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Crop rotation and seasonal effects on fatty acid profiles of neutral and phospholipids extracted from no-till agricultural soils

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Abstract

Analysis of phospholipids (PLFA) and neutral lipids fatty acids (NLFA) was used to characterize no-till productive agricultural soils associated with different crop rotation levels, replicated across a 400 km transect in the Argentinean pampas, during two sampling seasons, summer and winter. High rotation (HR) management consisted in maize–wheat–soybean intense rotation including cover crops. Low rotation (LR) management trend to soybean monocultures. Soils from nearby natural environments (NEs) were used as references. Fatty acids concentration in soils (nmol/g) decreased c.a. 50% from summer to winter differentially according to soil treatment being the smallest decrease in HR management 35%. Both PLFA and NLFA profiles showed strong potential to discriminate between different land uses. In winter samples, some rare or unknown fatty acids were relevant for the discrimination of agricultural practices while NLFA *20:0* appears to be a good marker of HR soils despite season or location. The PLFA-based taxonomic biomarkers for total bacteria, Gram-negative bacteria and arbuscular mycorrhiza showed a significant trend NE>HR>LR in the winter sampling. HR management was also characterized by high levels of NLFA in winter samples as if high crop rotation improves lipids reserves in soil during winter more than in monocropping soil management. In conclusion, PLFA and particularly NLFA profiles appear to provide useful and complementary information to obtain a footprint of different soil use and managements, improving soil biochemistry characterization tools.

Keywords: Agriculture, soil lipids, no-tillage, crop rotation, NLFA, PLFA

Abbreviations: PLFA, phospholipid fatty acids; NLFA, neutral lipid fatty acids; HR, high rotation management; LR, low rotation management; NE, natural environment.

Introduction

The production of soybean has increased in Argentina from 0.01 to 27 million hectares during the last 30 years, now being the third world producer of this legume as a consequence of biotechnology, no-till agriculture adoption and market pressure by commodities values (Meriles *et al.*, 2009). Reduced or no-tillage agricultural practices improve soil physical properties and reduce the risk of erosion or surface runoff if no-till were combined with crop rotation (Derpsch *et al.*, 2010). Soils under no-till agriculture in Argentina have been mostly chemically and physically characterized, but knowledge about biology and biochemistry of these soils is scarce (Wall, 2011). Different soil uses affect crops productivity and has been shown to produce different effects on soil microbial community

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structures and diversity (Figuerola *et al.*, 2012; Rodrigues *et al.*, 2013) without a clear link to soil function, a paradigm yet to be resolved (Stres & Tiedje, 2006). The use of certain fatty acids as biomarkers of different microbial taxons is a matter of debate, and it is not always accepted, especially when complex systems are analysed (Scott *et al.*, 2010; Frostegård *et al.*, 2011). Nevertheless, available data from culturable microorganisms are still used to support specific types of fatty acids as markers of given taxon for microbial communities characterization (Zelles, 1999; Scott *et al.*, 2010).

Despite of the relative low taxonomic resolution of fatty acid biomarkers when examining soil microbial communities, a comprehensive analysis of fatty acid profiles from different soil lipids may open new opportunities of systemic studies in relation to biochemical characterization of soil ecosystems.

Lipids extracted from soil can be fractionated into neutral, glyco- and phospho-lipids (Zelles, 1999). Because of their high turnover as biomolecules, phospholipid fatty acids (PLFA) are thought to provide information about the active soil microbial community (Tunlid & White, 1992), while neutral lipid fatty acids (NLFA), which mainly consist of energy reserve substances in living organisms (Larsen & Bødker, 2001), may provide information about the physiological status of soil biota. The PLFA methodology has been broadly used to characterize microbial communities in agricultural soils under different managements (Drijber *et al.*, 2000; Pankhurst *et al.*, 2001; Ibekwe *et al.*, 2002; Feng *et al.*, 2003; van Groenigen *et al.*, 2010; Montecchia *et al.*, 2011; Börjesson *et al.*, 2012), where smaller amounts of total PLFAs were generally found in agricultural sites with conventional tillage compared with no-till (Drijber *et al.*, 2000; Ibekwe *et al.*, 2002; Feng *et al.*, 2003). But comparisons between no-till soils with different rotation intensity were rarely reported.

Reports based on NLFA profiles are more scarce, and generally related to studies of arbuscular mycorrhizae and saprotrophic or pathogenic fungi (Olsson *et al.*, 1995; Olsson, 1999; Larsen & Bødker, 2001; Bååth, 2003).

Our hypothesis was that different soil managements, as different level of crop rotation, should drive soil biological diversity into different community structures modifying the profile of fatty acids from different lipid fractions. The differences in fatty acid profiles would open the possibility to detect particular fatty acids groups that could be useful as markers to differentiate soils with different history of use.

In this study, soils from two different and contrasting agricultural practices, using natural grasslands as references, and replicated across 400 km in the Argentinean Pampas, were characterized in terms of fatty acid profiles from the PLFA and NLFA fractions. Soil microbial community analysis based on taxonomic microbial biomarkers was also performed.

Materials and methods

Soil managements and experimental design

Two different agricultural soil managements were examined as treatments, in productive fields according to the adjustment, or not, to the definition of *good agricultural practices* stated by the principles of Certified Agriculture established by the Argentine No Till Farmers Association (AAPRESID, www.aapresid.org) and by the Food and Agriculture Organization (FAO). Briefly, *good agricultural practices* are based on high rotation (HR) management consisting of no-till maize–wheat–soybean intensive rotations, including winter cover crops, nutrient replacement by fertilizer application and integrated pest management with minimized use of herbicides, insecticides and fungicides. Low rotation (LR) management consisted in soybean monoculture with very scarce if any crop rotation and no winter cover crops, low nutrient replacement and large inputs of agrochemicals. Soils from natural environments (NEs) were used as treatment reference, consisting of natural grassland close to the cultivated plots, where no cultivation has been practised for at least the last 30 years. The blocks of soil treatments were replicated at four different locations (Bengolea, Monte Buey, Pergamino and Viale), across a west–east transect of 400 km with a gradient of soil texture (sandy soils in the west to clayey soils in the east) in the central part of Argentinean Pampas. More information regarding the sampling sites can be found elsewhere (Figuerola *et al.*, 2012; Duval *et al.*, 2013). Soil samples were extracted in February 2010 (summer) and September 2010 (late winter). Five subsamples (as repetitions) were taken from the top 10 cm of bulk soil at each location-treatment plot. Each subsample was a composite of 16–20 randomly selected cores from the top 10 cm of bulk soil within an area of 5 m^2. Composite soil samples were homogenized in the field. Each subsample was separated at least 50 m from each other, without following the sowing line in the field. Thus, a total of 60 soil samples [(3 soil management treatments × 4 locations (replicates of treatments) × 5 subsamples (repetitions)] were analysed for each season. Soil samples were transported at 5 °C to the lab, stored at −18 °C during 1 week, freeze dried, milled under liquid nitrogen and stored in the freezer (−20 °C) until further analysis.

Lipid analysis

Fatty acid profiles were analysed as follow: 3 g of soil were extracted with a 1:2:0.8 v/v/v chloroform–methanol–citrate buffer mixture, and the organic layer was fractionated through a solid phase extraction column filled with silica. Neutral, glyco- and phospho-lipids were eluted with chloroform, acetone and methanol, respectively. The fractions were then exposed to a mild alkaline

methanolysis, extracted with hexane, amended with 33.75 μg of the standard fatty acid *19:0* methyl ester (not found in environmental samples), evaporated under N_2 stream, and resuspended in 100 μL of hexane. Analytical details of the technique can be found in Frostegård *et al.* (1993). The chromatographic analysis of the extracted FAMEs was carried out according to the TSBA 40 MIDI system (Sasser, 1990). Briefly, 2 μL of lipid extract from each lipid fraction was injected in an Agilent 6890 plus gas chromatograph, with a flame ionization detector, using H_2 as carrier gas and an Ultra 2 5% phenyl methyl siloxane capillary column (25 m × 200 μm × 0.33 μm). The initial oven temperature of 170 °C was increased to 260 °C at 5 °C/min, followed by another increase to 310 °C at 40 °C/min. Each fatty acid concentration in the sample was estimated in relation to the internal standard *19:0* methyl ester, and expressed as nanomoles per gram of dried soil. In total, 73 different fatty acids from the PLFA and NLFA profiles obtained from the different soil samples were detected with the MIDI system (Table S1) and further analysed.

Lipid nomenclature and taxonomic group definition

Fatty acid nomenclature: Suffix *c* indicates *cis* isomer, *cy* means cyclopropyl ring; 2OH and 3OH represent hydroxyl substituted fatty acids in position 2 and 3 from the carboxyl end, respectively. Fatty acids methylated in C10 are represented as 10Me. Mono-unsaturated (MUFA) and poly-unsaturated fatty acids (PUFA) are designed by the omega (ω) nomenclature. Branched fatty acids in *iso* and *anteiso* position correspond to a lateral methyl group in the second and third C atom, respectively, from the methyl end. Fatty acids with the same retention time in the chromatogram are grouped as 'summed in feature'.

The fatty acids from the PLFA and NLFA profiles were then classified into 9 different chemical groups: straight chain, branched, MUFA, PUFA, cyclic, methylated, hydroxyl and hydroxy branched. Some PLFA were selected as specific biomarkers of taxonomic groups of microorganisms: *14:0iso*, *15:0iso*, *15:0anteiso*, *16:0iso*, *17:0iso* and *17:0anteiso* for Gram-positive bacteria (Hedrick *et al.*, 2010); *17:0cy* and *19:0cy* for Gram negative (Pankhurst *et al.*, 2001); *16:010Me*, *17:010Me* and *18:010Me* for actinomycetes (Kroppenstedt, 1985; Lechevalier & Lechevalier, 1988; Zelles, 1999); *16:1ω5c* for arbuscular mycorrhizal fungi (AMF) (Olsson, 1999; Larsen & Bødker, 2001; Grigera *et al.*, 2007); *18:2ω6,9c* for saprophytic fungi (Frostegård & Bååth, 1996; Pankhurst *et al.*, 2001; Ravnskov *et al.*, 2006; Grigera *et al.*, 2007) and *20:4 w6,9,12,15c* for protozoa (Gryndler *et al.*, 2006; Ravnskov *et al.*, 2006). The fatty acids *15:0*, *15:0iso*, *15:0anteiso*, *16:0iso*, *17:0*, *17:0iso*, *17:0anteiso*, *17:0cy* and *19:0cy* were considered biomarkers of bacteria (Pankhurst *et al.*, 2001).

Statistics

Two factors (locations and soil management treatments) analysis of variance (ANOVA) was used to assess the level of significance ($P < 0.05$) of location x soil management interaction. One-factor ANOVA was performed to compare soil managements as mean values among four replicates (locations), where each replicate was obtained as a mean value of five repetitions (subsamples). Means of fatty acid concentrations for each treatment were compared by the LSD Fisher test. Variance homogeneity was checked by means of the Bartlett test, and data were log transformed when necessary.

Three multivariate methods of ordination were used. Principal component analysis, based on correlation matrix, was used to observe the grouping of subsamples according to soil management or to location. Discriminant analysis was used to assess which parameters contribute mostly to the separation of treatments and was built with those fatty acids present in at least 20% of all samples. Classification and regression trees analysis was used to determine which fatty acids allow a better discrimination among treatments. All the analyses were performed with the INFOSTAT version 2012.

Results

Phospholipids fatty acids

PLFA profiles allowed the separation of samples by treatment. A discriminant analysis with the three soil managements as discriminant criteria showed a clear separation along the first axis (Figure 1). High crop rotation (HR) samples grouped between natural environment (NE) and low crop rotation (LR) samples either for summer (Figure 1a) or winter (Figure 1b) sampling. HR treatment clearly separated from NE and LR ones along the second axis. The discriminant function showed strong loadings of straight chain fatty acids like *14:0* and *20:0* in summer and *15:0* in winter (Table S2). Some rare fatty acids had also greater loadings associated with HR in both seasons, such as *15:1isoG* and *11Me18:1w7c*. It is worth noting that using season as random variable, discriminant analysis of individual PLFA data set including samples from both seasons also showed a good separation of treatments (Figure 1c). In the combined season data set, HR samples strongly correlated with *15:0*, *15:1isoG* and *11Me18:1w7c* (Table S2).

A principal component analysis for PLFA profiles did not show a clear-cut differential grouping among treatments in any of the seasons, although a trend to differential grouping of NE samples from agricultural soils samples was observed in both seasons (Figure S1).

The analysis of individual fatty acids within the PLFA profiles showed that, in summer, NE soil samples values

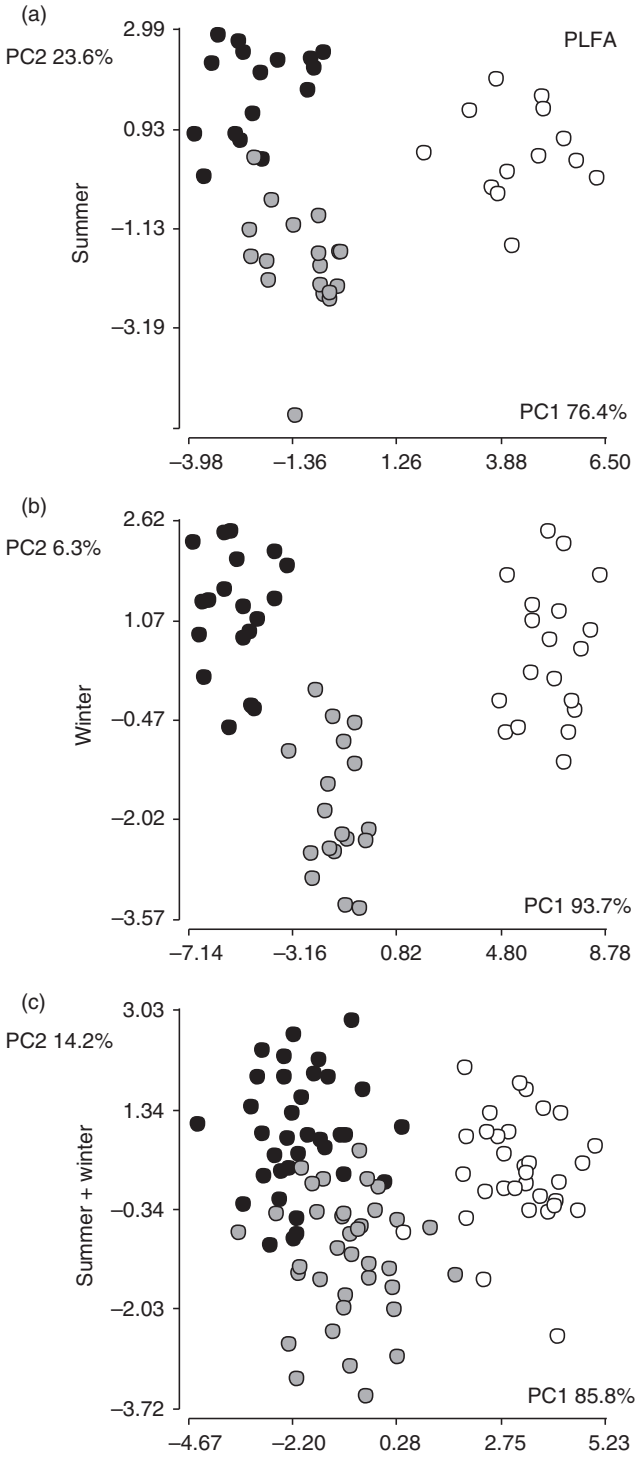

Figure 1 Discriminant analysis of individual PLFA for data from samples taken in summer (a), winter (b), and combining all samples from both seasons (c). Symbols' colours: white: samples from natural environments (NEs) sites, grey: samples from high crop rotation (HR) sites, black: samples from low crop rotation (LR) sites.

were generally greater than those from agricultural soils, and no differences were found between HR and LR treatments. However, in winter samples, it was possible to find particular fatty acids with significant variations between the three treatments. The fatty acids *16:0*, *16:0iso*, *16:1w5c*, *18:0* and *18:1w9c* showed decreasing concentrations in the direction NE>HR>LR, with significant differences ($P < 0.05$) among them (Table S3), but as strong location-soil management were found ($P < 0.001$) this comparison of treatment averaged among all locations should not be considered.

The total amount of soil PLFAs was greater in NE samples than in agricultural soils, regardless of season (Table 1). No differences were found between HR and LR in the summer sampling ($P = 0.984$), while the trend NE>HR>LR was present in the winter sampling. Nevertheless, a strong interaction was found in the two-factor ANOVA ($P = 0.0001$) suggesting the need of a location per location analysis.

The taxonomic groups estimated from PLFA data generally show greater values in NE than in agricultural sites, and values for HR were always greater than for LR samples at every location used as replication of treatments (data not shown). The two-factor ANOVA also showed strong interaction between treatment and location in winter samples (Table S4). The natural environments used as soil reference at different locations were quite different in terms of vegetation diversity and that fact seemed to be expressed in the dispersion of NE samples in the PCA (white dots in Figure S1). The variations of data in the site-by-site data analysis suggest that NE were more affected by location than agricultural sites (data not shown). Considering that the aim of this work was focused on the discrimination power of lipids profiles between different agricultural practices, and not the analysis of the natural environments *per se*, we ran a new two-way ANOVA excluding NE data. In this way, no interaction was found between treatments and locations either in summer or winter; thus, locations could be considered as true replicates of the treatments HR and LR. With this analysis, it was possible to find significant differences ($P < 0.05$) between HR and LR in winter samples for almost all taxonomic groups based on PLFA: total bacteria, Gram-positives, Gram-negatives, Arbuscular mycorrhiza fungi; actinomycetes and fungi groups showed greater values in HR than in LR sites, but the differences were slightly less significant ($P = 0.0627$ and 0.0932, respectively); Gram-positive bacteria were similar between NE and HR treatments in winter samples ($P = 0.116$). The fungal/bacterial ratio was similar for the three treatments in both seasons.

Discriminant analysis using taxonomic groups of PLFA showed an improved soil management separation in winter compared to summer, HR samples settled in the middle of LR

Table 1 Mean concentration (in nanomoles per gram of soil) of taxonomic groups of PLFA in sites under three agricultural treatments (NE: natural environment, HR: high rotation practices, LR: low rotation practice)

	Summer			Winter		
	NE	HR	LR	NE	HR	LR
Total PLFA	616b	396a	394a	296c	238b	185a
Total bacteria	213b	141a	129a	112c	90b	65a
Gram positive	134b	89a	83a	63c	54b	41a
Gram negative	75b	50a	43a	46c	35b	24a
Actinomycetes	71b	50a	50a	32c	29b	23a
Fungi	17b	9.8a	8.8a	6.2b	4.5a	3.3a
AMF	18b	10a	10a	8.5c	6.2b	4.5a
Protozoa	3.5a	3.0a	2.3a	0.3a	0.2a	0a
Fungi/bacteria ratio	0.09a	0.08a	0.07a	0.06a	0.05a	0.05a

Different letters indicate significant differences for $P = 0.05$.

and NE sites along the first discriminant axis. No separation between HR and LR was found in summer although these were separated from NE samples. Large data dispersion was also observed in the summer sampling (data not shown).

The classification and regression tree analysis based on taxonomic groups showed a seasonal variation for the PLFA pattern. In summer, HR treatment was associated with smaller levels of total bacteria compared to NE and high levels of protozoa compared to LR (if fungi level were also low) (Figure 2a). In winter, there was a dominant role of Gram-negative bacteria in the separation of treatments (Figure 2b), with high levels for NE, intermediate for HR and low for LR.

Considering fatty acid grouping based on chemical structure, the *branched chain* PLFA group was significantly greater in HR (58 nmol/g) than in LR (41 nmol/g) in the winter sampling. The *straight chain* PLFA had significant differences along NE>HR>LR (57, 44 and 36 nmol/g, respectively).

Neutral lipids fatty acids

Discriminant analysis of NLFA profiles separated soil managements either in summer (Figure 3a) or winter (Figure 3b). The separation between HR and LR treatments was achieved along the second axis, where the fatty acids with greater loadings for HR were *20:4* and *14:1w5c* in summer, *14:1w5c* and *17:1w8c* in winter and *16:0anteiso*, *17:0iso* and *17:0 10Me* when data from both seasons was pooled (Table S2).

A principal component analysis of NLFA profiles showed no clear-cut separation among agricultural managements (Figure S2) at any season, although NE samples appeared mostly scattered in the graph and slightly separated from agricultural soil samples.

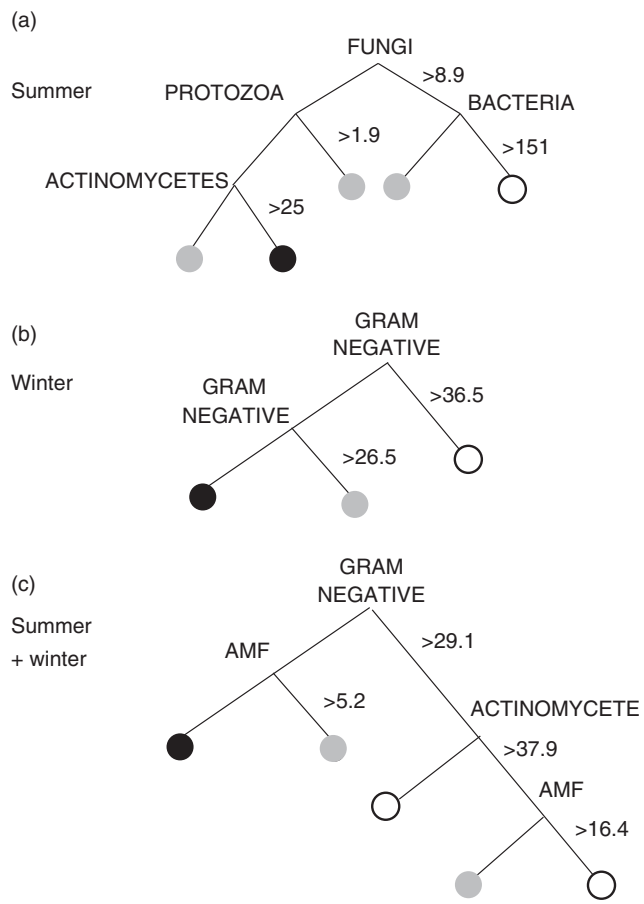

Figure 2 Classification and regression tree for the concentrations of taxonomic groups of PLFA. (a) summer; (b) winter (c) and combining all samples from both seasons; colour of symbols: white, natural environment (NE), grey: high crop rotation (HR), black: low crop rotation (LR).

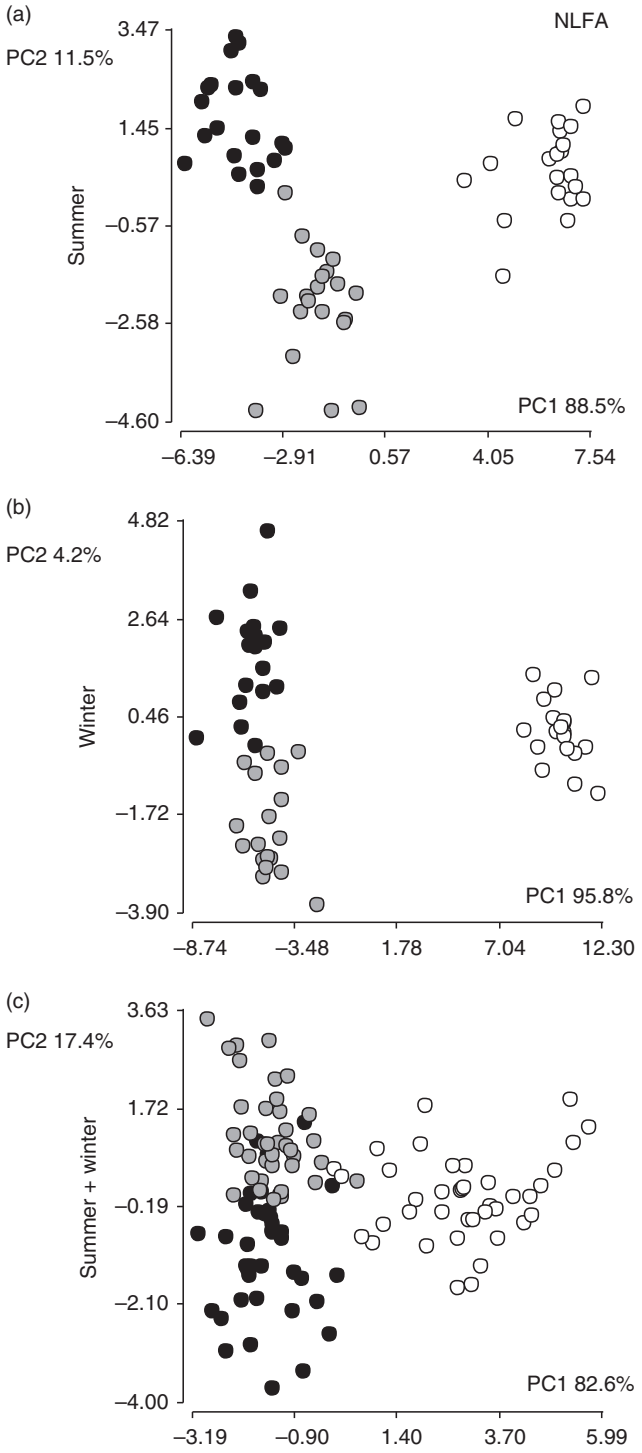

Figure 3 Discriminant analysis of individual NLFA for data from samples taken in summer (a), winter (b), and combining all samples from both seasons (c). Symbols' colours: white: samples from natural environments (NEs) sites, grey: samples from high crop rotation (HR) sites, black: samples from low crop rotation (LR) sites.

The analysis of individual fatty acids within the NLFA profiles showed that in summer, the fatty acid *20:0* was the only NLFA with significant different values between HR and LR (HR>LR). In winter, significant differences were also observed between HR and LR for common (*12:0iso*, *13:0*, *14:0iso* and *17:1w8c*) and rare (*15:1iso G*, *17:1anteisoB*, *18:1w7c11Me* and *unknown 13.565*) fatty acids (Table S2). The straight chain fatty acids *18:0* and *20:0* had a significant trend NE>HR>LR. In this way, *20:0* was the only fatty acid with a significant NE>HR>LR trend in both seasons (Figure 4), and this observation is highlighted by the fact that, for this fatty acid, no significant location–land-use interactions were found in summer ($P = 0.128$) and in winter ($P = 0.065$).

The mycorrhizal biomarker (*16:1w5c*) present in NLFA had significantly greater concentration in NE than in agricultural soils and no differences between HR and LR were found either in summer (60 and 59 nmoles/g, respectively, $P = 0.913$) or in winter (14 and 11 nmoles/g, respectively, $P = 0.243$).

Classifying and grouping NLFA based on their chemical structure (Table S1) improved the separation of treatments in a PCA (data not shown) compared to PCA of single NLFAs (Figure S2). NE sites were characterized by high levels of STRAIGHT chain and MUFA fatty acids in both sampling seasons (Table 2). The two-factor ANOVA of NLFA groups showed some significant interactions between treatment and location in both seasons (Table S5), while these interactions almost disappeared when NE data were excluded from the analysis in the summer samples and it was reduced to fewer groups in winter samples (Table S5). There were no significant differences between HR and LR

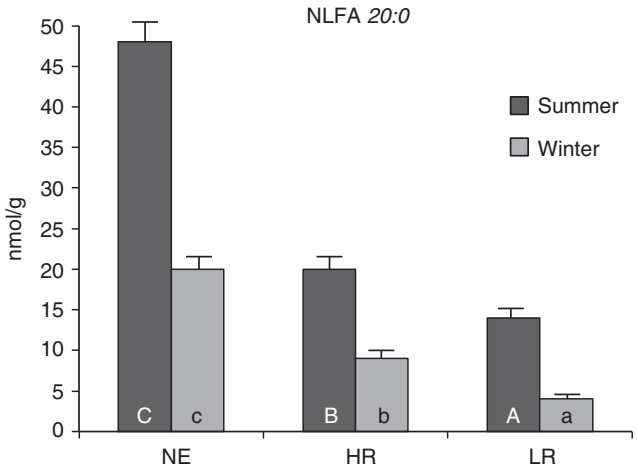

Figure 4 Concentration values of the fatty acid *20:0* present in the neutral lipids fraction. Averages for samples from summer (dark grey) and winter (light grey). Different letters indicate significant differences for $P = 0.05$ among treatments within each sampling season. Column bars correspond to the standard error.

treatments for single groups of NLFA but the NLFA ratios BRANCHED/PUFA, MUFA/PUFA and STRAIGHT/PUFA were significantly greater in HR than in LR, in the summer sampling (Table 2). A trend HR>LR was found in almost all NLFA chemical groups, especially in winter (Table 2).

In winter samples, the total amount of NLFAs was remarkably greater in HR treatment compared to LR, with a significant trend NE>HR>LR and no interaction in the two-factor ANOVA ($P = 0.059$). In summer, the total NLFAs were greater in NE than in agricultural treatments, with no difference between HR and LR.

These observations were also confirmed by the classification and regression tree analysis (Figure 5), where a strong dominance of STRAIGHT chain fatty acids in NE sites was observed. Low levels of PUFAs and high levels of BRANCHED fatty acids characterized HR sites in summer; while in winter HR sites were associated with high levels of relatively rare fatty acids ('OTHERS' in Figure 5b, Table 2). A discriminant analysis of NLFA grouped by their chemistry also showed a separation of HR and LR sites along the second axis, where HR sites correlated with CYCLO and STRAIGHT chain lipids in both seasons, and LR sites had high levels of PUFA and METHYL fatty acids groups (data not shown).

Discussion

The analysis of soil lipid fractions performed in the present study was sensitive enough to discriminate between different no-till agricultural managements at the level of crop rotation in different sites situated across a 400 km east–west transect in the central part of Argentinean best agricultural prairies, corroborating the hypothesis that different soil management would produce a differential soil lipid signature. The differences in soil fatty acids concentrations and profiles were found both in the PLFA and in the NLFA fractions, the latter being currently ignored in most studies about soil or soil microbial ecology.

The total amount of soil PLFAs is a good indicator of viable microbial biomass (White *et al*., 1998; Zelles, 1999), showing a suitable correlation with other methods of microbial biomass assessment like substrate-induced respiration (Bååth & Anderson, 2003), fumigation-extraction (Bailey *et al*., 2002), fumigation-incubation (Feng *et al*., 2003) and total adenine nucleotides (Zelles, 1999). In this work, the use of *good agricultural practices,* on the basis of high crop rotation under no-till, increased the soil microbial activity, although the difference was significant only in winter. Both bacterial and fungal biomarkers were greater under HR, but the fungal/bacterial ratio was similar for the three land-use treatments, which is in agreement with reports in no-till soils with soybean, sugarcane (Montecchia *et al*., 2011), and continuous cotton (Feng *et al*., 2003). Greater microbial biomass in reduced or no-tilled soils than in conventional tilled soils are usually reported (van Groenigen *et al*., 2010), but we have not found reports about differences between no-tilled soils varying in rotation intensity.

The fatty acid *16:1w5c* is broadly considered a mycorrhizal biomarker, although it can have a background origin from Gram-negative bacteria (Olsson, 1999). In our work, *16:1w5c* had a NLFA/PLFA ratio higher than 1 in all treatments (data not shown), supporting its mycorrhizal

Table 2 Comparison of concentrations of chemical groups of NLFA, among different land uses, as average among all locations

	Summer			Winter		
	NE	HR	LR	NE	HR	LR
Total NLFA	6032b	2925a	2851a	1553c	868b	564a
MUFA	781b	371a	365a	172b	80a	59a
PUFA	272b	117a	161a	22b	16a	12a
Hydroxy	27b	7.9a	7.5a	3.8b	0.8a	0.4a
Methylated	81b	49a	41a	29c	19b	10a
Cyclo	38b	27a	18a	18c	12b	4a
Straight chain	541b	235a	222a	154c	71b	48a
Branched chain	226b	138a	114a	83c	58b	33a
Others	49b	33a	24a	30b	28b	15a
Unknown	2.2a	1.0a	1.0a	7.9a	5.3a	5.1a
Branched /PUFA	0.99b	1.15b	0.77a	4.0b	3.4ab	2.7a
MUFA / PUFA	3.6b	3.2b	2.5a	7.9b	5.2a	4.7a
Straight /PUFA	2.4c	2.0b	1.5a	7.4b	4.5a	4.1a

Different letters indicate significant differences ($P = 0.05$). HR, high rotation practice; LR, low rotation practice. Values are expressed as nanomoles of FA per gram of dried soil.

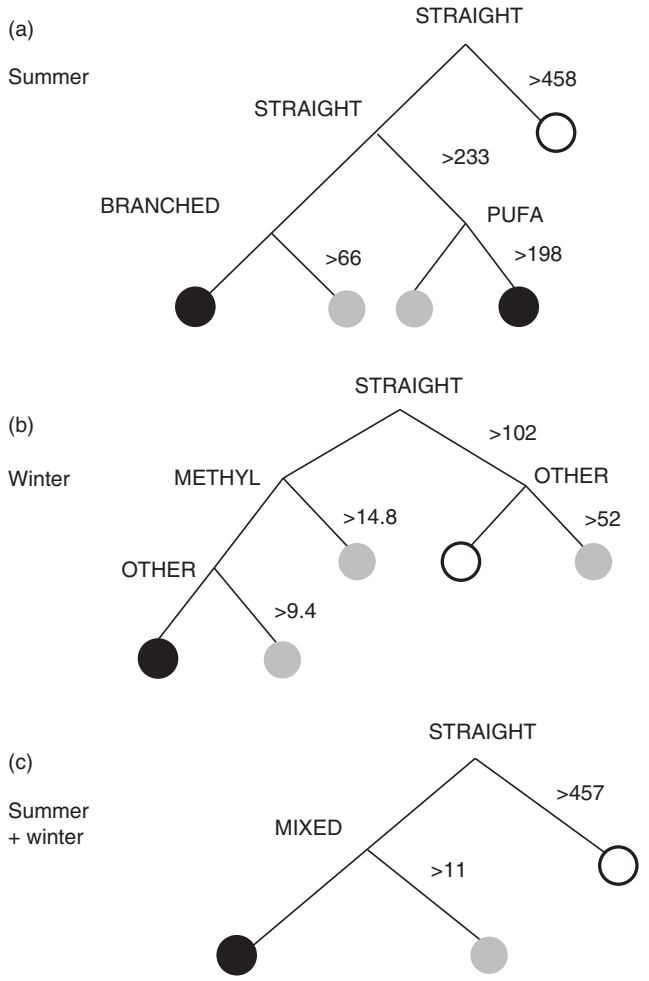

Figure 5 Classification and regression tree for the concentrations of chemical groups of NLFA. (a) summer; (b) winter (c) and combining all samples from both seasons; colour of symbols: white, natural environment (NE), grey: high crop rotation (HR), black: low crop rotation (LR).

origin (Joergensen & Wichern, 2008). *16:1w5c* regularly decreased in the direction NE>HR>LR in the PLFA profile, while no differences between HR and LR in the NLFA profile were found. This difference suggests a better development of active hyphae in HR (and even better in NE), while the development of storage structures (vesicles and spores) only increased in NE. The greater hyphal development in HR than in LR could be associated with a greater carbon input in HR. Gryndler *et al.* (2006), working with whole cell fatty acids analysis, found that organic fertilizer application increased AMF. Drijber *et al.* (2000) did not find differences in *16:1w5c* among tillage treatments, although they found differences between natural environment and agricultural sites.

Straight chain fatty acids, considered common to prokaryotic and eukaryotic organisms, were particularly relevant for land-use discrimination, especially *18:0* and *20:0*. The fatty acid *20:0* in the NLFA fraction, associated with eukaryotes or higher plants (MacNaughton *et al.*, 1999; Zelles, 1999), consistently decreased in the order NE>HR> LR in both seasons. Meriles *et al.* (2009) also found 20:0 associated with no-till but not with reduced-till; while Romaniuk *et al.* (2011) found the fatty acids 14:0, 15:0 and 20:0, with large loads in our discriminant function, as markers of soil use and soil quality in an analysis of whole cell fatty acids extracted from horticultural soils in the Argentinean Pampas. These data show great potential to be part of a soil management indicator for certified agriculture, although the value of this fatty acid as a biomarker of agricultural management could be of limited interpretation due its low specificity to a particular microbial group (Zelles *et al.*, 1992). Despite its small taxonomic potential, the arachidic acid (*20:0*) has been found to be abundant in collembola and nematodes (Chen *et al.*, 2001; Ruess *et al.*, 2005), and also in plant litter and throughfall (Dormaar & Willms, 1992). Thus, its high value would be a consequence of intensifying crop rotation as part of good agricultural management or a footprint of soil fauna increased by this practices.

Although differences between HR and LR were found for some fatty acids recognized as taxonomical biomarkers, significant differences were detected for unidentified fatty acids or relatively rare ones, such as *15:1isoG* and *11Me18:1w7c*. These uncommon fatty acids could be associated with soil microorganisms of the uncultivated microbial pool in the soil. In a complementary study of the same soil samples, using a metagenomic approach to analyse bacteria diversity on the basis of 16s sequence (Figuerola *et al.*, 2012), particular groups of bacteria were detected as markers or indicators of land use as Acidobacteria, *Rubellimicrobium*, Alphaproteobacteria, *Micromonosporaceae*, and some unclassified groups, most of them unculturable bacteria, that could be the source of this uncommon fatty acids that appear as marker of soil use. The use of molecular techniques could help the identification of these unknown fatty acids, as Ritchie *et al.* (2000) did with the rare fatty acid *16:0 N alcohol*.

Alternatively, the mentioned fatty acids as *18:0* and *20:0* and the rare ones that discriminate between high and low crop rotation managements could be also originated from organic matter decomposition and turnover (Otto & Simpson, 2006; Jandl *et al.*, 2007; Weisenberg *et al.*, 2010; Pereira de Assis *et al.*, 2011), which interpretation is more uncertain. However, the PLFA/NLFA procedure that we used releases less fatty acids from soil organic matter than the whole cell fatty acids procedure (Córdova-Kreylos & Scow, 2007). In this line of reasoning, it is worth noting that differential amounts of plant residues are accumulated between different crop rotation regimes, with the largest amounts of plant debris associated with HR managements (data not shown).

A seasonal effect in fatty acid profiles (Figure S3) and concentrations were also observed, with all fatty acids having greater concentrations in summer than in winter. Seasonal differences in lipid profiles are usually expected; as temperature, humidity and nutrient inputs from plants strongly affect soil biological parameters (Liebig *et al.*, 2005). We cannot discard that seasonal differences may be due to the interference of plant residues or rhizodeposition and to changes in humidity affecting the lipid extraction efficiency (Joergensen & Wichern, 2008). Nevertheless, the reduction of lipid concentration was different according to soil management (Table 1), being smaller in all high crop rotation soils, suggesting that the seasonal effect would be a dynamic characteristic of soil lipid footprint instead of a problem of lipid extraction efficiency.

Significant differences between HR and LR were observed only in winter, with LR sites experiencing a greater reduction in lipid concentrations than NE or HR, suggesting that HR management preserve the structure of the microbial community better than LR. The fact that land-use differences were only observed in winter, where the soil was almost naked, suggests a contribution of vegetation towards the soil lipid signature in summer. Feng *et al.* (2003) also found a significative tillage effect (conventional versus no-till) on PLFAs in the winter samples.

It is remarkable that in winter samples the total NLFAs were greater in crop rotation treatment (HR) than in monocropping (LR) suggesting a particular role of neutral lipids fraction in soil functioning. We can speculate that the intensification of soil use with the increase in crop rotation captures more solar energy through photosynthesis than monocropped managed soils, and part of this energy would be reserved as NLFA in soils, contributing to its function along seasons variations. Further studies comparing increasing levels of crop rotation indexes should be carried out to corroborate this idea.

In conclusion, we consider that quantitative and qualitative analyses of soil fatty acids from different lipid fractions provide useful and complementary information to obtain a footprint of different soil use and management, improving soil biochemistry characterization, either considering microbial communities structure (PLFA) or soil functioning (NLFA). The use of metagenomic analysis and isotope labelling linked to this kind of lipid studies at different and increasing indexes of crop rotation, and at different scales of soil fractions could shed more light into our knowledge of soil biological structure and functioning.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Principal Component Analysis of PLFA profiles for samples taken in Summer (A) or Winter (B).

Figure S2. Principal Component Analysis of NLFA profiles for samples taken in Summer (A) or Winter (B).

Figure S3. Principal component analysis of PLFA (A) and NLFA (B) profiles for a combined dataset from both sampling seasons; colour of the symbols: Black: Summer; Grey: Winter.

Table S1. List of individual fatty acids identified by the MIDI system in the PLFA and NLFA lipid profiles, classified into 10 chemical classes.

Table S2. Loading scores in the Discriminant analysis (PC2) for individual **PLFA** and **NLFA** correlated with the HR treatment, listed in decreasing order

Table S3. Comparison of winter mean concentrations of **selected** fatty acids from the NLFA and PLFA profiles, that show significant ($P < 0.05$) differences between HR and LR, as average among all locations.

Table S4. Two factor analysis of variance of taxonomic groups of **PLFA**. P values for location and land use factors and their interaction for three treatments (NE: natural environment; HR: high rotation; LR: low rotation); and the same analysis but excluding NE.

Table S5. Table 1: Two factor analysis of variance of chemical groups of **NLFA**. P values for location and land use factors and their interaction for three treatments (NE: natural environment; HR: high rotation; LR: low rotation); and the same analysis but excluding NE.