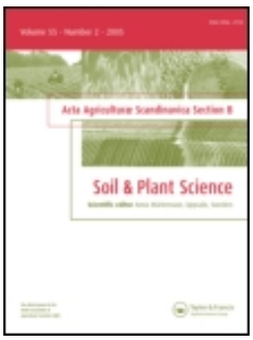

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Acta Agriculturae Scandinavica, Section B - Soil & Plant Science

ISSN: 0906-4710 (Print) 1651-1913 (Online) Journal homepage: http://www.tandfonline.com/loi/sagb20

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To cite this article: R.A. Verdenelli, C.B. Conforto, C. Pérez-Brandán, D. Chavarría, A. Rovea, S. Vargas-Gil & J.M. Meriles (2013) Integrated multivariate analysis of selected soil microbial properties and their relationships with mineral fertilization management in a conservation agriculture system, Acta Agriculturae Scandinavica, Section B - Soil & Plant Science, 63:7, 623-632, DOI: 10.1080/09064710.2013.837193

To link to this article: https://doi.org/10.1080/09064710.2013.837193

Published online: 05 Dec 2013.

Acta Agriculturae Scandinavica, Section B - Soil & Plant Science, 2013
Vol. 63, No. 7, 623–632, http://dx.doi.org/10.1080/09064710.2013.837193

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ORIGINAL ARTICLE

Integrated multivariate analysis of selected soil microbial properties and their relationships with mineral fertilization management in a conservation agriculture system

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(*Received 12 June 2013; final version received 16 August 2013*)

The effect of mineral fertilizer application on soil microbial community was investigated in a conservation agriculture system. The aim of this work was to evaluate how mineral fertilization with nitrogen (N), phosphorus (P), sulfur (S), and micronutrients (M) affects microbial community structure and function. A 10-year experiment, conducted on a typic Hapludoll using six mineral fertilizer treatments (control, CK; PS; NS; NP; NPS; and NPSM) was evaluated in central Argentina. Microbial community structure and function were characterized by phospholipid fatty acids and community-level physiological profiles, respectively. Soil microbial metabolic activity was determined by monitoring microbial respiration, fluorescein diacetate activity (FDA), dehydrogenase activity and phosphatase activity (PHA). NPS and NPSM treatments showed higher total microbial biomass and gram-positive and gram-negative bacteria, but lower fungal biomass than the remaining treatments. Fertilizer treatments without S (CK and NP) showed lower carbon source utilization and Shannon index than the other treatments. In addition, both FDA and PHA significantly increased under NPSM. An integrated PC analysis indicated that sensitive bioindicators were significantly associated with three carbon sources, one metabolic parameter, and six fatty acid bioindicators. These results provide information about the importance of balanced fertilization with P, N, S, and M in promoting microbial biomass, metabolic activity, and functional diversity.

Keywords: Argentina; CLPP; fertilization; microbial properties; PLFA

Introduction

Soil microorganisms play a central role in a wide range of biochemical processes related to soil quality, such as organic matter decomposition, humus formation, and plant nutrient cycling. Soil quality can be defined as "the continued capacity of soil to function as a vital living system, within ecosystem and land use boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal and human health" (Doran & Safely 1997). Variation in soil microbiological properties is usually detected earlier than physicochemical properties. Since soil microorganisms can respond rapidly to agricultural management system, they may be used as indicators of soil quality changes (Hill et al. 2000; Schloter et al. 2003). Examples of the most commonly used microbiological indicators include microbial biomass, respiration, and enzyme activities (Schloter et al. 2003). Another common approach in characterizing soil microbial communities is based on the physiological capability of the heterotrophic community. Community-level physiological profile (CLPP) reflects how microbial communities can

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use a range of carbon substrates, and may also be used to estimate soil functional diversity (Brackin et al. 2013). Changes in microbial community structure can be assessed by both molecular and biochemical approaches. Phospholipid fatty acids (PLFAs) analysis has been used as a culture-independent method for assessing the structure of soil microbial communities and determining gross changes that accompany different agricultural soil disturbances (Hill et al. 2000). The PLFA approach has been demonstrated to be more sensitive than nucleic acid-based techniques (Tscherko et al. 2005; Ramsey et al. 2006). Numerous studies on soil quality have shown the complexity of soil ecosystems and the need to integrate the extremely diverse microbiological properties to determine such quality (Doran & Safely 1997).

It has been widely demonstrated that soil microbial structure and function can be affected by anthropogenic disturbances. Land-use history has a significant impact on the microbial community and is a key component influencing soil community function and structure (Yao et al. 2000). Specifically, several studies have reported that the use of mineral fertilizers in conventional agricultural systems may have induced either direct or indirect changes in microbial biomass (Zhong & Cai 2007), metabolic activities (Liu et al. 2011), and microbial diversity (Wenhui et al. 2007). Zhang et al. (2007) found that mineral fertilizer application stimulated gram-positive bacteria population in paddy soil, as characterized by PLFA profiling. In contrast, Kong et al. (2008) did not find any significant differences in microbial biomass and functional diversity among mineral fertilizer treatments, including the control.

Sustainable agriculture systems are based on conservation management practices such as zero tillage, minimum tillage, organic matter addition, and crop rotation (Diosma et al. 2006). In recent years, most studies have focused on the comparative effects of both mineral fertilization and organic manure practices on the physical, chemical, and biological indicators in the soil. It is generally accepted that the addition of organic manure tends to increase the total microbial biomass through the response of specific microbial groups (Ai et al. 2012; Wakelin et al. 2012). Nevertheless, farmers usually find mineral fertilizers more affordable in response to the rapid economic agriculture development. Today, in many regions of the world, mineral fertilizers are favored over organic fertilizers (Gong et al. 2009). Crop rotation and zero tillage are alternative strategies which are used to increase organic matter and soil microbiological diversity by replacing organic manure practices. The increased organic matter content in soil associated with no-till practices not only improves soil aggregation and water retention but also serves as a substrate for soil microorganisms. However, there is little information about the effect of mineral fertilizers applied to the soil under conservation agriculture management. The aims of this study are to (1) evaluate the effect of different mineral fertilizing treatments on soil quality and (2) detect the microbiological properties that may serve as early and sensitive indicators of changes in soil microbial communities related to mineral fertilization management in a conservation agriculture system.

Materials and methods

Site description and soil collection

The fertilizer experiment was established in Teodelina, Santa Fe Province, Argentina (34°11′S, 61°31′W) in 2000. In the experimental area, average annual rainfall is 965 mm, with more than 60% of that concentrated between mid-October and late February. The average temperature is lowest (10 °C) in August and highest (25 °C) in January. The soil is classified as a typic Hapludol; it has a sandy loam texture with 35.1% sand, 53.2% silt, and 11.8% clay. The experimental design was a randomized block with three replicates. All fertilization treatments were applied to a soybean/maize rotation in a nontillage system. In all treatments, herbicides commonly used for no-till cropping systems were applied in equal amounts. Maize was planted in the beginning of the spring (September–October), whereas soybean was planted later in spring (November–December). Both crops were harvested at the beginning of autumn (March–April). Each treatment plot was 30 × 70 m in area. The six treatments were: PS (phosphorus, sulfur, 40 kg P ha^{-1} + 24 kg S ha^{-1}), NS (nitrogen, sulfur, 175 kg N ha^{-1} + 24 kg S ha^{-1}), NP (nitrogen, phosphorus 175 kg N ha^{-1} + 40 kg P ha^{-1}), NPS (nitrogen, phosphorus, sulfur 175 kg N ha^{-1} + 40 kg P ha^{-1} + 24 kg S ha^{-1}), NPSM (NPS + micronutrients: 12 kg Mg ha^{-1} + 1 kg B ha^{-1} + 2 kg Zn ha^{-1} + 2 kg Cu ha^{-1}), and CK (unfertilized control). Soil samples were collected during the soybean-growing season in April 2011. In each plot of the six treatments, six soil cores were randomly collected from the top 15 cm and mixed to make a composite sample. The random sampling method was used to ensure representative sampling from the different treatments. Soil was then passed through a 2-mm sieve and immediately stored in a cooler at 4 °C. In the laboratory the stone-free samples were thoroughly homogenized and divided into several subsamples, some of which were stored at −20 °C for PLFA.

Microbial community profiles

PLFA analysis was carried out according to the process described by Bossio and Scow (1998). Soil samples (10 g dry soil) were extracted overnight with 23 ml of a one-phase buffer containing 1:2:0.8 ratio of chloroform, methanol, and phosphate buffer (8.7 g K_2HPO_4 l^{-1}, pH 7.4). The total lipid extract was fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid chromatography. The polar lipid fraction containing the phospholipids was isolated and transesterified into fatty acid methyl esters (FAMEs) using a mild acid methanolysis reaction. FAMEs were analyzed by capillary gas chromatography with flame ionization detection on a PerkinElmer (Clarus 500 GC) using a 30 m nonpolar column (Col-Elite-5), where both the injector and detector were maintained at 290 °C. The column temperature was programmed with an initial temperature of 180 °C for 4 min and then ramped up at a rate of 4 – 280 °C. Methyl nonadecanoate was used as a quantitative internal standard. The separated FAMEs were identified and quantified by chromatography retention time, using standard bacterial acid methyl ester mix (Supelco, Supelco UK, Poole, Dorset, UK). For each sample, the abundance of individual FAMEs was expressed as nmol PLFA g^{-1} dry soil. Standard nomenclature was used, where the number before the colon represents the number of C-atoms and the number after the colon shows the number of double bonds and their location (ω). The prefixes "cy," "i," and "a" indicate cyclopropyl groups, and iso- and anteiso-branching, respectively. Cis ("c") and trans ("t") configuration in double bonds are indicated after the location. Total microbial biomass was estimated as the total extractable PLFAs. The branched fatty acids i15:0, a15:0, i16:0, i17:0, and a17:0 were chosen to represent gram-positive bacteria. The monoenoic and cyclopropane fatty acids 16:1ω9, 16:1ω11, cy17:0, 18:1ω9c, 18:1ω9t, and cy19:0 were chosen to represent gram-negative bacteria. The fatty acid 10 methyl 18:0 and the polyenoic 18:2ω6,9 were used as indicators of actinomycetes and fungal biomass, respectively.

CLPPs

Catabolic response profiles were estimated according to the maximum respiratory response technique (Montecchia et al. 2011). The selected carbon sources used accounted for a range of quality and complexity. Substrates consisted of six monosaccharides (L-glucose, D-glucose, D-mannose, D-galactose, D-xylose, and D-fructose), one disaccharide (D-lactose), four amino acids (DL-tryptophan, L-arginine, L-asparagine, and L-lysine), and one vitamin (thiamine). Briefly, stock solution was prepared with each carbon source in deionized water (3 g l^{-1}), filter sterilized, and stored at 4 °C in the dark. A basal medium consisted of K_2HPO_4 (21 g l^{-1}), KH_2PO_4 (9 g l^{-1}), $MgSO_4$ (0.3 g l^{-1}), $(NH_4)_2SO_4$ (1.5 g l^{-1}), $CaCl_2$ (0.03g l^{-1}), $FeSO_4$ (0.015 g l^{-1}), $MnSO_4$ (0.0075 g l^{-1}), and $NaMoO_4$ (0.0075 g l^{-1}). Five grams of each soil sample were suspended in 10 ml of filter-sterilized deionized water. Each individual microplate was prepared with stock solution (60 µl), basal medium (60 µl), and tretrazolium violet (0.0075%). Finally, soil suspensions (120 µl) were added into the wells, and plates were immediately incubated at 25 °C. Readings were obtained from the plate at 24, 48, and 72 h (Wallac 1420 Victor2 multi-label counter, Perkin Elmer Life Sciences). The rate between the absorbance data (590 nm) and the baseline value was used to estimate a normalized relative absorbance unit (NRAU). Statistical analysis was performed only on data obtained at 72 h. Negative scores were set to zero.

Microbial respiration and enzyme activities

Soil microbial respiration was determined as potentially mineralizable C (CO_2–C respiration) according to Alef (1995). The amount of CO_2 released was measured from chloroform-treated and untreated soil samples (ca. 20 g). Treated samples were previously fumigated with chloroform, inoculated with fresh soil, and incubated with NaOH 0.2 M at room temperature in the dark for no longer than two weeks. Released CO_2 was estimated using HCl 0.2 N. For the quantification microbial respiration, flasks that did not contain soil served as the control treatment.

Microbial activity was estimated by hydrolysis of fluorescein diacetate activity (FDA), according to Adam and Duncan (2001). Briefly, 2 g of soil and 15 ml of 60 mM potassium phosphate buffer pH 7.6 were placed in a 50-ml conical flask. Substrate (FDA, 1000 µg ml^{-1}) was added to start the reaction. The flasks were placed in an orbital incubator at 30°C and 100 rpm for 20 min. Once removed from the incubator, 15 ml of chloroform/methanol (2:1 v/v) was immediately added in order to terminate the reaction. The contents of the conical flasks were then centrifuged at 2000 rpm for 5 min. The supernatant was then filtered and measured at 490 nm on a spectrophotometer.

Dehydrogenase activity (DHA) was determined according to García et al. (1997). Briefly, 1 g of soil at 60% of field capacity was exposed to 0.2 ml of 0.4% INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride) in distilled water at 22 °C

for 20 h in the dark. The INTF (iodonitrotetrazolium formazan) formed was extracted with 10 ml of methanol by shaking vigorously for 1 min and filtered through a Whatman No. 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

Acid phosphatase was assayed using 1 g soil, 4 ml 0.1 M universal buffer (pH 6.5), and 1ml 25 mM p-nitrophenyl phosphate (Tabatabai & Bremner 1969). After incubation at 37 ± 1 °C for 1 h, the enzyme reaction was stopped by adding 4 ml 0.5 M NaOH and 1 ml 0.5 M $CaCl_2$ to prevent the dispersion of humic substances. Absorbance was measured in the supernatant at 400 nm. Enzyme activity was expressed as micrograms of p-nitrophenol-released per h^{-1} g^{-1} soil.

Statistical analysis

Data were analyzed using InfoStat Professional version 2012 (Universidad Nacional de Córdoba, Argentina). Differences between fertilization treatments for microbial biomass, respiration, enzyme activities, CLPPs, and PLFAs were evaluated by one-way analysis of variance (ANOVA) with least significant difference (LSD) post hoc test. A normality test was performed for all variables prior to ANOVA (Shapiro–Wilk test) and homogeneity of variances was examined by plotting residuals versus predicted values. CLPPs were generated by subtracting the basal response from each individual substrate response and then standardized by dividing this number by the total substrate response of each sample. Changes in the Shannon–Weaver diversity index of microbial communities in all treatments were estimated as follows:

$$H = -\sum P_i \log P_i$$

where P_i is the ratio of the activity on each substrate to the sum of activities on all substrates. The microbial groups estimated by individual fatty acids are reported as nmol% g^{-1} soil. This means that the sum of all the absolute amounts of lipid biomarkers totals 100%. This calculation was made to standardize the amounts of individual PLFAs (nmol g^{-1} soil), so that the differences observed between them would not simply be due to a difference in the amount of total PLFAs. Principal components analysis (PCA) of CLPPs and PLFA profiles was used to reduce the number of microbial variables, by extracting the most important principal components separately. In addition, soil microbial respiration and enzyme activities, community functions (CLPPs), and structure (PLFA) were also included in a single PCA in order to evaluate the interaction between these parameters and to pick out the microbial variables that show greater discrimination between the fertilization treatments. Finally, correlations between microbial indicators and PC coordinates were calculated to identify variables whose gradients were represented by PCs 1 and 2.

Results

Microbial community structure

The PCA of PLFA (nmol%) for 14 FAMEs explained 40% and 18% of the variance in PC1 and PC2, respectively (Figure 1). PLFA profiles from NPS and NPSM formed a single cluster that was clearly separated from the other treatments. PC2 separated NS from the other double-fertilizer treatments (PS and NP) and CK. PC analysis identified

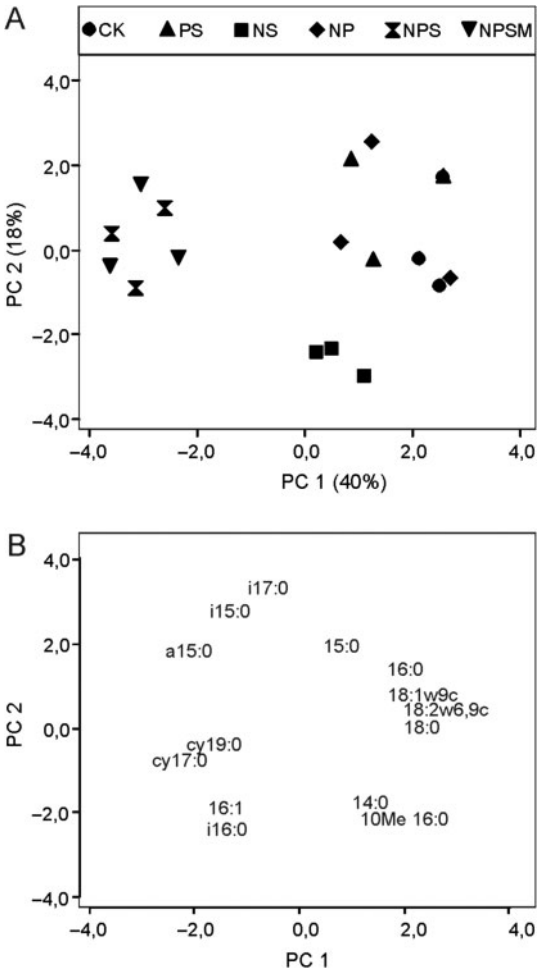

Figure 1. (A) Principal component analysis of phospholipids fatty acid (PLFA) profiles as influenced by mineral fertilization treatments. CK, unfertilized control; PS, application of P plus S fertilizers; NS, application of N plus S fertilizers; NP, application of N plus P fertilizers; NPS, application of N, P plus S fertilizers; NPSM, application of N, P, S plus micronutrients fertilizers. (B) PCA showing loading values of selected PLFAs of the treatments plots given in (A).

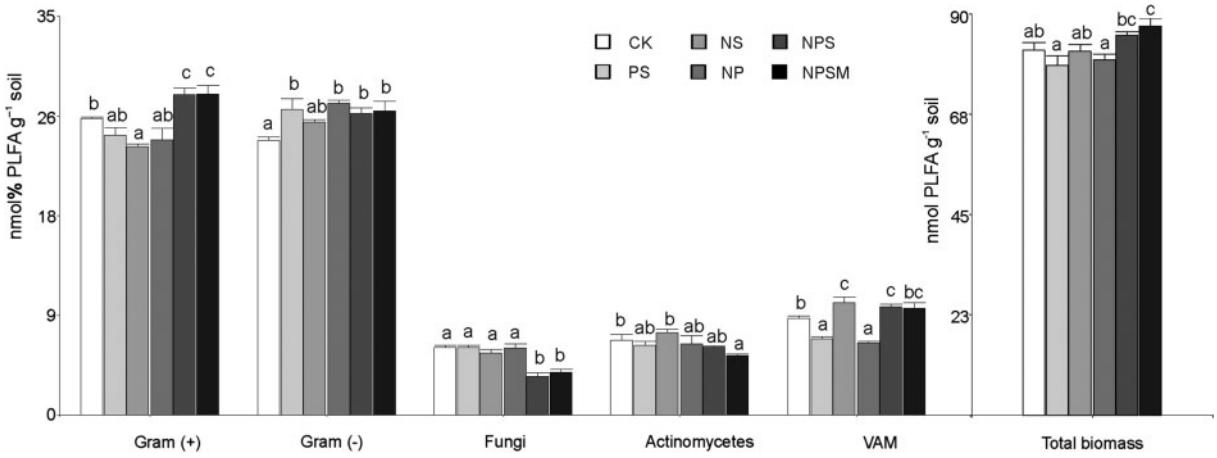

Figure 2. Microbial taxonomic groups (nmol% de PLFA g^{-1} soil) and total microbial biomass (nmol de PLFA g^{-1} soil) of soil as influenced by mineral fertilization treatments. Bars topped with the same letter are not significantly different according to the LSD test ($P \leq 0.0$). CK, unfertilized control; PS, application of P plus S fertilizers; NS, application of N plus S fertilizers; NP, application of N plus P fertilizers; NPS, application of N, P plus S fertilizers; NPSM: application of N, P, S plus micronutrients fertilizers.

fatty acids that were important in explaining the variability in PLFA profiles (Figure 1B). These data and loading values of selected PLFAs indicated that cyclopropane fatty acids (cy17:0 and cy19:0) and branched fatty acids (particularly a15:0 and i15:0) increased in NPS and NPSM treatments, whereas the unsaturated (16:2ω6,9 and 18:1ω9) and three saturated fatty acids (14:0, 16:0, and 18:0) increased in the other treatments and CK. The loading for PC1 and PC2 also revealed FAMEs responsible for group separation. Thus, the branched PLFAs associated with gram-positive bacteria were most abundant under NPS and NPSK treatments (Figures 1B and 2). Although less pronounced, a similar result was observed for gram-negative bacteria under the same treatments. The saturated fatty acids 14:0, 16:0, and 18:0 were not associated with any particular group of microorganisms. The fatty acid 18:2ω6,9, usually considered as a fungal biomass indicator, showed a strong positive correlation with PC1, indicating that fungal biomass decreased under NPS and NPSM treatments (Figures 1B and 2). The abundance of actinomycetes was also significantly reduced in the NPSM treatment in comparison with CK. The plots under NS, NPS, and NPSM showed a similar abundance of vesicular-arbuscular mycorrhizae (VAM). Total microbial biomass, estimated as total PLFAs, was significantly higher under NPSM (Figure 2).

Microbial functional profiling and diversity

CLPPs were also significantly affected by mineral fertilizer treatments (Figure 3). PCA of the functional profiles explained 47% of the total variation (PC1 29% and PC2 18%). CK and NP treatments were located on the left-hand side of the plot. NPSM also formed a separate cluster, located mainly in the first quadrant. The remaining treatments (PS, NS, and NPSM) formed a single cluster located in the lower left quadrant. The separation of the samples into distinct clusters was further explained by identifying those carbon sources that had the highest eigenvector in both PC dimensions (Figure 3B). Two carbohydrates (mannose and galactose) tended to show lower NRAU values in CK and NP than in the remaining treatments containing S (PS, NS, NPS, and NPSM) (Table 1). CK also showed the lowest values of lysine and thiamine. Microbial functional diversity (Shannon index) tended to be significantly higher under fertilization treatments than under CK (Table 1).

Microbial metabolic activities

The microbial metabolic parameters selected are shown in Table 2. A shift in soil microbial activities usually corresponds to changes in soil nutrient sources. In this study, the addition of several combinations of fertilizers caused significant changes in microbial metabolic activities. Our results showed that the microbial respiration was increased by NPS and NPSM fertilization treatments. Similarly, the highest FDA value was detected in NPSM and the lowest one in CK, with values ranging from 85 to 109 μg g^{-1} soil. There were no significant differences in DHA between CK and the other treatments. Finally, the treatments with NP (NP, NPS, and NPSM) significantly increased PHA in comparison to CK. Thus, the order of PHA was NPSM > NPS > NP, but the differences between these treatments were not statistically significant.

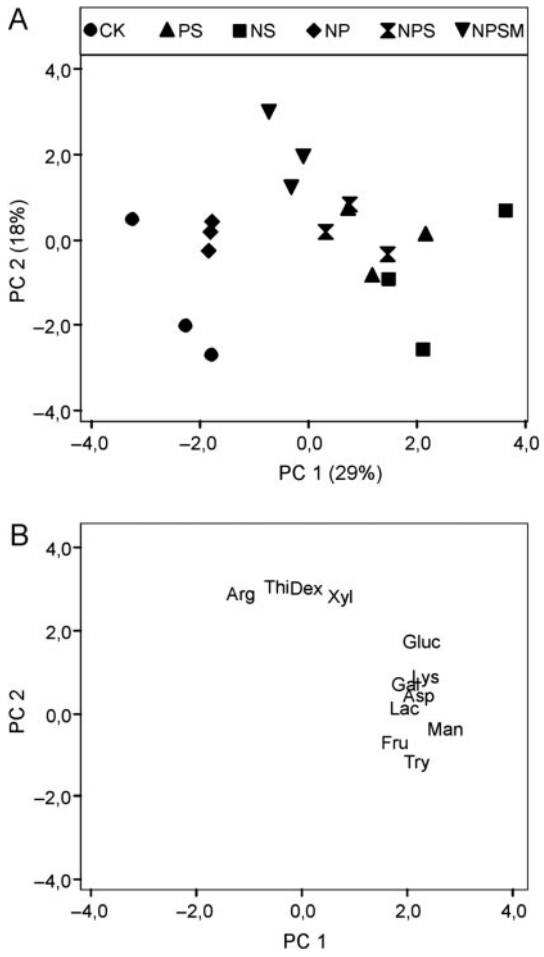

Figure 3. (A) Principal component analysis of community-level physiological profiles (CLPPs) as influenced by mineral fertilization treatments. CK, unfertilized control; PS, application of P plus S fertilizers; NS, application of N plus S fertilizers; NP, application of N plus P fertilizers; NPS, application of N, P plus S fertilizers; NPSM, application of N, P, S plus micronutrients fertilizers. (B) PCA showing loading values of selected carbon sources of the treatment plots given in Figure 1(A). Dex, dextrose; Man, mannose; Fru, fructose; Glu, glucose; Lac, lactose; Xyl, xylose; Gal, galactose; Try, tryptophan; Arg, arginine; Asp, asparagine; Lys, lysine; Thi, thiamine.

Integrated multivariate analysis

The PCA of the full-variable data-set clearly separated fertilizer treatments into three clusters, explaining 42% of the total variability (Figure 4). The first cluster included NPS and NPSM, while the second cluster included PS, NS, and NP. Finally, the third cluster included only CK. PC1 separated the first cluster from CK, PS, NS, and NP. PC2 separated CK from PS, NS, and NP double-fertilizer treatments. Microbial indicators with the highest correlations with scores along PCs 1 and 2 are presented in Table 3. These correlations enabled us to detect the variables that most clearly differentiated the fertilizer treatments. PCA was able to factorize the original data matrix into fewer dimensions. A total of 14 from the 37 original variables were significantly correlated to axes PC1 and PC2 ($P \leq 0.001$). PC1 was principally weighed by PLFA-related variables (a15:0; cy17:0; 18:2ω6,9; 18:1ω9, 18:0, and cy19:0), several microbial taxonomic groups (gram-positive bacteria, fungal biomass, and total microbial biomass) and microbial respiration activity. On the other hand, PC2 was principally weighed by the utilization of three carbon sources (lysine, mannose, and galactose), and Shannon index ($P \leq 0.001$). Microbial respiration activity, a15:0, cy17:0, cy19:0, gram-positive bacteria, and total microbial biomass tended to highly increase under NPS and NPSM.

Discussion

The results of this study support the hypothesis that mineral fertilization may alter the microbial community structure under a conservation agriculture system. The amounts of total microbial biomass, and gram-positive and gram-negative bacteria were highest in NPS and NPSM treatments, suggesting that the prokaryote communities might be stimulated by the application of balanced fertilization.

Table 1. Catabolic response profiles (NRAU)[a] as influenced by mineral fertilization treatments.

	Carbon sources[c]												
Treatments[b]	Dex	Man	Fru	Glu	Lac	Xyl	Gal	Try	Arg	Asp	Lys	Thi	Shannon
CK	6.03ab	0.88a	1.27ab	5.07a	0.61a	0.54a	0.83a	0.70a	5.30ab	4.98ab	0.84a	0.54a	0.89a
PS	6.20ab	1.37cd	1.36ab	5.47a	0.64a	0.59a	1.20c	0.82a	5.41ab	5.29bc	1.09b	0.63ab	0.92b
NS	5.91a	1.50d	1.39b	5.63a	1.06a	0.68ab	1.11bc	2.15a	4.96a	5.38c	1.15b	0.56a	0.93b
NP	5.99ab	1.04ab	1.11a	5.06a	0.70a	0.64a	0.94ab	1.04a	5.22ab	4.78a	1.03b	0.66ab	0.92b
NPS	6.41b	1.23bc	1.27ab	5.37a	0.71a	0.67ab	1.12bc	0.91a	5.29ab	5.38bc	1.07b	0.58ab	0.92b
NPSM	6.38ab	1.07b	1.23ab	5.62a	0.87a	0.89b	0.97ab	1.03a	5.56b	5.14bc	1.03b	0.75b	0.92b

Note: Different letters in the same column indicate significant difference at $P \leq 0.05$.
[a]The rate of the absorbance data and the baseline value was used to estimate a normalized relative absorbance unit.
[b]CK, unfertilized control; PS, application of P plus S fertilizers; NS, application of N plus S fertilizers; NP, application of N plus P fertilizers; NPS, application of N, P plus S fertilizers; NPSM, application of N, P, S plus micronutrients fertilizers.
[c]Dex, dextrose; Man, mannose; Fru, fructose; Glu, glucose; Lac, lactose; Xyl, xylose; Gal, galactose; Try, tryptophan; Arg, arginine; Asp, asparagine; Lys, lysine; Thi, thiamine.

Table 2. Soil organic matter, microbial respiration, and enzyme activities as influenced by mineral fertilization treatments.

Treatments[a]	Microbial respiration (mg CO_2 g^{-1})	FDA (μg fls g^{-1})	DHA (μg INTF g^{-1})	PHA (μg nph g^{-1})
CK	0.38a	85.10a	4.13ab	13.28a
PS	0.43a	90.46a	4.39ab	13.20a
NS	0.46a	94.41a	3.93a	14.53ab
NP	0.45a	88.93a	5.43b	17.03bc
NPS	0.57b	85.60a	3.89a	17.10bc
NPSM	0.67b	108.85b	4.46ab	17.56c

Note: Different letters in the same column indicate significant difference at $P \leq 0.05$.
[a]CK, unfertilized control; PS, application of P plus S fertilizers; NS, application of N plus S fertilizers; NP, application of N plus P fertilizers; NPS, application of N, P plus S fertilizers; NPSM, application of N, P, S plus micronutrients fertilizers. FDA, fluorescein diacetate activity, DHA, dehydrogenase activity, PHA, phosphatase activity; fls, fluorescein; INTF, iodonitrotetrazolium formazan; nph, p-nitrophenol.

Thus, the addition of mineral fertilizers could drive changes in microbial community composition through direct competition of microbes for substrates used for growth or respiration purposes (Clegg 2006). Accordingly, it has been frequently reported that soil bacteria, in particular gram-negative bacteria, seem to be a sensitive indicator of soil fertility and sustainability in a management system (Zhong et al. 2010). Zhang et al. (2007) indicated that the addition of NPK increased gram-negative fatty acid indicators (cy17:0 and cy19:0) in comparison to other double-fertilizer treatments. Other authors have also reported that the addition of the both mineral and organic fertilizers may increase total microbial biomass (Hu et al. 2011; Liu et al. 2011). Total PLFA has been found to be a sensitive indicator of perturbations to the soil microbial biomass, and has been found to decrease in soils following chloroform fumigation (Zelles et al. 1997).

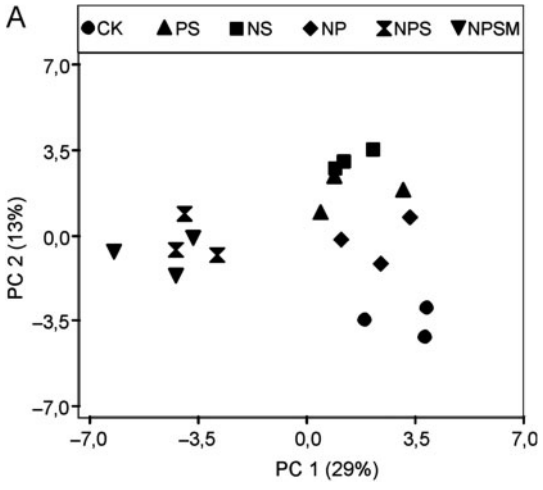

Figure 4. Full-variable multivariate analysis of soil as influenced by mineral fertilization treatments. CK, unfertilized control; PS, application of P plus S fertilizers; NS, application of N plus S fertilizers; NP, application of N plus P fertilizers; NPS, application of N, P plus S fertilizers; NPSM, application of N, P, S plus micronutrients fertilizers.

The increase in total observed microbial biomass in the present work may be due to the fact that fertilization promotes rice shoot and root growth and enhances root exudation to soil (Zhong et al. 2010). We had expected to find a positive relationship between treatments containing P (NP, PS, NPS, and NPSM) and VAM populations (Wakelin et al. 2012). However, VAM population was significantly increased only under NPS and NPSM, with no observed relationship existing between VAM populations and the remaining double-fertilizer treatments containing P. This result suggests that a balanced fertilization with N, P, and S may also play an important role in regulating VAM populations in a conservation agriculture system. The

Table 3. Microbial parameters significantly correlated with principal components 1 and 2 of full-variable PC plot.

	Correlation with	
Microbial parameter	PC1	PC2
Microbial respiration	−0.80**	0.04
Dextrose	−0.52*	−0.01
Xylose	−0.49*	0.16
Mannose	−0.02	0.89**
Lysine	−0.30	0.72**
Galactose	−0.12	0.73**
Shannon	−0.28	0.70**
a15:0	−0.76**	−0.30
cy17:0	−0.88**	0.20
18:2ω6,9	0.88**	0.14
18:1ω9	0.92**	−0.21
18:0	0.66**	−0.27
cy19:0	−0.75**	0.40
i16:0	−0.48*	−0.17
16:1	−0.56*	−0.20
16:0	0.60*	0.13
10Me 16:0	0.55*	0.22
Gram-positive	−0.74**	−0.55*
Fungal biomass	0.89**	0.09
Actinomycetes	0.58*	0.39
VAM	−0.53*	0.04
Total biomass	−0.77**	−0.25

*Significant correlation ($P \leq 0.05$).
**Highly significant correlation ($P \leq 0.001$).

amounts of fatty acid 18:2ω6,9, a fungal biomass indicator, decreased under NPS and NPSM treatments. Zhang et al. (2007) found that a triple-fertilizer application decreased fungal biomass in comparison with a double-fertilization system, which agrees, in part, with our result. However, the cause of this decrease is unknown and needs further examination to be elucidated.

Our results also indicate that NPSM application may reduce actinomycete populations. Studies on the relationship between fertilizer application and actinomycetes population have provided contradictory results. Zhang et al. (2007) reported that the abundance of actinomycete population was reduced under a double-fertilization treatment but relatively increased under an NPK treatment. Zhong et al. (2010) observed no relationship between actinomycete population and mineral fertilization with NPK combinations. The results of this present study suggest that actinomycete populations may be influenced by the application of M. Thus, the amounts of M in the soil may help to explain the inconsistencies observed in the previous works.

CLPPs is a fast-screening method used to detect differences in the utilization of carbon sources (Montecchia et al. 2011). This method has been subject to criticism, principally due to the arbitrary selection of carbon sources (Glimm et al. 1997). However, similar methods have been successfully used to evaluate the shift in soil microbial community in different soil management practices, including mineral and organic fertilizer treatments (Zhong & Cai 2007). Our results indicate that combinations of N, P, and S fertilizers may also alter the ability of the microbes to use soil carbon source and thus to modify functional diversity. In particular, fertilizer treatments without S (CK and NP) showed a reduced carbon source utilization and Shannon index in comparison with the other treatments. Although S is an important macroelement, excessive amounts of this fertilizer can be toxic to plants and can alter microbial function and structure (Zheng et al. 2011). Few studies have dealt with the effect of S fertilizer on biological properties; however, the incorporation of S to soil is a common practice in our region (Wyngaard et al. 2012). Our findings agree with those reported by Zhao et al. (2008), in that S fertilizer application affected S metabolism, and increased the amount of soil microorganisms and their metabolic activities. Other authors reported that the application of Biofer, an organic fertilizer with 7% S containing by-products, tended to increase arylsulphatase activity (Elfstrand et al. 2012). Similarly, Wu and Xiao (1999) demonstrated that S fertilizers incorporated into soils may be rapidly immobilized by the microbial biomass or transformed into soil organic fractions. The mechanisms through which inorganic S fertilizer induces changes in microbial functional diversity are not clear at present. However, we hypothesize that, at a microbiological level, S fertilizer would promote microbial conversion of soil organic matter with the concomitant increase in soil microbial activity and total microbial biomass (Zheng et al. 2011). The Shannon index provides information about the pattern of carbon source utilization by the soil microbial community (Harch et al. 1997). In our study, fertilization was found to alter the Shannon index with respect to unfertilized soil. Although fertilization resulted in microbial functional diversity increase, it was not possible to discriminate between fertilizer treatments.

Our data-set also suggests that the increase in nutrient contents may stimulate microbial activity and soil microbial respiration. A relationship between soil microbial respiration and nutrient content has been reported by other authors, who emphasized the sensitivity of this parameter in assessing soil quality (Masto et al. 2006; Li et al. 2007; Liu et al. 2011). Except for DHA, the impact of mineral fertilizers on enzyme activities was more obvious and consistent under NPSM fertilizer than under NPS and double-fertilizer treatments. Both FDA and PHA significantly increased when the NPSM was applied. It is widely accepted that FDA hydrolysis provides information about total microbial activity, as this enzyme involves both exoenzymes and membrane-bound enzymes (Adam & Duncan 2001). Thus, our results suggest that soil micronutrient content could play a major role in regulating these generalist enzymes. Although several studies have been conducted to evaluate the effect of M on crop growth and yield, the effect of M on soil microbial activities has been poorly addressed. Bilen et al. (2011) reported a positive correlation between boron application and several soil microbial activities; such correlation agrees in part with our results. The increase in soil enzymatic activity may be associated with the easily biodegradable organic matter, which promoted microbial biomass and soil enzyme activities (Perucci 1992). However, the effect of combinations of M on diverse soil environments is unclear and requires further investigation.

The aim of variable reduction is to make data-set analysis more manageable and straightforward (Ye & Wright 2010). The application of an integrated multivariate analytical method in our study suggested that the mineral fertilization may alter metabolic, functional, and structural aspects of the microbial community, especially in the NPS and NPSM treatments, when compared to double-fertilizers (PS, NS, and NP). Application of PCA

successfully reduced 37 biochemical variables to two principal components. According to the results of cluster analysis, the most sensitive bioindicators were related to three carbon sources, one metabolic parameter and six fatty acid bioindicators. Discrimination in microbial properties may have been directly related to variations in soil chemical properties. However, since soil microorganisms may respond rapidly, they usefully reflect anthropogenic disturbances and are, therefore, taken into consideration when monitoring soil quality (Schloter et al. 2003). Thus, microbial communities can provide an integrated approach to soil quality evaluation, an aspect that frequently cannot be obtained by estimating physical and chemical properties of the soil (Winding et al. 2005).

In conclusion, this study examined the consequences of mineral fertilizer applications on soil microbial community under a conservation agriculture system. The application of both NPS and NPSM treatments increased the total microbial biomass and several metabolic activities, principally microbial respiration. In addition, S deficiency appears to be associated with a reduction in microbial catabolic activity. The results obtained in this work contribute information about the importance of balanced fertilization with P, N, S, and M in promoting microbial biomass, metabolic activity, and functional diversity.

Acknowledgments

The authors thank the members of the Región CREA Sur de Santa Fe, in Teodelina, for all the support given during the development of this study. We also thank the Establecimiento Balducci for providing the field assay. We appreciate the valuable technical support provided by Pablo Molinero. This work was financially supported by CONICET PIP N° 114-200901-00041, by Instituto Nacional de Tecnología Agropecuaria (INTA) through the research projects AERN295582 and PN Suelos, by FONCyT through PICT N° 442, and finally by the Group Región CREA Sur de Santa Fe through the Convenio de Vinculación Tecnológica INTA (IPAVE)-AACREA.

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