDNA analysis using DNA extracted from control and amended soil samples was used to investigate the composition of the two microbial communities.

Materials and Methods

Soils and organic materials

The soil samples utilized in the experiment were collected from the Ap horizon (0-15 cm) of two arable sites typical of Central Italy (Perugia). The first soil (CS) with a silty loam texture, was collected from a Typic Ustifluvent (Soil Survey Staff, 2010a) located in 'Casalina' (42°57′10″N, 12°23′29″E). The second soil (PT) with a silty clay loam texture was collected from a Typic Haplustalf (Soil Survey Staff, 2010b) located in 'Petrignano' (43°05′25″N, 12°31′26″E). After the removal of vegetation and bigger roots, the soils were air dried and ground to <2 mm. The main characteristics of the soils are reported in Table 1. PT soil showed a finer texture, a higher CEC and lower $CaCO_3$ content with respect to the CS soil.
The organic materials used in the experiment included three livestock-derived organic materials and an urban solid waste compost. The livestock-derived organic materials were collected from a waste treatment plant managed by SIA S.p.A. located near Perugia, Italy. This plant receives untreated liquid wastes from neighbouring pig farms and processes them through a two-stage anaerobic digestion for the production of biogas. The resulting digestate is thereafter centrifuged to remove excess moisture, and after appropriate mixing with ligno-cellulosic waste, composted. In the experiment the untreated pig slurry (PSL), the solid fraction of the digestate from the anaerobic fermentation (AAS), and the aerobically stabilized compost (LDC) were used. These differently stabilized materials were also compared with an urban waste compost (UWC) collected from a municipal solid waste treatment plant managed by Gesenu S.p.A., Perugia, Italy. The main characteristics of the organic materials are shown in Table 2.

Incubation

The experimental design consisted of a factorial arrangement with 2 soils (CS and PT), 4 amendments plus an unamended control (PSL, AAS, LDC, UWC and CNT). All organic materials were applied to both soils at a dose equivalent to 340 kg N ha^{-1} (corresponding to 0.15 and 0.17 g N kg^{-1} soil for CS and PT soils, respectively, considering a soil depth of 15 cm). This dose was chosen in accordance with the limits prescribed by the European Nitrates Directive (91/676/EEC) for the protection of groundwater against pollution caused by nitrates from agricultural sources. For each treatment, a series of 12 cylindrical glass jars (250 mL) were filled with 200 g (dry weight) of soil

to allow for six destructive samplings at 0.13, 5, 12, 20, 30 and 45 d of incubation. Another set of samples (2 for each treatment) was prepared for monitoring CO_2 emissions during the incubation. AAS, LDC and UWC were thoroughly mixed with bulk soil samples in their dry form (obtained by freeze-drying and grinding) prior to the preparation of the jars. PLS was distributed directly on the soil surface in the glass jars. The amounts of each organic material added to the soil samples (expressed on a dry weight basis) and the equivalent quantities of C added are shown in Table 2. After amendment, soil samples were incubated (aerobically, non-leached conditions) for 45 days at 25°C and 60% of water holding capacity, to ensure good biomass activation. The water content was adjusted every alternate day to correct for any soil moisture lost through evaporation. After sampling, half of each sample was frozen at -20°C for soil microbial biomass, PLFA and DNA determinations, while the other half was air-dried and ground to <0.5 mm.

CO_2 emissions

During the incubation period, the CO_2 evolved from the amended soils and unamended controls was periodically measured on days 0.13, 1, 2, 4, 5, 7, 12, 18, 25, 32 and 39 after addition of the organic materials. The glass jars were sealed with a greased rubber ring and a lid having an inlet and outlet port. A CO_2-free air supply was provided at the inlet port at a flow rate of 20 mL min^{-1}. After flushing for 15 min, CO_2 in the outlet flow was trapped by bubbling through alkaline traps containing 50 mL of freshly prepared 0.1M NaOH. After 2 h the alkaline traps were removed, immediately sealed with rubber stoppers and then analysed for their inorganic C content using a elemental analyser, TOC-5000A, Shimadzu Corp., Tokyo, Japan. Blank runs (empty jars) were also carried out to correct for any CO_2 trapped in the alkaline solution during its preparation or during the connection of the sampling train. CO_2 fluxes, expressed in mg CO_2-C kg^{-1} soil d^{-1}, were calculated by using the following equation:

$$\mathrm{CO_2\ flux} = \frac{(IC_s - IC_0) \times V \times 24}{M \times t} \quad (1)$$

where IC_s and IC_0 were the concentrations of inorganic C in mg C l^{-1} from the sample and blank runs, respectively; V was the volume in mL of NaOH used; M the dry mass in g of sample in the jars; and t the time in h over which evolved CO_2 was trapped. The emission of CO_2, expressed in mg CO_2-C kg^{-1} soil, was calculated as the product of the mean CO_2 flux and the time between successive measurements, assuming that the emission of CO_2 changed linearly between the nearest sampling dates. The cumulative quantities of CO_2-C evolved during the incubation period were fitted into a double first-order exponential model, typically used to describe the decomposition of different pools of organic matter (Wang et al., 2004):

$$C_t = C_a\left(1 - e^{-k_a t}\right) + C_s\left(1 - e^{-k_s t}\right) \quad (2)$$

In the above reported equation, C_t is the cumulative amount of C mineralized (mg C kg^{-1}) at time t; C_a and C_s are the sizes of the active and slow pools of mineralizable C (mg C kg^{-1}), respectively; while k_a and k_s correspond to the mineralization rate constants (d^{-1}) for each pool.

Water-extractable organic C, microbial biomass and microbial community structure

Water-extractable organic matter (WEOM) was extracted from the dry soil samples with deionized water (solid to water ratio of 1:2 w/w) for 24 h at 200 rpm in a horizontal shaker at room

temperature. The suspensions were then centrifuged at 10,000 rpm for 10 min and filtered through a 0.45 μm membrane filter. The water extracts were analysed for organic C (WEOC). Before determination of organic C, the inorganic C was removed by adjusting the solution to pH 2 with concentrated H_3PO_4 and sparging with CO_2-free synthetic air at a flow rate of 50 mL min^{-1} for 2 min. No flocculation of organic matter was observed on acidification of samples. UV absorption at 254 nm of the water extracts was measured using a Genesys 10 UV/Vis spectrophotometer (Thermo Electron, Waltham, MA) Before measurement, all solutions were diluted to organic C concentrations <50 mg/L. The measured absorbance was normalized to the concentration of dissolved organic C giving the specific UV absorption ($SUVA_{254}$) as an estimate for the content of aromatic structures in the water-extractable organic C (Dilling and Kaiser, 2002).

Soil microbial biomass C content (C_{mic}) was determined using a modified chloroform fumigation-extraction method (Vance et al., 1987). In brief, soluble C was extracted with 40 mL of 0.5 M K_2SO_4 from 10 g wet weight of soil from fumigated and non-fumigated samples by shaking for 1 h on a mechanical shaker at 200 rpm. The samples were then filtered through Whatman No. 42 filter and the organic C determined using a TOC analyser after removal of inorganic C as described above. The C_{mic} was calculated as the difference in soluble organic C expressed on a dry weight basis between the fumigated and non-fumigated extracts, divided by 0.45 (Wu et al., 1990).

In order to verify the effect of the different organic amendments on changes in the soil microbial community structure, PLFA analysis was carried out on soil samples (both CS and PT soils) collected after 20 d of incubation. This sampling time was chosen because at this point the influence of amendment on microbial community structure was expected to be best expressed enabling adequate comparison between treatments. PLFAs were extracted from control and amended soil samples using the procedure described by Bossio et al. (1998) based on the Bligh and Dyer (1959) method. Briefly, soil samples (3 g) were extracted twice with a one-phase extraction mixture containing chloroform:methanol:phosphate buffer (1:2:0.8 v/v/v). After joining the extracts and forcing the separation of phases, the organic phase was removed and evaporated to dryness under a gentle stream of N_2. Phospholipids were separated from neutral and glycolipids on silica (0.5 g) solid phase extraction columns (Phenomenex, USA) and the polar lipid fraction subsequently derivatized into fatty acid methyl esters (FAMEs) by mild alkaline methanolysis. The FAMEs were extracted with two 2 mL aliquots of hexane:chloroform (4:1 v/v), the combined aliquots dried under N_2 at room temperature and redissolved in hexane containing methyl nonadecanoate (19:0) as an internal standard. The FAMEs were separated and quantified by capillary gas chromatography equipped with a flame ionization detector and a 100% dimethyl-polysiloxane non-polar column (50 m, 0.25 mm i.d. and 0.25 µm film thickness). Preliminary peak identification was carried out by comparison of retention times with known standards. The identification of peaks was confirmed by GC-MS analyses on a GC-MS system (Saturn 2100T, Varian, Walnut Creek, CA, USA) equipped with a multiple-ion detector and Saturn Workstation software, using the same column previously described. Mass spectra were generated by electron ionization (electron voltage at 70 eV) and scans were taken at 50-650 amu. The transfer line and injector temperatures were 225 and 260 °C, respectively. The oven was temperature-programmend from 60 °C (2 min) to 300 °C (2 min) at rate of 8 °C min^{-1}. The carrier gas was ultrapure helium at flow rate of 1 mL min^{-1}. The sample volume was 1µL with split injection (1:10). Fatty acids were designated according to the standard notation (total number of carbon atoms: number of double bonds, followed by the position of the double bond from the methyl end of the molecule) as described by Peacock et al. (2001). The fatty acids

i15:0, a15:0, 15:0, i16:0, 16:1ω7c, i17:0, a17:0, cy17, 17:0 and 18:1ω9c were chosen to represent bacterial biomass while 18:2ω6 was taken to represent fungal biomass (Frostegård and Bååth, 1996). PLFAs were also subdivided into groups possibly indicative of Gram-positive bacteria (i14:0, i15:0, a15:0, i16:0, i17:0, a17:0, br18:0), Gram-negative bacteria (cy17, 16:1ω7c, 18:1ω9c) and Actinomycetes (White et al., 1996; Bossio et al., 1998; Zelles, 1999).

A 16S rDNA sequence analysis approach was adopted on CNT and UWC of the CS soil collected after 20 days of incubation, in order to provide a more detailed insight on the influence of organic amendment on changes in microbial community structure. DNA analysis was carried out on a single amended sample, which showed evidenced variations in their PLFA profiles with respect to the unamended controls. Soil samples were extracted using the Powersoil™ DNA isolation kit (MoBIO, Carlsbad, CA) following the manufacturer's instructions. Because of its specific characteristics the kit allows an efficient DNA extraction from various organisms (i.e. bacteria, fungi, algae and Actinomycetes). DNA concentration was assessed through optical density reading (DU650 spectrophotometer, Beckman) and confirmed by agarose gel electrophoresis. Bacterial 16S rDNA from CNT and UWC extracts was amplified using the universal primers 27f (5'-agagtttgatcatggctcag-3' at nucleotide positions 8–27 of the *Escherichia coli* numbering) and 1492r (5'-ggttaccttgttacgactt-3' at positions 1510–1492).[29] PCR reactions were performed in a 50 µl volume containing 20 ng template DNA, 1x PCR buffer (Invitrogen), 200 µM of dNTPs, 20 µg of Bovine Serum Albumin (BSA), 10 pMol of each primer and 1.75 U Taq DNA polymerase (Invitrogen). Reactions were performed using the following cycling parameters: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 63°C for 30 s and 72°C for 1 min with a final step of 72°C for 10 min. Ten µL of PCR products were electrophoresed on 1.5 % (w/v) agarose gel and visualized by ethidium bromide staining. Two µL of each amplification product were ligated into the TOPO TA Cloning® vector (Invitrogen) following the manufacturer's instructions. The ligated products were transformed into *E. coli* TOP10 One Shot® Chemically Competent cells generating two clone libraries. For each library eighty colonies, grown over night on nutrient agar medium supplemented with 50 µg mL^{-1} of Kanamycin, were randomly chosen. Plasmids were than purified from sixty positive colonies for each sample, sequenced using Big Dye Terminator v3.1 chemistry (Applied Biosystems) and run on a ABI 3130xl DNA Analyzer. Obtained sequences were analyzed using *Classifier* from the Ribosomal Database Project to assess their phylogenetic affiliations (Wang et al., 2007). The *Classifier* automatically estimates the classification reliability using bootstrapping and allows classification of both bacterial and archaeal 16S rRNA sequences. The two libraries were also analyzed using the library compare tool (Wang et al., 2007). Accumulation curves were produced using bootstrap richness estimator (mean among runs) for both control and amended samples. Standard deviations among randomizations of sample order were also calculated.

Statistical analysis

Three-way ANOVA was used to compare the CO_2 cumulative fluxes, WEOC and microbial biomass C as a function of organic amendments, incubation time and soil type. Two-way ANOVA was conducted for the comparison of PLFA profiles (mole percents of bacteria, Gram-positive, Gram-negative, fungi and Actinomycetes) as a function of amendments and soil type. Homogeneity of variances was verified by graphical analysis of residuals, while all significant effects were assessed by means of the Tukey's Honest Significant Difference (HSD) test at $P = 0.05$ for

comparison of means for C biomass and PLFA profiles (mole percents of individual or grouped fatty acids). The analyses of variance assumes that the variances of the design are identical.
Factor analysis was used on PLFA data to reduce the original set of variables into a smaller set of non-correlated factorial components to allow evaluation of changes in community structure. The factorial loadings of the data were analysed after application of varimax normalized rotation and two principal components extracted. An ANOVA was performed on the factor scores and Tukey's HSD at $P = 0.05$ was used to identify significant differences between treatments.

Results

CO_2 emissions

Variations in CO_2 fluxes with time after the application of organic materials to both soils are reported in Fig. 1 (inlays). Whereas control soils showed relatively constant emission rates throughout the incubation period, the addition of organic materials generally resulted in greater CO_2 fluxes in both soils with respect to unamended controls, particularly in the first days following amendment. Among the soil samples treated with livestock-derived organic materials (i.e. PSL, AAS and LDC), highest emission rates were observed for soils treated with PSL within 1 d after amendment (36.3 and 58.7 mg C kg^{-1} d^{-1} for CS and PT soils, respectively). With time CO_2 fluxes tended to decrease steadily reaching relatively constant values similar to those obtained for unamended controls within 10 d. Peak CO_2 emission in AAS treated soils were slightly delayed with respect to PSL treated soils. In fact, maximum fluxes were observed after 5 d for CS (27.9 mg C kg^{-1} d^{-1}), and after 2 days for PT (40.2 mg C kg^{-1} d^{-1}). Successively, emissions decreased to values similar to those obtained for the controls after about 32 and 18 d for CS and PT, respectively. In contrast, LDC treated soils did not show any significant differences in emission rates with respect to the unamended controls throughout the incubation period in CS and PT. Highest rates of CO_2 emissions were obtained for both UWC treated soils. In fact, the addition of UWC resulted in peak emissions within 2 days from amendment (maximum fluxes of 67.4 and 84.0 mg C kg^{-1} d^{-1} for CS and PT, respectively) which thereafter decreased steadily with time. Nevertheless, even after 40 d of incubation the CO_2 fluxes from UWC treated samples were significantly higher than those obtained for the unamended controls ($P < 0.01$) in both soils.
Fig. 1 also shows the cumulative emissions of CO_2 after the application of organic materials to the two soils and comparison to the unamended controls. For both soils cumulative CO_2 emissions at the end of the incubation period increased in the order CNT<LDC<PSL<AAS<UWC. In the PT soils the cumulative emissions were significantly higher than in CS soils ($P < 0.01$), with a total emission of CO_2 that was 22% higher in the former after 39 d of incubation. The double exponential model (Eq. 2) fit the cumulative C mineralization data adequately with a regression coefficient of $R^2_{adj} > 0.998$ for all data series. The estimated C mineralization parameters for the two amended soils are reported in Table 3. The mean mineralization rate constants for the active (k_a) and slow (k_s) organic matter pools in amended soils at 25°C and under optimal moisture conditions were 0.094 and 0.005 d^{-1}, respectively. Estimates for the pool sizes revealed that the active pool (C_a) represented approximately 1 and 5% of the total organic C for amended CS and PT soils, respectively. On the other hand, the slow pool (C_s) represented 8 and 30% of total C for amended CS and PT soils, respectively. In both soils, size and rate constants of the active pool were strongly influenced by amendment. For both the soils amended with livestock-derived organic materials, C_a decreased in the order PSL>AAS>LDC, whereas UWC amended soils showed a varying pool size

in the two soils. However, in both cases treatment with UWC resulted in the greatest values for k_a. Since application doses were based on the N content of the organic materials, the application of organic materials having different C content consequently resulted in a different input of organic C. For this reason C_a values were also normalized to the organic C added with each treatment (Table 3). The results obtained suggest that on average the C_a pool represents about 34% of the C added to both soils with PSL, whereas this value decreased to about 20% for soils treated with AAS and 11% for soil treated with LDC or UWC.

Water-extractable organic C

The application of organic materials to the two soils significantly enhanced the concentrations of WEOC with respect to the unamended controls, particularly in the days immediately after organic materials application (Fig. 2). Initial WEOC concentrations in amended soils were 1.2-2.8 times greater than control soils in CS and 1.2-2.3 in PT, increasing in the order PSL≈AAS<LDC<UWC for both soils. Three-way ANOVA showed for both soils changes in the concentration of WEOC over time and differences between treatments were significant ($P < 0.01$). In fact PSL, LDC and UWC in both amended soils showed a decreasing trend in WEOC concentrations with incubation time, whereas AAS amended soils showed an initial increase during the first 20 d of incubation followed by a gradual decrease thereafter. By the end of the incubation period soils amended with PSL had WEOC contents that were significantly different from the unamended control ($P < 0.05$) in both soils, as well as AAS, LDC and UWC treated soils still showed significantly higher concentrations ($P < 0.05$). No significant differences in WEOC concentration were found between the two tested soils. Values for $SUVA_{254}$ also evidenced changes in the quality of WEOC as a result of organic amendment, particularly for PT soil. In fact, amendment of PT with PSL, AAS, LDC and UWC resulted in higher mean $SUVA_{254}$ values (0.85, 0.98, 1.23 and 1.00 L mg^{-1} m^{-1}, respectively) with respect to the unamended control (0.70 L mg^{-1} m^{-1}). In contrast, only amendment with LDC resulted in higher $SUVA_{254}$ values for CS soil (1.63 and 1.09 L mg^{-1} m^{-1} for amended and unamended soils, respectively) while all other treatments had values similar to the unamended control. Moreover, the $SUVA_{254}$ in CS soil was significantly higher ($P < 0.01$) than in PT soil during all the experiment.

Microbial biomass C and community structure

Fig. 3 shows the variations in microbial biomass C (C_{mic}) with time after organic amendment of the two soils under investigation. Three-way ANOVA showed that for both soils, changes in C_{mic} over time, differences between treatments, and the interaction between the three factors were significant ($P < 0.01$). Values of C_{mic} for the two unamended controls were generally constant over time with mean values of 147 and 195 mg C kg^{-1} for CS and PT soils, respectively. Organic amendment generally enhanced C_{mic} values during the incubation period. The application of UWC to both soils resulted in the greatest increase in C_{mic} values with respect to unamended controls ($P < 0.01$), with highest values obtained after 12 and 20 d of incubation for CS and PT soils, respectively. The application of livestock-derived organic matter to CS soil also resulted in an increase in C_{mic} and significant differences were generally observed among treatments. In the PT soil the C_{mic} throughout all the experiment was significantly ($P < 0.01$) higher than in the CS soil, for CNT and treated plots.

The PLFA approach was used to study if the different kind of organic materials added with each treatment led to changes in the microbial community structure with respect to unamended soils. PLFA analysis identified a suite of fatty acids of which, only 20 comprised >1% of the total PLFA in any given treatment. The types of fatty acids identified included normal saturated, terminal (iso- and anteiso) branched saturated, mid-chain branched saturated, monounsaturated, cyclopropyl and polyunsaturated fatty acids. Total PLFA contents in the different soil samples correlated well with the C_{mic} values ($r = 0.8525$; $P < 0.01$) and tended to increase as a result of amendment by 1.1-1.9 fold and 1.4-2.4 fold with respect to unamended controls for CS and PT soils, respectively. Figure 4 reports the influence of organic amendment and soil type on the distribution of PLFAs among the different functional groups. Organic amendment generally resulted in a relative decrease in the percentages of mid-chain branched PLFAs with respect to unamended controls, and a slight increase in total monounsaturated PLFAs particularly for PT soils. No significant differences were observed in the relative contents of normal and terminal branched saturated PLFAs for soils treated with livestock-derived organic materials, whereas addition of UWC lead to a significant increase in the former and decrease in the latter with respect to the unamended controls. For both soils, organic amendment had no effect on the relative contribution of cyclopropyl and polyunsaturated PLFAs.

Factor analysis made with the total PLFA content, and mole percents of 20 individual PLFAs (abundance > 1%) provided two factors that accounted for 41 and 17% of the variance in the data. A plot of the first and second factors for all treatments and both soils is shown in Fig. 5. Moreover, factor analysis identified PLFAs that were highly correlated (loading >|0.7|) in describing the similarities and differences between treatment PLFA profiles. The increase of Factor 1 indicated a great presence of the PLFAs i15:0, i17:0, a17:0, br18:0 and 10Me18:0 principally indicative of Gram-positive bacteria, whereas negative contributors included total PLFA content, the more ubiquitous *n*-saturated PLFAs (particularly 14:0, 16:0 and 18:0) and 18:1ω9c indicative of Gram-negative bacteria. On the other hand, Factor 2 was most strongly influenced by the PLFA 16:1ω7c also indicative of Gram-negative bacteria and the fungal biomarker 18:2ω6c. Points plotted in the principal component factor space (Fig. 5), suggest that among samples treated with livestock-derived organic materials, PSL and AAS amended soils showed significant changes in their PLFA profiles with respect to unamended controls as a result of the contribution of Factor 2 for both soils, and Factor 1 for PT soil only. In fact, both soils amendment with PSL and AAS evidenced a consistent increase in Factor 2. Moreover, Fig. 5 also showed a clear segregation of the soils amended with UWC relative to all other treatments, primarily as a result of the contribution of Factor 1. In contrast, LDC amendment did not show consistent changes in their PLFA profiles with respect to unamended controls, indicating that this treatment did not result in significant differentiation of the microbial community.

Further insight into the important changes in microbial community structure resulting from UWC addition was provided by DNA analyses. A total of 104 16S rDNA clones (50 from the CNT and 54 from the UWC libraries) were successfully sequenced. Using the *Classifier* tool it was possible to obtain a phylogenetic affiliation with a confidence threshold of 80% for 88 16S rDNA sequences (84.6 %; Fig. 6). In particular, more than 75% of the analyzed sequences (76% for the control soil and 81.4% for the treated one) belonged to three major Phyla: Bacteroidetes, Acidobacteria and Proteobacteria. For all these clones the Subphylum was also assessed. All the Bacteroidetes sequences belonged to the Subphylum Sphyngobacteria (with just one exception); Acidobacteria sequences were classified into 3 Subphyla (Group 4, 6 and 7); Proteobacteria sequences were

almost equally distributed into the α, β, γ and δ Subphyla (Fig. 6). The remaining sequences belonged to Actinobacteria, Chloroflexi, Firmicutes, Nitrospora, Planctomycetes and Verrucomicrobia Phyla. It is worth nothing that whereas Bacteroidetes, Acidobacteria, Proteobacteria, Actinobacteria, Chloroflexi and Firmicutes were found in both CNT and UWC soil samples, the others were specific of either CNT (Nitrospora and Planctomycetes) or UWC (Verrucomicrobia) soil samples (Fig.6).

Discussion

Influence of organic amendment on labile C sources and microbial activity

The increase in soil microbial respiration after organic amendment of arable soils is expected to depend on both the quantity and quality of organic matter added, as well as intrinsic soil properties such as the clay content of the receiving soils (De Neve et al., 2003; Bustamante et al., 2010). The former inevitably depend on the source and composition of the organic materials being added to the soil. Application of organic amendments to the two soils generally resulted in an increase in WEOC concentrations, microbial biomass C and CO_2 emissions during incubation, the extent of which differed among organic materials.

The application of differently stabilized livestock-derived organic amendments to the two soils led an increase in microbial respiration, particularly in the first days after their application. This increase in the microbial activity may have been due to a significant input of labile organic matter contained in the livestock-derived organic materials, a source of C and energy for soil microorganisms (Said-Pullicino and Gigliotti, 2007). This behaviour was observed particularly in soils amended with PSL in which the easy degradable organic constituents added with PSL led to an increase in CO_2 emissions after one day from the treatment, particularly in PT amended soil. In fact, amended and unamended PT soils showed greater CO_2 fluxes than CS suggesting that soil properties could have also influenced microbial activity. The different $CaCO_3$ contents of these two soils (Table 1) may suggest that Ca^{2+} bridging could have partially stabilized the labile organic constituents added to CS thus reducing their availability for microbial degradation (lower C_a pool) and resulting in a lower respiration rates.

The lower initial rates of respiration observed for AAS treated soils with respect to those receiving PSL was probably due to the removal of easily biodegradable C fractions from pig slurries during their anaerobic fermentation. Similarly, further stabilization of the anaerobic digestate during the aerobic composting process could explain the lower initial rates of respiration observed for LDC treated soils with respect those receiving PSL and AAS. These variations in respiration rates with increasing stability of the livestock-derived organic amendments was in line with the decreasing proportion of added C in the active, potentially mineralizable pool (i.e. C_a parameter of the double first order model) and increasing aromaticity of the soluble C fraction. Stability-dependent respiration rates were reported by various authors for soils amended with composted organic materials (e.g. Bernal et al., 1998; Sánchez-Monedero et al., 2004; Guerrero et al., 2007; Bustamante et al., 2010), most of which also observed peak CO_2 emissions in the first few days following amendment with an intensity related to the contents of water-extractable and microbial biomass C. In fact, it is well known that organic amendment can change amount and quality of dissolved organic matter present in the soil solution (Chantigny, 2003), and being an easily available organic matter fraction for soil microorganisms, has important implications on microbial activity and soil respiration (Marschner et al., 2003). Moreover, Said-Pullicino et al. (2007a) have

shown that the soluble C fraction of organic amendments tends to decrease with organic matter stabilization.

Although amendment of soils with AAS resulted in a relatively lower addition of potentially mineralizable C with respect to PSL, cumulative CO_2 emissions over the whole incubation resulted in greater values for the former in both soils. This was attributable to higher respiration rates in the later stages of the incubation period, probably due to the greater contribution of AAS to the C pool having a slower turnover, as evidenced by the greater C_s parameter of the double first order model. Although anaerobic degradation of organic matter results in the loss of labile organic substrates, the digestate may be enriched in more complex ligno-cellulosic constituents, due to their relative recalcitrance under anaerobic conditions. Nonetheless, these compounds may be subject to aerobic decomposition following soil amendment, possibly also leading to the release of soluble organic constituents. The delayed peak in CO_2 emissions, the important contribution to the C pool having a slower turnover, as well as the initial increase in WEOC concentrations with incubation time after application of AAS to the two soils lend support to this inference.

When compared to the other organic amendments, addition of UWC to both soils resulted in the greatest increase in WEOC contents, microbial biomass C and CO_2 emissions with respect to the unamended controls, even though this material was previously stabilized through a composting process. In fact, the wider C/N ratio of UWC warranted a quantitatively greater input of organic C to the soils with respect to the other organic amendments. Nevertheless, although UWC amended soils received nearly 2-fold more C than LDC treated soils, the proportion of added C in the active pool was comparable for both composts. These results highlight the dual role quantity and quality of added organic matter have on soil microbial activity and, consequently, on CO_2 emissions.

With regard to the quality of WEOC, the $SUVA_{254}$ values showed differences between the two soils. The higher $SUVA_{254}$ values observed in CS than in PT soils (in all amended and unamended) might be related to the WEOM mineralization that seemed to be lowest in the first one as shown by CO_2 cumulate flux data. This phenomena was also demonstrated by Kalbitz et al. (2003), who stated that the biodegradation is closely related to chemical WEOM properties, obtaining significant correlations between SUVA and the rate of WEOC mineralization.

The higher potential adsorption capability of PT soil with respect to CS soil is testify by the higher CEC, indirect index of adsorption capacity of a soil, that in PT soil was the double with respect of CS soil.

Changes in the soil microbial biomass after amendment

It is widely accepted that soil management options such as the application of organic materials to enhance soil fertility, can significantly impact soil biological and biochemical properties. Since in most mineral soils, the activity and growth of the soil microbial biomass is limited by the availability of substrate, organic amendment is a key factor that governs the size and activity of the microbial biomass. In fact, in both soils studied the introduction of labile organic matter with amendment was immediately accompanied by an increase in microbial biomass C and total PLFA content. This was particularly true for PT soils that generally showed greater microbial biomass C contents and activity with respect to CS, possibly due to a combination of factors including the intrinsic microbial community structure and activity, as well as mechanisms involved in the stabilization of the added organic matter (e.g. cation bridging). Stimulation of microbial growth after organic amendment has been reported during long-term experiments (Marschner et al., 2003;

Toyota and Kuninaga, 2006; Bastida et al., 2008) as well as in short-term microcosm experiments (Saison et al., 2006; Calbrix et al., 2007; Plaza et al., 2007). Kuzyakov and Bol (2006) demonstrated that after the addition of an easily available substrate to the soil, the most active part of the microbial community benefits first from the added substrate (microbial activation) and, if the substrate amount is sufficient, the microbial biomass strongly increases. However, our results have also shown that entry of labile organic matter into the soil with amendment did not only result in an increase in microbial biomass and activity, but also generally led to a rapid shift in microbial community structure within 20 d from amendment in both soils. Saison et al. (2006) also reported rapid changes in community structure within 4 d after amendment. They also suggest that microorganisms present in the organic materials added to soil are rapidly outcompeted by soil-derived microorganisms and therefore only marginally influence changes in community structure.
PLFA profiles of treated and control soils have evidenced a significant increase in all bacterial biomarkers after organic amendment with a generally lower relative proportion of Gram-positive and a higher relative proportion of Gram-negative bacteria (Table 4). This behaviour was observed in both soils, demonstrating the relative influence of soil characteristics in microbial community structure. Also the relative proportion of biomarkers attributable to Actinomycetes tended to decrease after amendment, while fungal biomarkers tended to increase with the introduction of organic matter (Table 4). These results are in line with the findings of Kramer and Gleixner (2008) that have shown that Gram-negative bacteria are better adapted to grow on recently added, easily degradable C sources with respect to Gram-positive bacteria that are able to use more complex C sources. Similarly, Griffiths et al. (1999) reported declining proportions of Gram-positive bacteria and Actinomycetes, and increasing proportions of Gram-negative bacteria with increasing substrate loadings. A similar shift in microbial community structure was most evident in both UWC treated soils; these soils treated with UWC received the greatest amount of exogenous organic C, however having the lowest degradability (i.e. lowest proportion of added organic C in the active pool) with respect to the other organic amendments. DNA analysis, carried out on two selected samples confirmed that it is possible to investigate the changes in microbial community composition previously observed by PLFA analysis. Although a single comparison between a control and amended soil (CNT and UWC) was carried out for the DNA analysis, our preliminary data based on 104 sequences showed that Bacteroidetes, Acidobacteria and Protobacteria were the most abundant classes. Moreover, our data suggest that amendment addition slightly influenced the relative abundance of microbial classes (Fig. 6). Despite being slight, we observed an increase of Gram-negative Bacteroidetes and Acidobacteria and a decrease of Gram-positive Actinobacteria, Chloroflexi and Firmicutes after amendment. For less abundant classes (i.e. Nitrospira and Planctomycetes), the absence of sequences in the amended soil sample could be due to the relative low amount of sequences produced. However, the accumulation curves obtained indicated that, using our applied scheme, the observed richness was not a marked underestimation of the actual one. In fact, as is clear from the Fig. 6 B and C, the curves approach the asymptote. Therefore, it was demonstrated that the DNA analysis carried out only on a single samples (CNT and UWC) was a valuable method to evidence differences on the microbial community structure in a laboratory incubation experiment.These findings indicate that short-term changes in the structure of the microbial community can be induced by quantitatively increasing organic matter input, and are not only governed by the quality of the available substrate. In fact, treatment of soils with LDC having a degradability similar to the organic matter added with UWC (≈ 11% of added C in the active

pool), but resulting in the addition of a similar amount of exogenous C as PSL and AAS treated soils, showed small changes in the microbial community structure or activity with respect to the unamended controls in both soils studied. One plausible explanation could be that short-term microbial community shifts following the input of important quantities of organic matter could be primarily controlled by the addition of labile constituents as well as N availability. In fact, the greatest shifts in PLFA profiles were obtained for UWC treated soils that also had the greatest values of WEOC. N is often a key limiting factor for soil organisms and addition of available forms can change microbial biomass, activity and species composition (Sarathchandra et al., 2001). In the present study, the addition of PSL did not contribute large amounts of soluble C, but its elevated inorganic N content suggests that N availability could have been responsible of the shift in community structure. Calbrix et al. (2007) showed that in organically amended soils, the modification of the structure of bacterial communities was quality dependent, with the effects being proportional to the mineralizable organic C content of the added materials. However, in this work different treatments resulted in similar organic C additions, probably limiting the appreciation of dose-dependent modifications of the community structure. In another study, Saison et al. (2006) observed a positive and dose-dependent effect of compost amendment on microbial activity and community structure.

Whereas the bacterial community structure was influenced by the addition of labile organic constituents and N availability, a different factor was probably responsible for the observed trend in the fungal component. In fact, results evidenced that although all organic amendments generally enhanced the relative proportion of fungal biomarkers, addition of AAS resulted in the greatest positive shift in the fungal biomass with respect to unamended controls (Table 4). Griffiths et al. (1999) showed a faster increase in fungal biomass with respect to bacteria after addition of substrate, particularly at higher substrate loadings. Moreover, the stimulation of fungi after the addition of organic matter poor in N (wide C/N ratio) is well known (Bastida et al., 2008). However, these do not reasonably explain the higher contribution of fungal biomass in AAS treated soils with respect to the other organic amendments, since AAS neither resulted in the greatest addition of soluble organic constituents or active C pool, nor had the highest C/N ratio. AAS is particular in that this organic material was subjected to an anaerobic stabilization process, possibly resulting in a relative enrichment in the lignin content due to the lower degradation efficiency under anoxic conditions. Subsequent application of AAS to CS soil could have stimulated fungal growth as these organisms, amongst which white-rot Basidiomycetes, are known to be the most efficient lignin degraders (Martinez et al., 2005), thus resulting in the lowest bacteria:fungi ratios with respect to the other amended and unamended soils. The increasing trends in WEOC concentrations of AAS treated soils in the first weeks of incubation, suggesting a faster release of soluble constituents with respect to their mineralization, could also be attributed to a bacterial to fungal microorganism succession favouring the decomposition of a lignin-rich residue in an aerobic environment (Said-Pullicino et al., 2007b).

In conclusion, the application of organic amendments to arable soils may have a strong influence on the short-term variations, particularly evidenced in microbial activity and structure. Therefore, it is important to know how the microbial community is involved after different organic amendments, because it may have important implications on organic matter turnover and subsequent release of plant available nutrients. Results obtained suggest that the degree of stability of the organic

materials, is expected to govern soil microbial processes in the short-term, influencing over time several ecological functions.

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Table 1: Characteristics of the soils used in the experiment.

	Casalina (CS)	Petrignano (PT)
Total organic C (g kg^{-1})	9.6	10.5
Total N (g kg^{-1})	1.1	1.1
C/N ratio	8.7	9.5
pH (H_2O)	8.3	8.4
CEC† ($cmol_{(+)}\ kg^{-1}$)	13.0	26.0
$CaCO_3$ (g kg^{-1})	20.8	2.9
Bulk density (g cm^{-3})	1.55	1.31
Field capacity (% w/w)	24.4	30.0
Soil texture		
% sand	22	13
% silt	52	48
% clay	26	39

† CEC, cation exchange capacity

Table 2: Main characteristics of the different organic materials utilized and their respective application doses.

Parameter		PSL	AAS	LDC	UWC
Total organic C (g kg^{-1})		345 ± 32	342 ± 4	318 ± 10	327 ± 0
Total N (g kg^{-1})		44.2 ± 6.7	29.0 ± 0.2	31.8 ± 1.4	20.1 ± 0.3
C/N ratio		7.8	11.8	10.0	16.3
Ammonium-N (g kg^{-1})		16.8	7.7	6.3	<0.5
Total P (g kg^{-1})		21.6 ± 0.0	23.9 ± 0.0	27.1 ± 0.1	4.7 ± 0.0
Application rates					
to soil CS	(g kg^{-1} soil)	3.3	4.4	4.7	8.2
	(g C kg^{-1} soil)	1.1	1.5	1.5	2.7
to soil PT	(g kg^{-1} soil)	3.9	5.1	5.4	9.8
	(g C kg^{-1} soil)	1.3	1.8	1.7	3.2

All data are expressed on a dry weight basis. PSL, pig slurry; AAS, anaerobic sludge; LDC, livestock-derived compost; UWC, urban waste compost.

Table 3: Estimated C mineralization parameters of the double first order model.

	C_a (mg C kg^{-1})	k_a (d^{-1})	C_s (mg C kg^{-1})	k_s (d^{-1})	C_a (% added C)
Soil CS	145	0.039	965	0.005	–
+ PSL	373	0.079	1487	0.002	33.2
+ AAS	301	0.056	2007	0.005	19.8
+ LDC	164	0.081	1090	0.005	11.0
+ UWC	183	0.211	1391	0.013	6.8
Soil PT	201	0.007	1401	0.007	–
+ PSL	399	0.089	2069	0.001	35.6
+ AAS	339	0.073	2263	0.004	19.4
+ LDC	234	0.039	1562	0.005	13.8
+ UWC	442	0.124	2945	0.004	13.8

C_a and C_s, active and slow pool sizes respectively; k_a and k_s mineralisation rate constants for each pool. PSL, pig slurry; AAS, anaerobic sludge; LDC, livestock-derived compost; UWC, urban waste compost.

Table 4: Proportion of selected PLFAs (mol%) indicative of notional microbial groups (see text), extracted from amended and unamended soils.

	Bacteria	*Gram +ve*	*Gram –ve*	*Fungi*	*Actinomycetes*
Soil CS	46.9	31.6	17.4	2.7	12.5
+ PSL	48.8	31.0	18.7	3.3	10.7
+ AAS	48.3	30.9	18.7	5.3	9.3
+ LDC	48.3	33.9	17.4	1.9	11.5
+ UWC	46.3	25.4	22.0	2.6	9.1
Soil PT	45.9	34.7	15.8	1.7	12.1
+ PSL	47.6	33.5	18.1	2.7	10.4
+ AAS	47.4	33.1	16.9	3.5	10.2
+ LDC	47.4	33.5	17.5	3.4	10.9
+ UWC	46.0	27.6	20.2	3.0	7.8

PSL, pig slurry; AAS, anaerobic sludge; LDC, livestock-derived compost; UWC, urban waste compost.

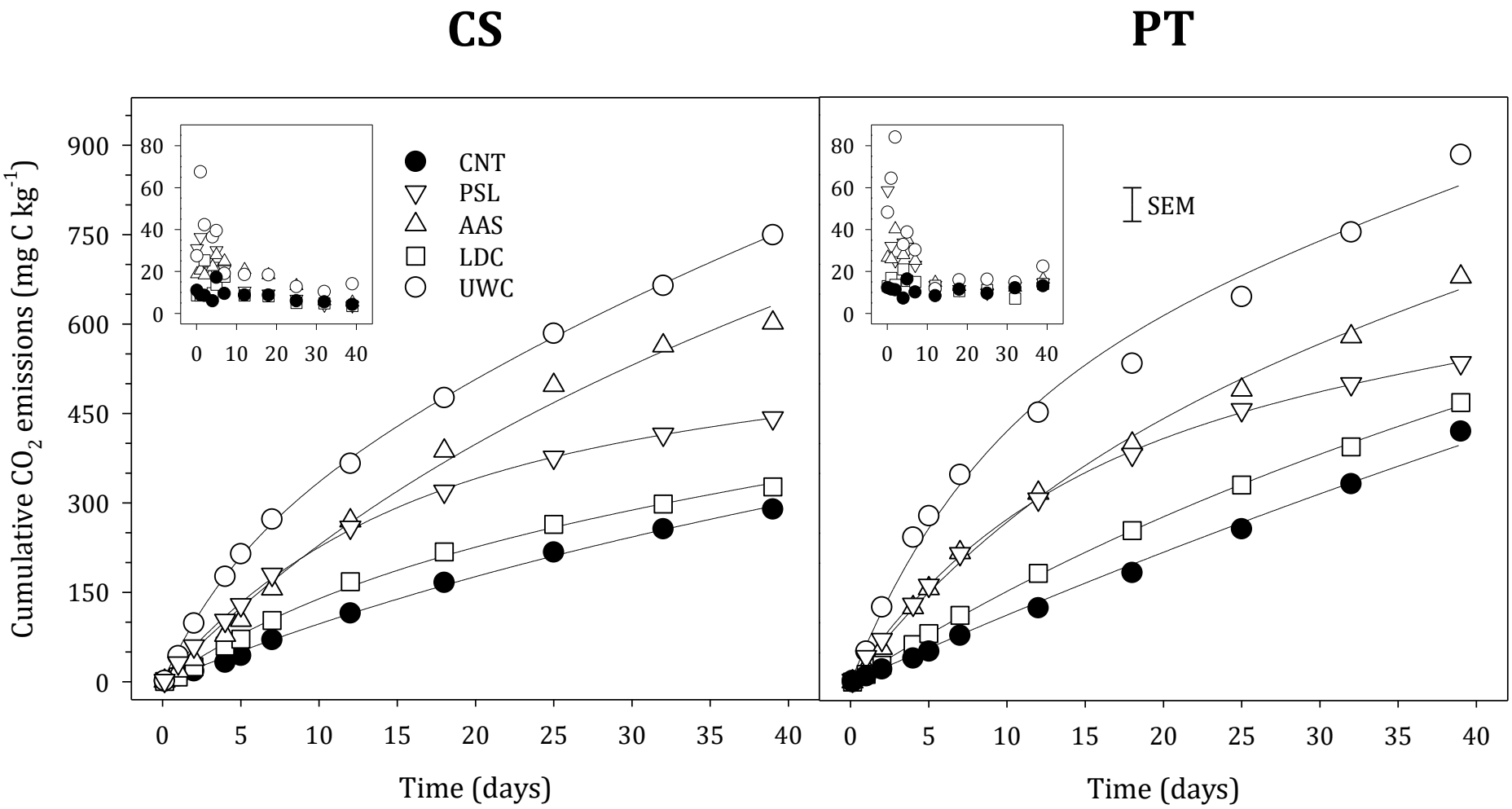

Figure 1. Cumulative emissions of CO_2 after the application of organic materials (open symbols) to CS and PT soils, and comparison to the unamended controls (closed symbols). Lines represent the fitting of the double first-order exponential model while error bar represents the standard error of the mean. Inlays represent changes in the rate of CO_2 emissions expressed in mg C kg^{-1} d^{-1} with incubation time for both amended and non-amended soils. (CNT: control; PSL: pig slurry; AAS: anaerobic sludge; LDC: live-stock derived compost; UWC: urban waste compost).

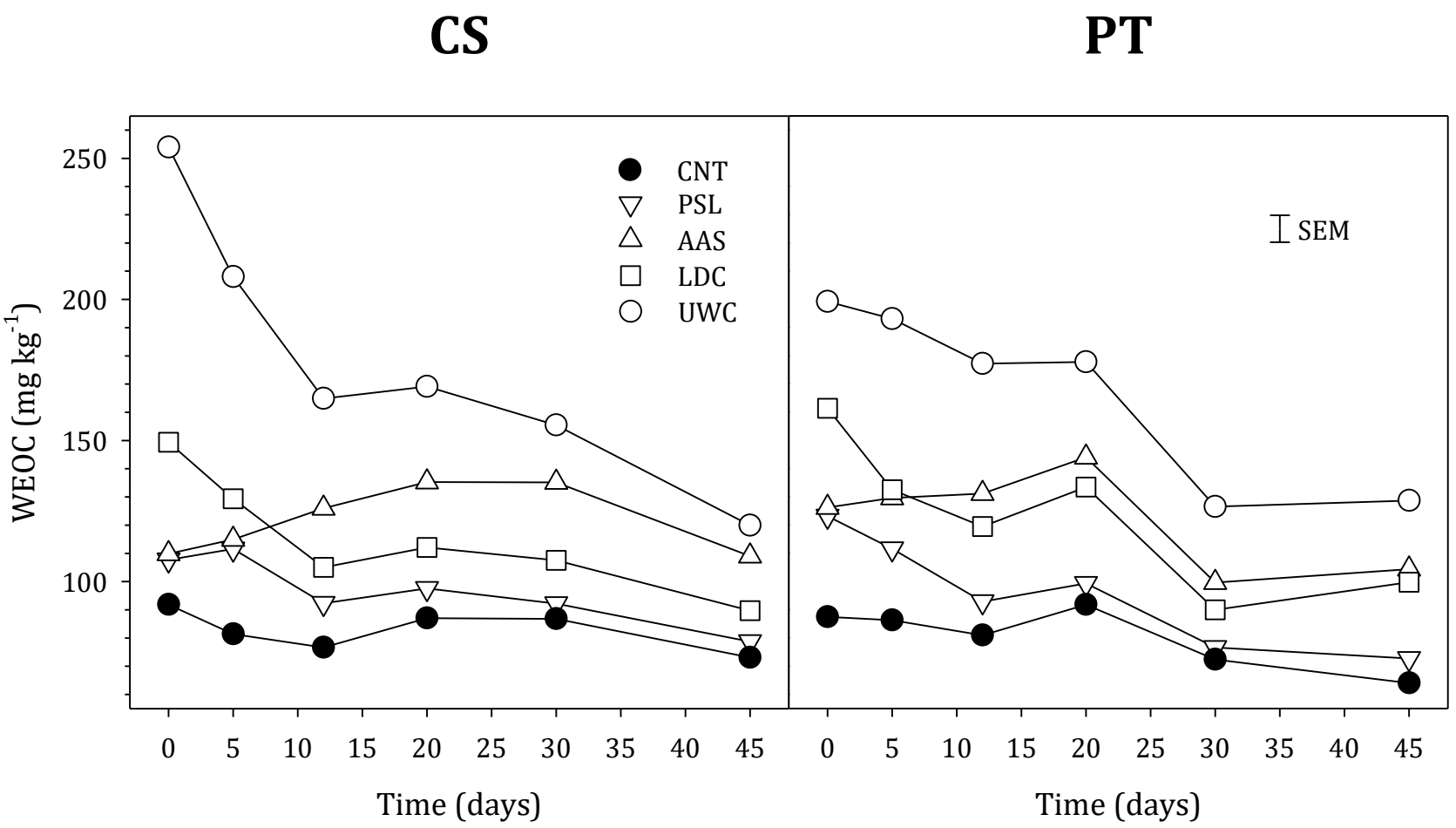

Figure 2: Variations in the mean concentration of water-extractable organic matter with time after the application of organic materials (open symbols) to CS and PT soils, and comparison to the unamended controls (closed symbols). Error bar represents the standard error of the mean. Series labels as for Fig. 1.

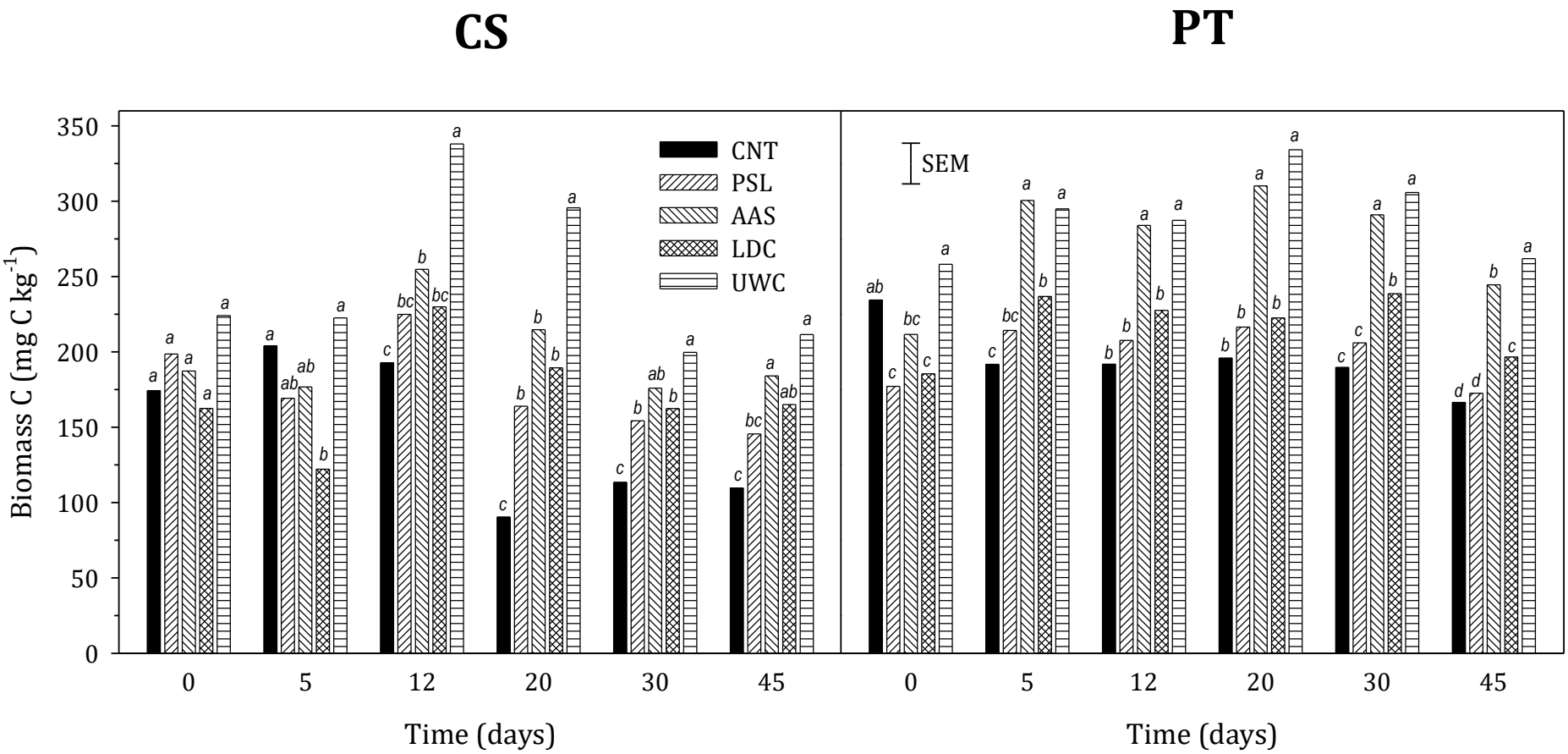

Figure 3: Variations in mean microbial biomass C with time after the application of organic materials to CS and PT soils, and comparison to unamended controls. Error bar represents the standard error of the mean while different letters indicate a significant difference ($p \leq 0.05$) between treatments within same sampling time for each soil. Series labels as for Fig. 1.

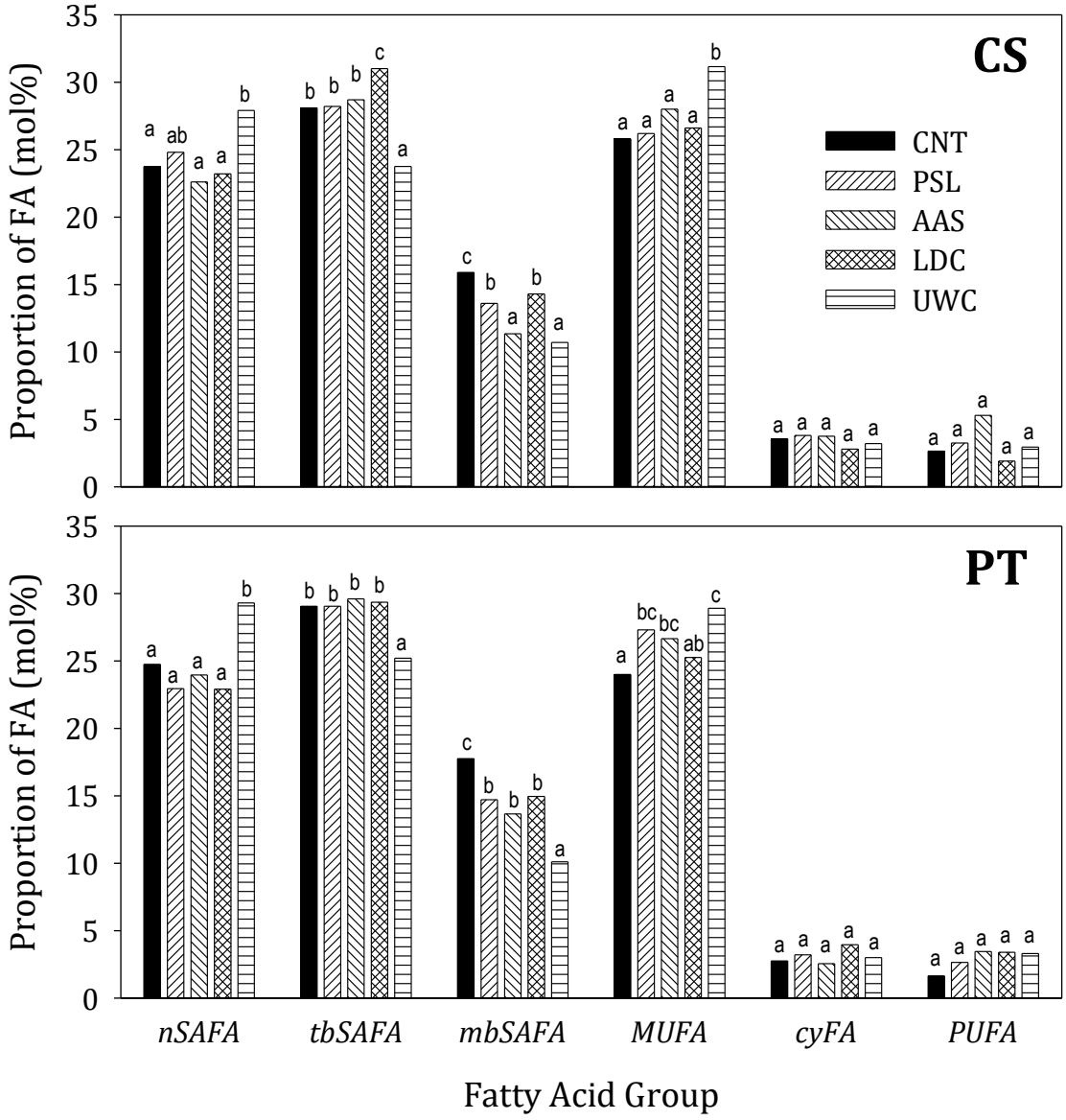

Figure 4: Proportion of PLFA in different functional groups in amended and unamended soils. Abbreviations: nSAFA, normal saturated; tbSAFA, terminal branched; mbSAFA, mid-chain branched; MUFA, monounsaturated; cyFA, cyclopropyl; PUFA, polyunsaturated fatty acids. Different letters indicate a significant difference ($p \leq 0.05$) between treatments within same sampling time for each soil. Series labels as for Fig. 1.

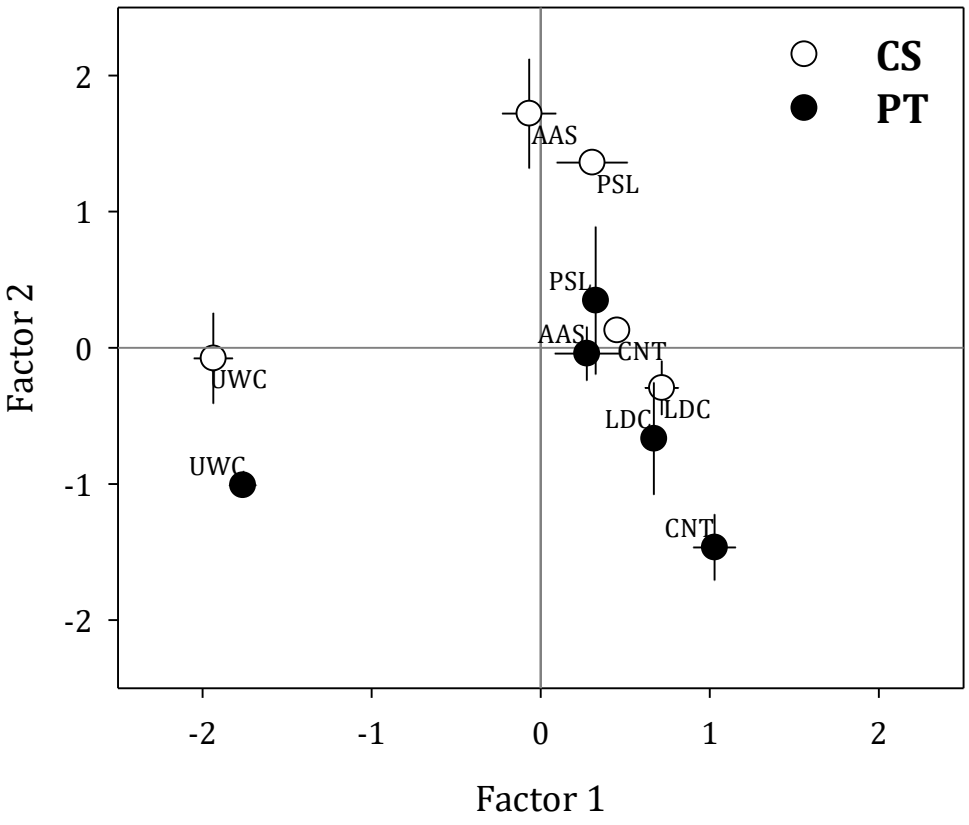

Figure 5: Plot of first and second principal components of PLFA data from amended and unamended soils. Points show means ± standard error. CNT: control; PSL: pig slurry; AAS: anaerobic sludge; LDC: live-stock derived compost; UWC: urban waste compost.

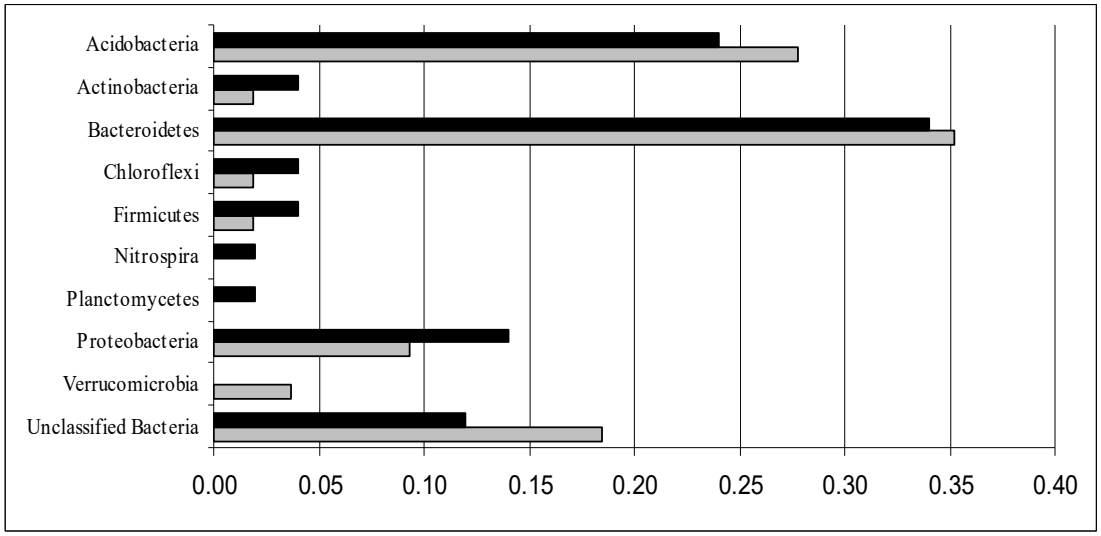

Figure 6. Phyla bar chart (confidence threshold 80%) of relative abundance: black bars are relative to control soil, gray bars to treated soil. The phylogenetic affiliations of the 104 clones were deduced using Classifier from the Ribosomal Database Project.