Bioresource Technology 101 (2010) 1348–1354

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

A new combination of extraction and derivatization methods that reduces the complexity and preparation time in determining phospholipid fatty acids in solid environmental samples

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ARTICLE INFO

Article history:
Received 17 July 2009
Received in revised form 10 September 2009
Accepted 14 September 2009
Available online 2 October 2009

Keywords:
Microbial biomarkers
Manure
Compost
Vermicompost
Soil

ABSTRACT

Combinations of three extraction methods (modified Folch method, modified Bligh and Dyer and microwave-assisted extraction (MAE)) and two derivatization methods (alkaline methanolysis and derivatization with trimethylsulfonium hydroxide (TMSH)) are compared for determining phospholipid fatty acids (PLFAs) in soil and solid organic samples (animal manures, compost and vermicompost). The modified Folch method rendered the greatest total amount of PLFAs and the highest yields of individual PLFA biomarkers; the effect was most apparent in the vermicompost samples. MAE rendered similar extraction yields as the modified Bligh and Dyer method (the most commonly used extraction method), although MAE is much simpler and faster. The highest conversion yields of PLFAs into fatty acid methyl esters were achieved with TMSH as the derivatization agent. The modified Folch method together with derivatization with TMSH was the least complex and time consuming method of determining microbial community structure in solid environmental samples.

1. Introduction

Analysis of phospholipid fatty acid (PLFA) composition is one of the most commonly used culture-independent tools for investigating microbial populations in ecological studies (Øvreås, 2000). The methodology provides qualitative and quantitative insights into the structure of the microbial community and indicates the main groups of microorganisms present, their abundance and nutritional status (Smith et al., 2000).

Phospholipids consist of a glycerol linked to one phosphatidyl head group and two fatty acyl side-chains. The head group is responsible for the different polarity of phospholipids, and the fatty acyl side-chains vary in composition (i.e., length, alkyl-branches and number of double bonds) between eukaryotes and prokaryotes, as well as among many prokaryotic groups (Joergensen and Wichern, 2008). Thus, PLFAs are used as biomarkers to determine the presence and the abundance of specific microbial groups in their habitats (Zelles, 1999). Since PLFAs are rapidly synthesized during microbial growth and are quickly degraded upon microbial death, they represent a 'fingerprint' of the viable microbial community (Evershed et al., 2006) and do not function as storage compounds. Nielsen and Petersen (2000) estimated that no more than 5–10% of fatty acids (FAs) in PLFA analysis are derived from non-microbial sources.

Phospholipid fatty acids are commonly extracted from environmental samples by use of modifications of the Bligh and Dyer method followed by purification with silicic acid chromatography (Tunlid and White, 1992). Bligh and Dyer (1959) used a one-phase mixture containing chloroform, methanol and water for total extraction of lipids from fish muscle. The most widely used modifications of this method include use of a buffer (rather than water) in the extraction mixture. White et al. (1979) added a phosphate buffer to study microbial populations in marine and estuarine sediments and Frostegård et al. (1991) used a citrate buffer to study soil microbial populations.

Fatty acids from the phospholipidic fraction are usually transformed into their less polar methyl ester derivatives (FAMEs) by mild alkaline methanolysis (Dowling et al., 1986; White and Ringelberg, 1998) for analysis by gas chromatography. The TMSH agent and other hydroxides such as tetramethylammonium hydroxide (TMAH) and trimethylphenylamine hydroxide (TMPAH) have also been used to convert FAs into FAMES in solid environmental samples (Jandl et al., 2005; Lores et al., 2006; Aira et al., 2009).

Although the methods usually used for soil samples have been applied in the analysis of PLFAs in organic samples such as compost (Steger et al., 2007; Kato and Miura, 2008), it is important

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doi:10.1016/j.biortech.2009.09.047

to determine the best methods of extraction and derivatization for these types of samples. In a previous study, we compared extraction and derivatization methods for determining total FA profiles in environmental samples (Gómez-Brandón et al., 2008). Although total FAs can be used to evaluate shifts in the microbial community structure of different environmental samples (Lores et al., 2006; Quezada et al., 2007; Mechri et al., 2008), most of the current knowledge regarding the study of microbial populations in environmental research has been obtained from PLFA profiles. Therefore, in the present study, we compared combinations of three extraction and two derivatization methods for determining PLFAs in soil and solid organic samples (animal manures, compost and vermicompost).

2. Methods

2.1. Chemicals

For the identification and quantification of PLFAs, the following analytical grade standards were used: 37-component FAME mix #47885-U (1000 μg mL^{-1}, Supelco, Bellefonte, USA). Mixture BR1 #90-1051 (1000 μg mL^{-1}); methyl 13-methyltetradecanoate #21-1413 (250 μg mL^{-1}); methyl vaccenate #20-1812-9 (100 μg mL^{-1}) (Larodan Lipids, Malmö, Sweden). The internal standard methyl nonadecanoate (19:0, 230 μg mL^{-1}) and the derivatization agent TMSH (~0.25 mol L^{-1} in methanol) were purchased from Sigma–Aldrich (Madrid, Spain).

Chloroform, methanol, *n*-hexane and acetone were obtained from Scharlab (Barcelona, Spain), and methyl *tert*-butyl ether from Sigma–Aldrich (Madrid, Spain). All reagents were HPLC-grade. Sodium sulphate (Na_2SO_4) and potassium hydroxide (KOH) were supplied by Scharlab (Barcelona, Spain), and the dibasic potassium phosphate (K_2HPO_4) used to prepare the phosphate buffer was purchased from Sigma–Aldrich. Milli-Q water was obtained by purifying demineralized water in a Milli-Q system (Millipore, Madrid, Spain).

Laboratory glassware was soaked overnight in phosphate-free Extran MA O3 5% (v/v) aqueous solution (Merck, Mollet del Valles, Barcelona, Spain), and washed thoroughly in tap and deionised water.

2.2. Environmental samples

Twelve solid environmental samples were analyzed in the study: soils collected from three different ecosystems (pasture, pine forest and chestnut forest) in Bertoa (Galicia, NW Spain); three composts obtained from different organic materials (a mixture of pruning waste, litter and grass clippings; gorse (*Ulex europaeus*); and organic household waste); three animal manures (cattle, horse and rabbit) collected from farms in Pontevedra and Ourense (Galicia, NW Spain); and the corresponding vermicomposts produced in the laboratory with the earthworm specie *Eisenia andrei*. Some of the physicochemical characteristics of the samples are summarized in Table 1. All samples were lyophilized before PLFA analysis.

2.3. Extraction methods

Total FAs were extracted from 200 mg of each substrate with 60 mL chloroform–methanol, 2:1 (v/v), in 100 mL sterilized plastic jars. The jars were shaken vigorously for 30 min and the mixture was allowed to separate at room temperature for 24 h. The supernatant was filtered, collected in a glass test tube and then evaporated to dryness under a stream of oxygen-free N_2 gas. We designated this as the modified Folch method because it involves use of the same extraction mixture as described by Folch et al. (1957) but without the washing step.

Table 1
Some physicochemical properties of the different solid environmental samples.

	Organic matter content (%)	pH	Electrical conductivity (mS cm^{-2})
Soil			
Pasture	18 ± 1	4.6 ± 0.3	0.05 ± 0.0
Pine	19 ± 2	4.1 ± 0.1	0.05 ± 0.0
Chestnut	16 ± 4	4.0 ± 0.1	0.07 ± 0.0
Manure			
Cattle	85 ± 1	7.6 ± 0.0	0.20 ± 0.0
Horse	90 ± 2	7.3 ± 0.0	0.10 ± 0.0
Rabbit	76 ± 3	8.2 ± 0.1	0.20 ± 0.0
Compost			
Pruning	35 ± 2	4.4 ± 0.3	0.10 ± 0.0
Gorse	37 ± 7	4.4 ± 0.0	1.10 ± 0.0
Household	53 ± 3	6.9 ± 0.0	1.90 ± 0.3
Vermicompost			
Cattle	72 ± 3	6.7 ± 0.0	0.09 ± 0.0
Horse	85 ± 4	6.7 ± 0.1	0.08 ± 0.0
Rabbit	54 ± 3	7.6 ± 0.1	0.30 ± 0.0

Values are means ± standard error (n = 3).

Total FAs were extracted from 200 mg of each substrate by addition of a mixture 1:2:0.8 (v/v/v) containing chloroform, methanol and phosphate buffer (50 mmol L^{-1}, pH 7.4) according to Bligh and Dyer (B and D) method, as modified by White et al. (1979). The suspension was mixed vigorously for 30 min, and then left for 2 h at room temperature. The samples were then centrifuged for 15 min at 2500 rpm. The extraction step was repeated three times. The organic phase was filtered, transferred to a glass test tube and evaporated to dryness under a stream of oxygen-free N_2 gas.

Total FAs were extracted from 200 mg of each substrate with 10 mL *n*-hexane–acetone, 1:1 (v/v) in Teflon vessels (López-Ávila et al., 1994). These vessels were tightly sealed and placed in a scientific microwave oven (CEM Corporation MDS-2000) operating at 2450 MHz and 630 W maximum output, and irradiated at medium power (60% of maximum output, manufacturer's setting) for 20 s, three times, with 1 min cooling between each irradiation; these conditions were adjusted in order to obtain the most accurate response for this type of samples containing large amounts of organic matter (Young, 1995). The samples were cooled for approximately 30 min and then allowed to settle. The supernatants from each vessel were separated, dried with Na_2SO_4, and collected in a glass test tube. The washing solutions (2 × 5 mL *n*-hexane–acetone, 1:1 (v/v)) were also dehydrated through Na_2SO_4 (Batista et al., 2001). The combined solutions were evaporated to dryness under a stream of oxygen-free N_2 gas.

2.4. Lipid fractionation

Total lipid extracts, obtained with the three extraction methods described above, were dissolved in chloroform (3 × 1 mL) and fractionated into neutral lipids, glycolipids and phospholipids, with chloroform (5 mL), acetone (10 mL) and methanol (5 mL), on silicic acid columns (Strata SI-1 Silica (55 μm, 70 Å), 500 mg/6 mL). FAMEs from the phospholipid fraction of each method were prepared with two different derivatization procedures, described as follows:

2.5. Derivatization procedures

Phospholipid fractions of each method were subjected to alkaline methanolysis (Dowling et al., 1986). Briefly, phospholipid extracts were vortex-mixed for 30 s in 2 mL of a mixture of

methanolic KOH solution (0.2 mol L^{-1}) and toluene–methanol, 1:1 (v/v), incubated at 37 °C in a water bath for 15 min, cooled, and neutralized with acetic acid (1 mol L^{-1}). The FAMEs were then extracted as follows: 2 mL of hexane–chloroform, 4:1 (v/v) and 2 mL of Milli-Q water were added to the samples, which were then centrifuged for 5 min at 2000 rpm. The organic phase containing the FAMEs was transferred to a glass test tube and the aqueous phase was again processed with hexane–chloroform, 4:1 (v/v) (2 mL × 2). The combined supernatants were then evaporated under a stream of oxygen-free N_2 gas to a volume of 0.5 mL. Prior to gas chromatography–mass spectrometry (GC/MS) analysis, 10 μL methyl nonadecanoate (19:0, 230 μg mL^{-1}) was added as an internal standard to 150 μL of the extract of FAMEs.

Phospholipid fractions of each method were subjected to derivatization with TMSH (Batista et al., 2001). Briefly, phospholipid extracts were redissolved in 500 μL of methyl *tert*-butyl ether. This solution (100 μL) was placed in a screw-cap vial with 50 μL of the derivatization agent (TMSH), vortex-mixed for 30 s and allowed to react for 30 min; 10 μL of the internal standard methyl nonadecanoate (19:0, 230 μg mL^{-1}) was then added to the extract of FAMEs.

2.6. Gas chromatography–mass spectrometry analysis

FAMEs were separated on a CP-SIL 88 Varian Select FAME FS (50 m × 0.25 mm × 0.2 μm) capillary column in a Varian 3800 gas chromatograph equipped with a Saturn 2000 mass spectrometer (Varian Chromatography Systems, Walnut Creek, CA, USA). The detailed GC–MS experimental conditions have been described elsewhere by the authors (Gómez-Brandón et al., 2008). The PLFA 18:1ω7c, not included in the previous study, was detected at 30.150 min and quantified with *m/z* 55, 69, 83, 97 and 264.

To identify the FAMEs, the retention times and the mass spectra were compared with those obtained with standards. FAMEs were quantified by an internal standard calibration procedure (see Gómez-Brandón et al. (2008)). The calibration levels of the FAMEs varied in the range 0.4–250 μg mL^{-1}. The coefficients of determination (R^2) were higher than 0.99 for all calibration curves. FAMEs were described by the standard ω-nomenclature A:BωC (IUPAC-IUB, 1977).

2.7. Statistical analysis

A three-way ANOVA was used to examine the effects on the PLFA yields of the following: type of sample, considering four levels (soil, animal manure, compost and vermicompost); the extraction method, considering three levels (modified Folch; modified B and D; and MAE), and the derivatization method, considering two levels (alkaline methanolysis and derivatization with TMSH). Post hoc comparisons of means were performed by a Tukey HSD test at $\alpha = 0.05$. All statistical analyses were carried out with SPSS v.14 software.

The response variables analyzed were the total amount of PLFAs (total PLFAs) expressed as the sum of the concentrations (μg g^{-1}) of all identified PLFAs; and the concentration (μg g^{-1}) of the individual PLFAs used as biomarkers of specific microbial groups, i.e., the iso/anteiso i15:0, a15:0, i16:0 and a17:0 for Gram-positive bacteria (G+ bacteria); the 16:1ω7c, 17:1ω7c and 18:1ω7c for Gram-negative bacteria (G− bacteria) and the fungal biomarkers 18:1ω9c and 18:2ω6c (Frostegård and Bååth, 1996; Zelles, 1997).

3. Results

Twenty-two saturated and unsaturated PLFAs, ranging from 10 to 19 carbon atoms were identified and quantified in the studied samples. Their corresponding retention times, molecular weights and identification and quantification ions are reported in Gómez-Brandón et al. (2008).

The total amount of PLFAs (total PLFAs) and the yields of all the PLFA biomarkers differed greatly depending on the type of sample

Table 2
Main effects of the type of sample (soil, manure, compost and vermicompost); the extraction methods (modified Folch, modified B and D and MAE) and derivatization methods (alkaline methanolysis and TMSH) on total PLFAs and specific biomarker PLFAs. The second order interactions between the main factors are also shown.

Dependent variables	Main effects			Interactions		
	Sample (A)	Extraction method (B)	Derivatization method (C)	AB	AC	BC

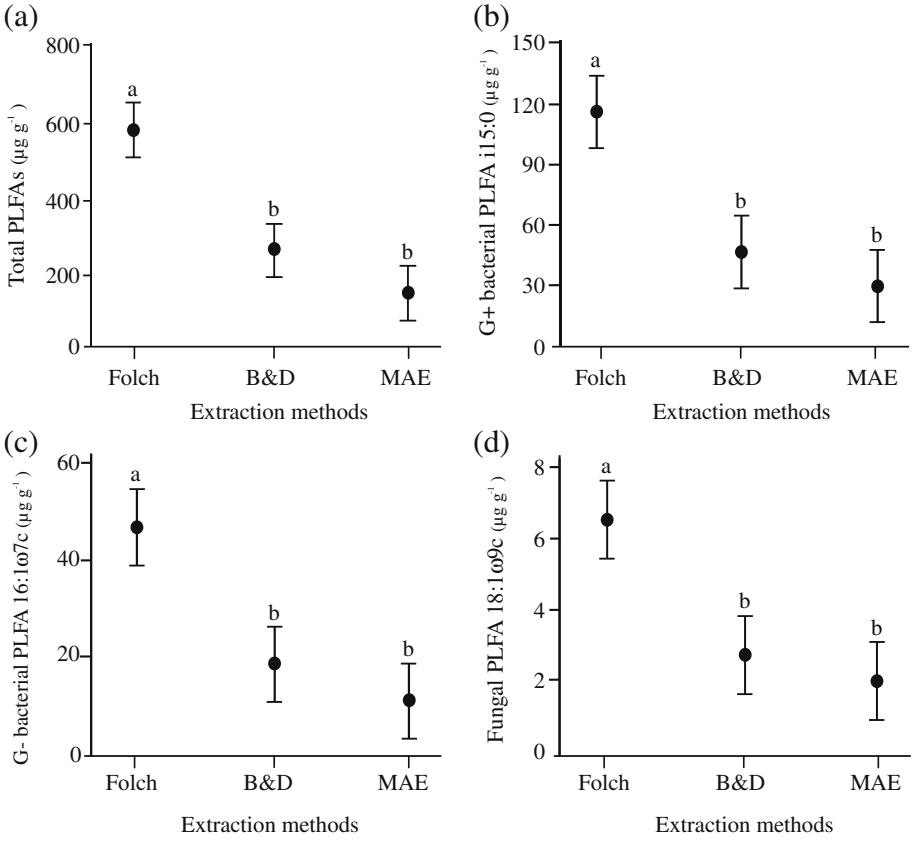

Fig. 1. PLFA yields (μg g^{-1}) of (a) total PLFAs, (b) G+ bacterial biomarker i15:0; (c) G− bacterial biomarker 16:1ω7c; and (d) the fungal biomarker 18:1ω9c obtained with the three extraction methods tested: the modified Folch method, the modified Bligh and Dyer method and microwave-assisted extraction. Values are means ± standard error. Different letters above the error bars indicate significant differences at $P < 0.05$ (Tukey's HSD test).

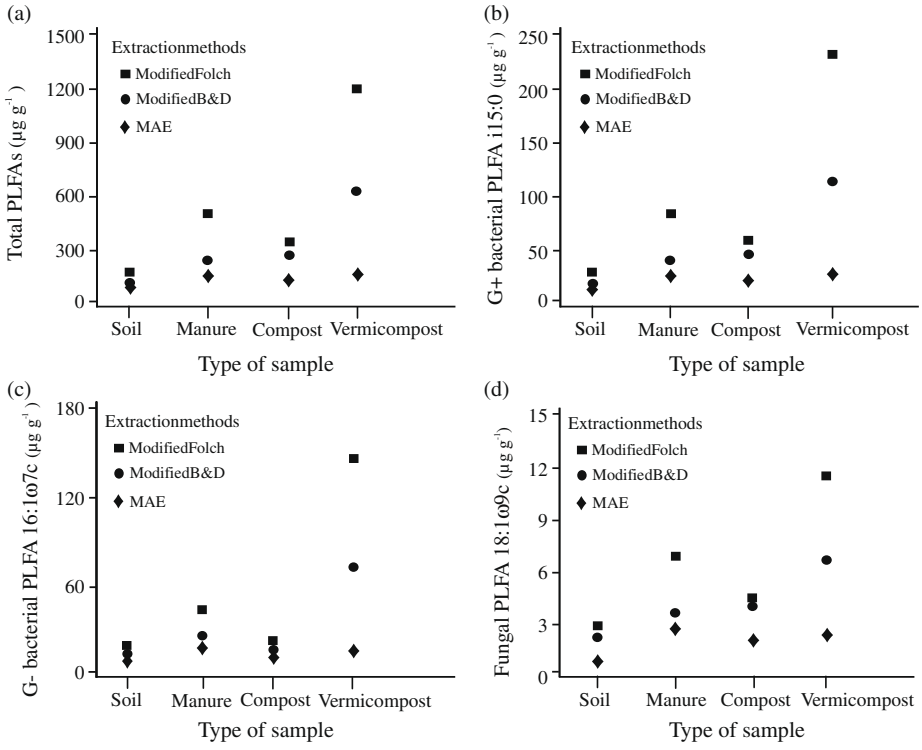

Fig. 2. PLFA yields (μg g^{-1}) of (a) total PLFAs, (b) G+ bacterial biomarker i15:0, (c) G− bacterial biomarker 16:1ω7c and (d) fungal biomarker 18:1ω9c in the solid environmental samples processed by the three extraction methods tested: the modified Folch method (■), the modified Bligh and Dyer method (●) and microwave-assisted extraction (♦).

(Table 2). The extraction method had also different effects on total PLFAs, and PLFA biomarkers for Gram-positive and -negative bacteria and fungi (Table 2). The derivatization method also rendered significantly different PLFA yields from the samples (Table 2); this factor greatly affected the total amount of PLFAs, and the yields of PLFAs characteristic of fungi and bacteria, with the exception of G+ bacterial biomarkers a15:0 and i16:0 and G− bacterial markers 16:1ω7c and 17:1ω7c (Table 2). Besides determining the influence of each factor on PLFA yields, we also evaluated the second order interactions (Table 2). The efficiency of the extraction method depended on the type of sample for total PLFAs and all PLFA biomarkers, except for the G+ bacterial PLFAs a15:0 and a17:0 (Table 2). A significant interaction between the type of sample and the derivatization method was also found for total PLFAs, and the G+ and G− bacterial PLFAs i15:0 and 18:1ω7c (Table 2). However, there was no a significant interaction between the derivatization procedure and the extraction method (Table 2).

The extraction yields of total PLFAs and bacterial and fungal PLFA biomarkers were much higher with the modified Folch method than with the modified B and D and the microwave extraction (Fig. 1 and Table 2). The extraction efficiency depended on the type of matrix, with the highest extraction yields obtained with the vermicompost samples (Fig. 2 and Table 2).

The conversion of PLFAs into FAMEs was higher when TMSH was used as the derivatization agent than by alkaline methanolysis

Fig. 1

svrminomposwamples

The main difference between this method and the B and D method as modified by White et al. (1979) is the addition in the latter method of a phosphate buffer to the extraction mixture; other widely used methods of extracting PLFAs from environmental solid samples also included a citrate buffer (Frostegård et al., 1991) and even an acetate buffer (Zink and Mangelsdorf, 2004) in their extraction mixtures. Use of a buffered solvent system is recommended for samples containing large amounts of salts (e.g. sediments with high mineral content) to prevent ionic adsorption effects (Christie, 1993). Otherwise, phospholipids may be ionized and consequently retained during phase separation in the aqueous phase and not recovered in the organic phase. However, in the present study the addition of a buffer did not improve the extraction yield of PLFAs, mainly because nine of the samples studied are highly organic, and as such the buffer effect was not significant.

The yields of PLFAs extracted with MAE were also much lower than those obtained by the modified Folch method. The lower extraction efficiency of the MAE method may be attributed to the extraction solvent mixture (*n*-hexane–acetone instead of chloroform–methanol), rather than the extraction process. For efficient extraction of FAs derived from cell membrane lipids (i.e. phospholipids) a polar solvent mixture is required; according to Kates (1986), the inclusion of an alcohol in the extraction mixture is necessary to dissolve polar lipids in the cell membranes, thereby facilitating the extraction of FAs. However, even with these limitations, MAE rendered similar extraction yields as the modified B and D method, the most commonly used extraction method in environmental research. Indeed MAE is much simpler and faster (see Methods section and Gómez-Brandón et al. (2008)).

Once extracted, PLFAs are transformed into FAMEs for analysis by gas chromatography. In this study the highest conversion yields of PLFAs into FAMEs were obtained with TMSH as the derivatization agent. This effect was dependent on the type of sample and was greatest in compost and vermicompost samples. Although derivatization with TMSH was much more efficient than alkaline methanolysis for the compost and vermicompost samples, the efficiency was similar for manure and soil samples. Esterification with TMSH is a simple and a non-time consuming procedure that can be performed at room temperature in a fast and single-step reaction; trimethylsulfonium salts are formed by deprotonation of FAs, and then thermally decomposed by heating, to produce FAMEs and dimethyl sulphide. The by-products of this reaction (water and dimethyl sulphide) elute with the solvent peak (during the delay period), and do not interfere with the chromatographic separation of the analytes (Yamauchi et al., 1979). Trimethylsulfonium salts decompose at lower temperatures than other derivatization products obtained with hydroxides (e.g. TMAH and TMPAH), thus minimizing degradation/isomerization side-reactions (Akoto et al., 2008). Interestingly, despite being a much slower and more tedious procedure, alkaline methanolysis is still the most common derivatization method.

The combination of the modified Folch extraction method with TMSH derivatization was also the best option for determining total fatty acids (FAs) in different solid environmental samples (soil, manure, compost and vermicompost) (Gómez-Brandón et al., 2008).

Some studies have shown that the microbial communities in compost and vermicompost are very different, and that relative to the initial substrate they are more diverse in vermicompost than in compost (Fracchia et al., 2006; Vivas et al., 2009; Sen and Chandra, 2009). Similarly, in the present study the analysis of PLFA profiles indicated that the structure of the microbial community differed between compost and vermicompost samples. In addition, the viable microbial biomass measured as the total content of PLFAs, and the abundance of both bacteria and fungi were much higher in vermicompost samples than in manure and compost samples.

5. Conclusions

The combination of the modified Folch extraction method with TMSH derivatization worked well in a wide range of microbial biomass in the samples, ranging from soil to vermicompost, and proved to be the least complex and time consuming method of determining microbial community structure in soil and animal manures, as well as in processed materials resulting from both composting and vermicomposting processes.

Acknowledgements

This research was financially supported by the Xunta de Galicia (Project No. 07MRU023383PR) and from the Spanish Ministerio de Ciencia e Innovación (CTM2009-08477). María Gómez Brandón is financially supported by a FPU fellowship from the Spanish Ministerio de Educación.

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