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RESEARCH ARTICLE

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Influence of aerobic treated manure application on the chemical and microbiological properties of soil

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Abstract

Aim of study: This study evaluated the effect of the application of liquid aerobic treated manure (continuous liquid composting, CLC) on physical, chemical and biological soil properties, with the objective of monitoring changes induced by soil management with CLC as a biofertilizer.

Area of study: Colonia, Uruguay (lat. 34,338164 S, long. 57,222630 W).

Material and methods: Soil's chemical properties, including nitrogen mineralization potential (NMP) and 15 microbiological properties (microbial biomass carbon, MBC; mesophylic aerobic bacteria; actinobacteria; filamentus fungi; fluorescein diacetate hydrolysis; dehydrogenase; with NMP; acid and alkaline phosphatase; cellulolose degraders; P-solubilizing bacteria; nitrifying; denitrifying and free-living N-fixing microorganisms; glomalin; and soil-pathogenicity index, SPI) were evaluated in two sites with similar cropping history, with one and three years of respective CLC application.

Main results: CLC application had significant effects on soil microbial biomass ($p<0.05$), soil enzyme ($p<0.1$) and functional groups activity ($p<0.05$). SPI decreased in both sites with CLC application. No significant variations were detected for the chemical variables, with the exception of NMP, which was significantly high ($p<0.05$) in soil treated with CLC at both sites.

Research highlights: The improved biological soil properties analyzed (MBC, soil enzyme activities and SPI, together with NMP) emerged as reasonable indicators to assess and monitor the effects of CLC application.

Additional keywords: organic fertilization; microbiological indicators.

Abbreviations used: AcPh (acid phosphatase); AlPh (alkaline phosphatase); CLC (continuous liquid composting); DHA (dehydrogenase); FDA (fluorescein diacetate); GRSP (glomalin related soil protein); MAA (actinobacteria); MAB (mesophylic aerobic bacteria); MBC (microbial biomass carbon) MFF (filamentous fungi); NMP (nitrogen mineralization potential); NCLC (No-CLC); MPN (most probable number); PCA (principal component analyses); $S1_Y$ (site one year); $S3_Y$ (site three years) SOC (soil organic carbon); SPI (soil pathogenicity index).

Authors' contributions: AM: concept and design of the experiments, coordination and supervision of the research project, interpretation of results and drafting of the manuscript. NR, SV, CS and LN: laboratory and field technical assistance, data acquisition. SZ: statistical analysis and critical revision of the manuscript.

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Introduction

In order to achieve a sustainable agriculture, it is necessary to reduce the need for mineral fertilization input. The FAO Voluntary Guidelines on Sustainable Soil Management (VGSSM) encourage the adoption of agricultural practices that build and retain soil organic matter (FAO, 2017). The total or partial replacement of mineral fertilizer with organic amendments is a good practice to solve the problems of the excessive use of mineral fertilizer while improving physical and chemical soil properties, carbon stocks and soil biodiversity (Gattinger *et al.*, 2012; Sradnick *et al.*, 2013). The application of manure and compost on

agricultural lands has shown a positive increase in water and nutrient retention, nutrient cycling, carbon transformation, soil biodiversity, soil structure and soil aggregation while enhancing soil organic matter content (Treonis *et al.*, 2010; Nair & Ngouajio, 2012) and suppressing soil-borne pathogens (Zaccardelli *et al.*, 2013). The supply of manure to agricultural soils is an ancient practice and a well-tested strategy to increase soil organic matter (SOM), replenish basic plant nutrients, improve yield response to fertilizers and restore soil productivity in degraded areas (Schröder, 2005; Rufino *et al.*, 2007; Bogaard *et al.*, 2013; Nezomba *et al.*, 2015). At the same time, excessive manure applications and inefficient manure storage practices can have detrimental effects on the environment at multiple scales, such as the contamination of water and soil resources at local and regional levels, and the increase of greenhouse gases emissions (GHG) at a global level (Sutton, 2011; Tubiello *et al.*, 2013). Manure is rich in nitrogen, phosphorus and carbon (Pagliari & Laboski 2012) and can contribute to increase global climate change through the emission of methane and nitrous oxide (Leytem *et al.*, 2011). Thus, while the recycling of livestock manure within agricultural systems is necessary to improve and maintain soil health, its efficient management is also important to reduce the environmental impact of farming activities. Therefore, manure treatment becomes a focal issue in relation to current national policies on environmental, climate and renewable energy matters.

Uruguayan agriculture has intensified during the last 15 years, with a large input of mineral fertilizer and other agrochemicals. In recent years, the application of manure has attracted increased attention, and nowadays a group of Uruguayan farmers is incorporating a new technology based on an *in situ* aerobic process of the manure treatment that consists of continuous liquid composting (CLC) in aerated tanks. There are 52 tanks installed in the country, and the liquid manure composting is applied throughout ~ 8,000 ha. The assessment of soil quality after CLC application is essential to track changes in soils as a result of management practices. Within the framework of sustainable agricultural production, a high soil quality and health should maintain a high productivity without significant soil or environmental degradation (Govaerts *et al.*, 2006; Griffiths *et al.*, 2010; Bünemann *et al.*, 2018). A set of biological soil indicators must be selected in order to advise farmers as to whether this management strategy yields the positive changes they anticipated.

Soil biological indicators are considered good soil quality indicators due to their sensitivity and ability to reflect soil management effects (McGuire & Treseder, 2010; Bowles *et al.*, 2014; Benintende *et al.*, 2015). Generally, soil health has been related to the soil organic matter content (Gao, 2006), soil fertility and structure, total N and C/N ratio (Murphy *et al.*, 2011), but these soil properties do not necessarily change as a result of changing external conditions or use (Chodak & Niklińska, 2010; Preem *et al.*, 2012; Muscolo *et al.*, 2014), and hardly reflect short-term changes in soil processes associated to new environmental threats. Changes in soil characteristics or environmental conditions induce rapid changes on microbial biomass, community composition and activity (Schloter *et al.*, 2003; Gil-Sotres *et al.*, 2005; Nogueira *et al.*, 2006; Qin *et al.*, 2010). In some cases, changes in microbial communities and activity can precede detectable changes in soil physical and chemical properties, thereby providing an early sign of soil improvement or an early warning of soil degradation (Muscolo *et al.*, 2015).

However, there is still a lack of consensus on what to measure for the evaluation of soil biota linked to soil health and the ensuing prediction of sustainability or productivity (Saha *et al.*, 2008; Dong *et al.*, 2014). Most of the studies about biological soil quality indicators propose measuring key functional microbial groups, as well as specific biological properties such as soil enzyme, microbial biomass carbon or N, basal respiration, FAME profile, genomic analysis, and glomalin concentration (Huang *et al.*, 2014; Dose *et al.*, 2015). These parameters can be used for the evaluation of soil health and as early indicators of soil degradation. However, the characterization of multiple microbiological soil parameters to enhance the understanding of the correlation between soil biota and positive or negative effects on microbial functions and ecosystem services has not been as widely regarded.

The objectives of this study were: (i) to evaluate the influence of aerobically treated liquid manure (CLC) on soil microbial properties on two different agricultural sites with short- and medium-term CLC application (1 and 3 years, respectively), and (ii) to identify a minimal data set of microbiological parameters to monitor early changes induced by soil management with CLC as a biofertilizer.

Material and methods

Biofertilizer: liquid manure composting

The biofertilizer used in the experiment was obtained through the *in situ* aerobic treatment of livestock manure according to the method developed at the University of São Paulo, Brazil (D´Andrea & Medeiros, 2002). In brief, the liquid biofertilizer was obtained by a continuous system of aerobic biodegradation in aerated

tanks filled with 15% livestock manure, 80% water and 5% micronutrients and commercially available additives (Microgeo®). Microgeo is a Brazilian patented biofertilizer (#PI0207342 A2-0), which shall be named continuous liquid composting (CLC) for the purpose of this paper.

The N-P-K content of CLC was 2.7 N - 0.90 P – 7.4 K mg L^{-1}. Standard methods were used to analyse total N (4500-NC), total P (4500-PB) and total K (3500-KB).

Experimental area and design

The studied area was located in Colonia, Uruguay (lat. 34,338164 S, long. 57,222630 W). The climate of the area is classified as humid subtropical, with an average temperature of 24 °C and an annual mean precipitation of 1200 mm. The soil is a *Eutric Cambisol* (FAO, 1998) or *Brunosol Eutrico* according to Duran´s soil classification (Durán, 1991). Within this area, two adjacent sites were selected with one year (S1y) and three years (S3y) of CLC application and a similar crop sequence (Table 1). At each site, the experiment had a completely randomized block design with four replicate plots (144 m^2 each) of two treatments: CLC amendment (CLC) and no CLC application (NCLC) plots. CLC was applied in two doses post crop sowing, half in September, after the summer crop emergence (2-3 leaves), and the other half in autumn, after the winter crop emergence, completing a total rate of 300 L ha^{-1} $year^{-1}$. Within each block, the distance between plots was 12 m. All treatments received mineral fertilization. The mineral fertilization in both sites during maize cultivation was: 50 kg ha^{-1} of P, 50 kg ha^{-1} of N in the form of urea (50% at maize seeding and 50% at the 4^{th} leaf stage), and 50 kg ha^{-1} of KCl.

Soil sampling

Soil samples were collected from the two sites in September 2015, after the maize harvest. Composite soil samples from eight plots of 144 m^2 were collected from each site, four with CLC application and four without it (NCLC). Composite soil samples consisted of 10 soil cores (20 cm depth, 10 cm diameter) from each plot. Each composite soil sample was well homogenized in sterile plastic bags and divided into two parts, for chemical and microbial analysis. Soil samples for microbial analysis were sieved through a 2 mm mesh and stored at -20°C for microbial biomass and enzyme analyses, and at 4°C for culturable biodiversity and functional groups assessment. Soil samples for chemical analysis were sieved through a 2 mm mesh and air dried.

Table 1. Crop sequence and application of continuous liquid composting (CLC) before spring 2015 in the two sites selected for this study: S1y (site with one year of CLC application) and S3y (site with three years of CLC application). NCLC: no application.

Year/Season	S3y	S1y
2013 Autumn	Cover crop / CLC	Cover crop / NCLC
2013 Spring	Sorghum / CLC	Sorghum / NCLC
2014 Autumn	Wheat / CLC	Wheat / NCLC
2014 Spring	Soybean / CLC	Soybean / CLC
2015 Autumn	Cover crop / CLC	Wheat / CLC
2015 Spring	Maize / CLC	Maize / CLC

Physicochemical analysis of soil

The pH of a 1:2.5 (w/v) soil/water suspension and the total organic carbon (SOC) (Walkley & Black, 1934), available PO_4^- (Bray & Kurtz, 1945), inorganic N ($N\text{-}NO_3$) (Gelderman & Beegle, 1998) and exchangeable K (Toth & Prince, 1949) in the soil were determined. The soil's water content was measured after drying soil at 105°C for 24 h. The N-mineralization potential (NMP) was determined according to Waring & Bremner (1964). In brief, 20 g of soil were incubated under waterlogged conditions for 7 days at 25 °C in stoppered 50 mL tubes. After the incubation period, N in the resulting slurry was extracted with a 4 M KCl solution (1:10 extraction ratio). Ammonia was distilled, trapped in boric acid solution (2%) and titrated with sulfuric acid (0.005 N). The NMP was determined by subtracting the initial NH_4+ -N content in the soil samples from the concentration of NH_4+ determined after the incubation (Waring & Bremner, 1964).

Biochemical and microbiological properties

Soil microbial biomass

Microbial biomass carbon (MBC) was determined by a chloroform-fumigation extraction method (Vance *et al.*, 1987). An extraction efficiency coefficient of 0.35 was used to convert soluble C into biomass C (Sparling *et al.*, 1990).

Culturable mesophilic aerobic microorganisms

Total mesophilic aerobic bacteria (MAB), filamentous fungi (MFF) and actinobacteria (MAA) were evaluated by means of a plate counting technique. Plates were incubated at 28°C and the results were expressed as colony-forming units (cfu) per gram of dry soil. MAB were enumerated on TY (Beringer, 1974) with 1 mL amphotericin B (1.5 mg mL^{-1}); MFF, on rose bengal agar with 1 mL of streptomycin (3 mg mL^{-1}) (Frioni, 2011) and MAA, on actinomycetes isolation

agar (Frioni, 2011). MAB were enumerated at 48 h of incubation, whereas MFF and MAA, after 72 h.

Microbial functional groups related to soil fertility

The populations of microbial functional groups involved in carbon (soil-borne cellulose degraders: cellulolytic population), phosphorus (P-solubilizing microorganisms) and N (free living N-fixing, ammonium-oxidizing and denitrifying microorganisms) were analyzed. The quantification of the cellulolytic population was determined by a most-probable-number (MPN) count technique. Tubes with minimal medium with a 50 mg piece of Whatman 1 paper as the sole C source were incubated at 28°C, 140 rpm for 20 days (Frioni, 2011). For P solubilizing microorganisms, tenfold dilutions were spread on duplicate plates containing National Botanical Research Institute's phosphate growth medium (NBRIP) (Nautiyal, 1999). To yield 1:10 soil dilution, 10 g of soil was added to 90 mL of sterile phosphate buffer (10 mM, pH =7) with two drops of 2.5% Tween and agitated 15 min at 200 rpm. Serial dilutions were produced by taking 1 mL (1:10 dilution) in 9 mL of phosphate buffer. Plates were incubated at 28°C and the results were expressed as cfu g^{-1} of dry soil. P solubilizing activity was considered if a solubilization halo was present on the respective culture medium. The MPN technique was used for quantifying the nitrifying, denitrifying and free-living N-fixing microaerophilic organisms (Schmidt & Belser, 1994; Schinner *et al.*, 1996).

Soil enzyme activity

Enzymatic activities of fluorescein diacetate hydrolysis (FDA), dehydrogenase (DHA), and alkaline and acid phosphatase enzymes (AlPh and AcPh) were assayed based on the colorimetric determination of the product released by the enzyme. Enzyme activities were expressed as micrograms of product per gram of dry soil per specified time. FDA hydrolysis reaction was determined according to the methods of Adam & Duncan (2001); DHA activity, according to the procedure of Casida *et al.* (1964). AlPh and AcPh enzymes were determined with p-nitrophenyl phosphate by the methodology described by Eivazi & Tabatabai (1977).

Glomalin

The extraction of glomalin-related soil protein (GRSP) was performed by adding 8 mL of 50 mM trisodium citrate dehydrate solution at pH 8 in a centrifuge tube and then autoclaving it at 121°C for 60 min. After each extraction, the sample was centrifuged at 3220 rpm for 15 min and the supernatant containing glomalin was collected and stored at 4°C. At least four sequential extractions were carried out until the supernatant showed yellow pale color indicating the absence of glomalin. GRSP was then quantified by means of the Bradford dye-binding assay (Wright & Upadhyaya, 1998).

Soil suppressive capacity

The soil pathogenicity index (SPI) is an indicator of soil suppressive capacity. Ten surface-sterilized soybean seeds were placed on 40 mL soil and 10 mL sterile water placed on paper tissue and rolled. Each pack was placed in a sterile plastic bag. Sterile and unsterile soil was evaluated, counting the number of seeds that germinated and emerged in the dark after 10 days at 22 °C. The SPI was calculated as follows: SPI = (N° of seeds emerged in sterile soil – N° of seeds emerged in unsterile soil) / N° of seeds emerged in sterile soil (Altier & Zerbino, 2012). SPI values vary from zero to one, zero being no soil pathogenicity.

Statistical analysis

The sites were treated as independent experiments, and the main treatment for statistical comparison was presence (CLC) and absence (NCLC) of CLC application. All data were tested for normality using the Shapiro-Wilk test and variance homogeneity was assessed by means of the Levene test. Data of celluloses degraders, FDA, N-fixation, and NH_4^+ oxidizers were log transformed to meet the normality assumption of the statistical tests. The effect of CLC application on soil microbiological and chemical properties was analyzed using a factorial analysis of variance (ANOVA). Fisher's LSD was used to identify significant differences ($p<0.05$ or $p<0.1$). The relationship between variables was determined by multiple correlation analysis. The correlation analysis between soil pH, moisture and microbiological parameters was carried out using Spearman's correlation coefficient. Principal Component Analyses (PCA) were performed to analyze the ordination of the treatments according to physicochemical and biology soil properties. Statistical analyses were performed using the software InfoStat (Di Rienzo *et al.*, 2016) with the interface software R (http://www.r-project.org/).

Results

Fifteen different microbial variables related to several soil biological functions and seven physicochemical parameters were analyzed (Table 2). The study showed variations in most of the evaluated microbiological properties of treated and untreated soil in each site

Table 2. Mean values (n=4) and standard deviation of soil physicochemical parameters for each site biofertilized with continuous liquid composting (CLC) and not biofertilized (NCLC).

Physicochemical parameters	S3y		S1y	
	NCLC	CLC	NCLC	CLC
pH	5.5 (0.15)	5.4 (0.01)	5.7 (0.17)	5.6 (0.05)
Humidity (%)	19.0 (1.88)	18.8 (1.42)	18.9 (1.92)	20.5 (1.29)
SOC (%)	5.3 (0.37)	4.5 (0.21)	2.5 (0.51)	2.5 (0.11)
$N\text{-}NO_3$ (μg N g^{-1})	10.6 (3,54)	12.2 (3,15)	12.2 (4.72)	10.8 (3.26)
P (μg P g^{-1})	45 (4.20)	54 (5.77)	10.8 (3.86)	9.3 (4.11)
K (mg 100 g^{-1})	0.6 (0.21)	0.9 (0.08)	0.4 (0.03)	0.4 (0.03)
NMP (μg N g^{-1})	25* (0.99)	33* (0.14)	32 * (6.55)	52* (0.11)

SOC: total soil organic carbon. $N\text{-}NO_3$: inorganic nitrogen. P: soil available PO^{4-}. K: exchangeable potassium. NMP: N-mineralization potential. S3y: site with three years of CLC application; S1y: site with one year of CLC application. Significance differences recorded among NCLC and CLC treatments at each site according to the LSD test (* $p< 0.05$).

(S1y and S3y, with one and three years of CLC application, respectively) (Table 3). S1y and S3y are adjacent sites with *Eutric Cambisols*. According to the physicochemical parameters analysed (Table 2), the two sites showed differences in SOC% and P content before the application of CLC. To avoid site effect, the main treatment for statistical comparison was presence (CLC) and absence (NCLC) of CLC application within each site. The statistical analysis of the main soil physicochemical properties within each site did not show changes with CLC application (Table 2). However, NMP was statistically higher ($p<0.05$) in CLC amended plots at both sites.

MBC increased with the application of CLC in both sites. The increase was 13.0% in S3y and 23.3% in S1y; the increase was statistically significant only in the latter case (Table 3). The application of CLC had positive and negative significant effects on the mesophilic aerobic culturable microorganisms. Increases in cfu g^{-1} of MAB at S1y ($p<0.1$) and MAA at S3y were recorded, as well as a decrease in MFF at S3y ($p<0.05$, Table 3). These results indicate that the effects of the application of CLC on the recorded increases or decreases in the mesophilic culturable population were not consistent.

In both sites (S1y and S3y), there was a significant increase in the MPN of the functional groups as a result of the application of CLC (Table 3). In both sites, the application of CLC caused an increase in the values of cellulolytic and diazotrophic microorganisms. However, the CLC and NCLC treatments were only statistically different in S3y ($p<0.05$). The density of P solubilizing bacteria was lower in CLC treatment in both sites, but the differences were only significant in S3y ($p<0.1$). MPN of denitrifying microorganisms increased with CLC application in both sites (Table 3). In addition, we recorded significant positive correlations between MFF and P solubilizing microorganisms; MAB and NH_4 oxidizers; MAA, N-fixing microorganisms and denitrificants (Table 4).

The activities of four enzymes (FDA, DHA, AlPh, AcPh) increased in both sites with the application of CLC (Table 3). However, the changes in FDA and DHA activities were not statistically significant. AcPh activity increased significantly in CLC treated plots at both sites (S1y, $p<0.05$; S3y, $p>0.1$).

In the case of glomalin, contrary to what was expected, our results did not show a significant change in GRSP with CLC treatment in neither site (Table 3). Glomalin was positively correlated with MAA and negatively correlated with enzyme activities ($p<0.05$, Table 4).

SPI decreased with CLC treatment, although the differences were only significant in S3y ($p<0.05$, Table 3). Therefore, a medium-term application of CLC (3 years) significantly increased the suppressing capacity of soil pathogens. Positive and negative significant correlations were found between SPI, enzymes and some functional groups (Table 4).

The results of the PCA carried out with physicochemical and biological soil properties indicate that the two first components (PC1 and PC2) accounted for 88.3% of the total data variation (Fig. 1). The first axis opposed sites S1y and S3y (61.5% of variance explained). The biological soil properties DHA, AcPh and FDA, associated with S1y, and denitrifying, SPI, MAA and glomalin, associated with S3y, were important for the ordination of treatments in PC1. The second axis grouped CLC and NCLC treatments at each site (26.8% of variance explained). On the other hand, P solubilizers and MFF, associated with NCLC,

Table 3. Mean values (n=4) of the selected microbiological variables measured in plots biofertilized and not biofertilized with CLC (CLC and NCLC, respectively).

MICROBIAL PARAMETERS	S3y		S1y	
	NCLC	CLC	NCLC	CLC
MBC (µg C 100-g^{-1})	244.26 (123.77)	279.12 (62.10)	69.45 ** (22.92)	90.56 ** (4.03)
Mesophilic aerobic microorganism (cfu g^{-1} dry soil)				
MAB	$4.7 \cdot 10^6$ (0.47)	$4.4 \cdot 10^6$ (0.78)	$5.5 \cdot 10^6$ ** (0.54)	$7.7 \cdot 10^6$ ** (1.60)
MAA	$1.9 \cdot 10^5$ * (0.30)	$2.4 \cdot 10^5$ * (0.18)	$1.5 \cdot 10^4$ (0.69)	$1.2 \cdot 10^4$ (0.67)
MFF	$19.84 \cdot 10^4$ * (5.02)	$9.2 \cdot 10^4$ * (0.38)	$1.0 \cdot 10^5$ (1.2)	$2.0 \cdot 10^5$ (1.4)
Functional groups				
Cellulolose degraders (MPN g^{-1} dry soil)	$1.8 \cdot 10^3$* (1.4)	$23.2 \cdot 10^3$* (9.9)	$5.5 \cdot 10^3$* (0.7)	$15.6 \cdot 10^3$* (0.06)
P-solubilizing (cfu g^{-1} dry soil)	$10.6 \cdot 10^4$ ** (6.6)	$4.1 \cdot 10^4$** (0.6)	$7.3 \cdot 10^4$ (0.9)	$7.0 \cdot 10^4$ (4.3)
N-fixation (MPN g^{-1} dry soil)	$9.2 \cdot 10^3$* (7.2)	$39.6 \cdot 10^3$* (19.3)	$7.7 \cdot 10^2$ (6.3)	$35.9 \cdot 10^2$ (34.7)
NH_4^+ oxidizers (MPN g^{-1} dry soil)	$3.86 \cdot 10^1$** (1.4)	$1.03 \cdot 10^1$** (0.1)	$3.87 \cdot 10^2$ (1.8)	$6.3 \cdot 10^2$ (3.4)
Denitrifying microorganism (MPN g^{-1} dry soil)	6.3 (2.0)	12.5 (6.9)	0.4 (0.02)	0.73 (0.3)
Soil enzymes activity				
FDA (µg FDA g^{-1}.0,5-h^{-1})	9.17 (0.74)	9.52 (0.84)	42.43 (36.94)	53 (47.79)
DHA (µg INTF g^{-1} 2-h^{-1})	29.81 (10.76)	40.31 (11.86)	196.72 (43.56)	208.74 (28.20)
AlPh (µg pNP g^{-1} h^{-1})	5.63 (3.68)	6.21 (4.55)	10.04 (2.25)	10.18 (1.13)
AcPh (µg pNP g^{-1} h^{-1})	29.16 * (2.31)	38.23 * (4.15)	61.87 * (16.82)	89.60 * (21.33)
SPI	0.76 * (0.07)	0.63 * (0.03)	0.36 (0.01)	0.32 (0.12)
GRSP (µg BSA g^{-1} dry soil)	69.8 (4.3)	69.06 (2.5)	5.61 (1.9)	5.17 (1.01)

S3y: site with three years of CLC application. S1y: site with one year of CLC application. MBC: microbial biomass carbon. MAB: mesophylic aerobic bacteria. MAA: actinobacteria. MFF: filamentous fungi. MPN: most probable number; FDA: fluorescein diacetate hydrolysis. DHA: dehydrogenase. INTF: iodonitrotetrazolium formazan; AlPh: alkaline phosphatase. AcPh: acid phosphatase. pNP: p-nitrophenyl phosphate; SPI: soil pathogenicity index. GRSP: glomalin-related soil protein; BSA: bovine serum albumin. Asterisks indicate significant differences between biofertilized and non-biofertilized plots (** for $p< 0.05$ and * for $p<0.1$).

Table 4. Matrix of Spearman correlation coefficients (r).

	MBC	MAB	MFF	MAA	FDA	DHA	AlPh	AcPh	Cel	P Sol	NH_4 ox	Den	N fix	SPI	Glom	H
MAB	ns															
MFF	ns															
MAA	ns	-0.78**	ns													
FDA	ns	0.63**	ns	-0.65**												
DHA	-0.49*	0.74**	ns	-0.80**	0.66**											
AlPh	ns	0.53**	ns	-0.48 **	0.71**	ns										
AcPh	ns	0.79**	ns	-0.64**	0.81**	0.72**	0.58**									
Cel	ns	ns	-0.62*	ns	ns	ns	ns	ns								
P –Sol	ns	ns	0.59*	ns	ns	ns	ns	ns	-0.55**							
NH_4 ox	ns	0.75**	ns	-0.76**	0.67**	0.71**	0.44*	0.69**	ns	0.57**						
Den	ns	-0.54**	ns	0.82**	-0.76**	-0.72**	-0.43*	-0.55**	ns	ns	-0.71**					
N-fix	ns	-0.52**	ns	0.66**	-0.50**	-0.54**	ns	-0.43*	0.17	ns	-0.64**	0.82**				
SPI	ns	-0.59**	ns	0.65**	-0.82**	-0.77**	ns	-0.79**	-0.44*	ns	-0.62**	0.74**	0.50**			
Glom	ns	-0.74**	ns	0.79**	-0.80**	-0.72**	-0.62**	-0.75**	ns	ns	-0.82**	0.72**	0.43*	0.75**		
H	ns	ns	ns	ns	ns	-0.14	ns	0.44*	ns	ns	ns	ns	ns	ns	ns	
pH	-0.69**	0.70**	ns	-0.67**	ns	0.71**	0.61**	0.48*	ns	ns	0.77**	-0.62**	ns	-0.55**	-0.77**	-0.77**

MBC: microbial carbon biomass. MAB: mesophylic aerobic bacteria. MFF: filamentous fungi. MAA: actinobacteria. FDA: fluorescein diacetate hydrolysis. DHA: dehydrogenase. AlPh: alkaline phosphatase. AcPh: acid phosphatase. Cel: cellulolose degraders. P Sol: P-solubilizing microorganisms. NH_4 ox: NH_4^+ oxidizers. Den: denitrifying microorganisms. N fix: diazotrophs. SPI: soil pathogenicity index. Glom: glomalin. H: humidity. Significant correlations at ** ($p<0.05$), * ($p<0.1$), ns (not significant), n=16.

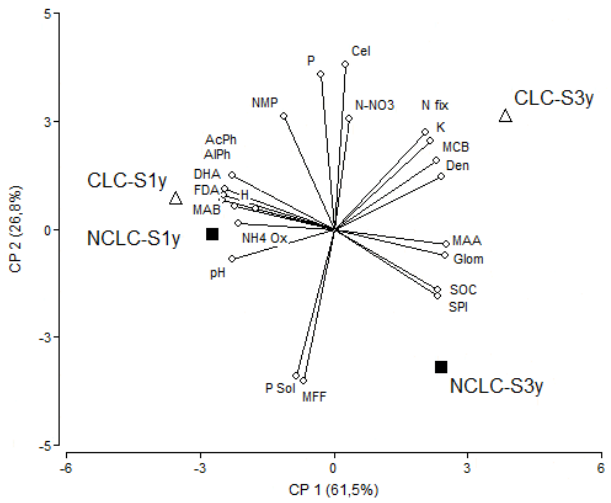

Figure 1. Results of principal component ordination including 21 of the measured physicochemical and biological soil properties. S3y: site with three years of CLC application. S1y: site with one year of CLC application. CLC: application of continuous liquid composting in spring 2015. NCLC: control without application of CLC in spring 2015. AlPh: alkaline phosphatase. AcPh: acid phosphatase. Cel: cellulolose degraders. Den: denitrifying microorganisms. DHA: dehydrogenase. FDA: fluorescein diacetate hydrolysis. Glom: glomalin. H: humidity. MBC: microbial carbon biomass. MAA: actinobacteria. MAB: mesophylic aerobic bacteria. MFF: filamentous fungi. N fix: diazotrophs. NH_4 Ox: NH_4^+ oxidizers. P Sol: P-solubilizing microorganisms. SPI: soil pathogenicity index. K: exchangeable potassium. N-NO_3: nitrates. NMP: N-mineralization potential. P: available phosphorus. SOC: total soil organic carbon. ■: Plots without CLC application; Δ: Plots with CLC application.

and P, associated with CLC, were important for the definition of PC2. The results also indicated that the differences between the amended and non-amended soils were more pronounced in S3y than in S1y (Fig. 1). For this reason, and for the purpose of establishing the relationships between soil properties after three years of CLC application, another PCA was performed with the data from S3y (Fig. 2). The results indicate (Table 3) that the soil with three years of CLC application was characterized by higher values of N fixation, AcPh enzyme activity, actinobacteria and cellulolytic microorganisms, and lower values of P solubilization, MFF, oxidizing NH_4+, and SPI than NCLC.

Discussion

This research provides insight into how microbial communities function and how composition responds to a short-term (S1y) and long-term (S3y) application of CLC. In accordance with other authors (Giacometti *et al.*, 2014), long-term manure fertilization increases MBC. In this study, MBC was sensitive to CLC application and to the length of time of the application. The results obtained in this study showed that CLC application modifies some soil microbial properties and, in some variables, the effect becomes significant with time. MBC is considered an active component of the soil organic pool. Ren & Stefano (2000) suggested that Cmic:Corg ratios obtained over long-term treatments also represent C equilibrium in the soil. The results obtained at both sites indicate that CLC application increased MBC but not total soil SOC (Table 2). Thus, the changes in soil microbial biomass carbon measured over relatively short periods can indicate the trends in total organic matter content long before these can be detected by chemical analyses. Therefore, MBC as a biological parameter involved in soil C mineralization needs to be monitored together with SOC to predict SOC stability. Although many authors have reported positive correlations among the activities of soil enzymes, the microbial biomass and organic matter contents (Wang *et al.*, 2011), it was not confirmed in this study. NMP increased significantly (p<0.05) in both sites with CLC amendments (Table 2). Those differences were not likely due to different soil N content but rather to differences in microbial activity stimulated by CLC application (Qiu *et al.*, 2008) warranting further investigation.

In this study, the abundance of functional groups was influenced by CLC amendments. Organic amendments, including manure application, modify the C/N ratio and the P demand for the development of microbial biomass (Qiu *et al.*, 2008; Luo *et al.*, 2018). CLC application caused increases in NMP of N-fixing organisms and in acid phosphatase enzyme (AcPh) activity. On the other hand, P-solubilizing abundance decreased. Both variables, P-solubilizing and AcPh, are related to microbial activity and P availability, but linked to different soil P pools. P solubilizing was positively correlated with filamentus fungi (MFF) and AlPh to mesophilic aerobic bacteria (MAB) population. This may explain the different activity and origin of P availability in the soil. The majority of the variation in potential enzyme activity and functional groups could be explained by nutrient availability related to CLC application. In both sites, MBC was higher in CLC treatment than in NCLC, but differences were only significant at S1y (p<0.1). Soil fertility is related to the activity of functional groups of microorganisms, with the ability to directly or indirectly supply essential plant nutrients because they are linked to N, P and C biogeochemical cycles. Trends in microbial biomass and functionality were not accompanied by a change in the size of the microbial culturable mesophilic

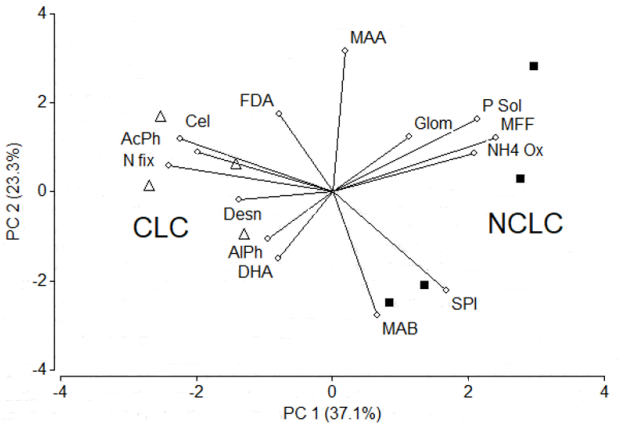

Figure 2. Results of principal component analyses with the 14 main soil microbial variables at the site with three years of CLC application (S3y). CLC: application of continuous liquid composting. NCLC: control without application of CLC. AlPh: alkaline phosphatase. AcPh: acid phosphatase. Cel: cellulolose degraders. Den: denitrification. DHA: dehydrogenase. FDA: fluorescein diacetate hydrolysis. Glom: glomalin. MAA: actinobacteria. MAB: mesophylic aerobic bacteria. MFF: filamentous fungi. N fix: N-fixation. NH_4 Ox: NH_4^+ oxidizers. P Sol: P-solubilizing. SPI: soil pathogenicity index. ■: Plots without CLC application; Δ: Plots with CLC application.

population of bacteria, fungi and actinomyces (Table 3). The relationship between inorganic N and soil microbial activity, enzymes or functions is not straightforward because the number of microorganisms represents the number of culturable mesophilic microbial organism and not necessarily the active or functional groups in the soil. In general, microbial activity changes more quickly in response to management than to community composition (Burger & Jackson, 2003). These results indicate that the number of mesophilic aerobic culturable organisms do not show consistent changes with CLC treatment, although quantitative changes of functional groups were recorded in both sites. Cellulolytic microorganisms break cellulose chains into small units and the treatment-enhanced activity of the cellulolytic community could indicate higher litter or root turnover in the soil and therefore a higher C pool. At the same time, the treatment also increases diazotrophic organisms with. An increase in microbial N demand due to CLC fertilization is expected. Furthermore, N_2O and N_2 production is further stimulated by the addition of labile C sources typically added with organic-based fertilizers such as manure (Zhou *et al.*, 2013). Increased N fixation could be a consequence of increased N_2 emission with CLC application (Asgedom *et al.*, 2014; Gao *et al.*, 2016); thus, microbial activity would regulate N input and output from the system. In this study, microbial community composition explained little variation compared to potential enzyme activity and functional groups. The phenotypic plasticity of the microbial community in response to treatment may be high, as suggested by the relatively large variation in soil microbial community functions. There were no statistically significant differences in soil pH among treated and untreated soils (Table 2). Many authors have suggested that manure amendments may decrease soil pH. It is known that soil pH affects microbial biomass and activity, as well as the relative proportions of bacteria and fungi (Pietri & Brookes, 2008, 2009). In this study, soil pH was positively correlated with enzyme activity and bacteria (MAB) and negatively correlated with actinobacteria (MAA) (Table 4). This positive correlation suggests that soil enzyme activity is mainly a function of pH and of the total present MAB population for potential synthesis (Zhou *et al.*, 2019). The soil bacteria community abundance and composition were well adapted to soil pH for optimum enzyme activity (Nannipieri *et al.*, 2017).

The suppressing capacity of soil pathogens (SPI) increased with CLC treatment in both sites. Actinobacteria (MAA) had a strong relationship with SPI, together with glomaline, diazotrophs and denitrifying organisms (p<0.05, Table 4). Actinobacteria have gained special relevance as the most potent source of antibiotics (Kandasamy *et al.*, 2012) and other bioactive secondary metabolites (Solecka *et al.*, 2012) as potential biocontrol agents.

The activities of four enzymes (FDA, DHA, AlPh, AcPh) increased in both sites with the application of CLC. Soil enzyme activities are important indicators of microbiological and biochemical processes because they are involved in soil organic matter decomposition, nutrient cycling and availability, and in the biodegradation of toxic organic pollutants. Recent studies in temperate environments have suggested that measurements of soil enzyme activity are generally the most sensitive indicators of changes in the below-ground microbial community from different management practices (Nanipieri *et al.*, 2002; Chaer *et al.*, 2009; Bowles *et al.*, 2014; Raiesi & Beheshti, 2014). Activities of specific enzymes may change depending on the composition of the organic amendments and the relative availability of nutrients, as well as other factors, such as soil type. Our results indicate that CLC application tends to increase soil enzyme activity and this pattern could be the result of higher microbial activity stimulated by CLC amendment. At the same time, given the relatively constrained level of soil nutrients, enzymatic activity might enhance the availability of the most limiting nutrients in order to meet microbial metabolic demands (Sinsabaugh *et al.*, 2008; Mooshammer *et al.*, 2014), which could

explain the significant increases of AcPh enzyme activity as a result of CLC application in both sites (p<0.05, Table 3). In order to adapt to environmental constraints, microorganisms have to compensate the regulation of extracellular enzyme production with a C and nutrients acquisition strategy or by enhancing microbial metabolism for its nutrient use efficiency. In this study, the AcPh activity (linked to organic P), together with MPN of diazotrophic (N-fixation), celulolitic microorganism (C) and saprophytic fungi (MFF) increased significantly with CLC treatment. This could indicate a microbial response to P organic deficiency and changes of C/N ratio with treatment for the development of microbial biomass. These may partially help resolve the constraint of resource availability and explain the increase in enzyme activity. In this study, all enzyme activities increased; DHA showed a particular increase with CLC treatment in both sites. Among the various soil enzymes, DHA is of great relevance, as its activity levels are considered an indicator of overall microbial activity, due to their intracellular presence in all living microbial cells, and an indicator of biological redox systems.

Contrary to what was expected, our results showed no significant change in GRSP with treatment. According to Zhang *et al.* (2014), organic amendments enhance soil aggregate stability through the positive effect on soil binding agents, including GRSP. Recent results have described glomalin as a primary soil component in the formation of micro-aggregates and the maintenance of soil structure (Wright & Anderson, 2000; Emran *et al.*, 2012). Results of Wang H *et al.* (2017) and Wang CY *et al.* (2017) showed that the enzyme activities after a 23-year manure amendment led to an increase in the macro-aggregates (> 1 mm) but not in the micro-aggregates. The latter could explain the negative correlation between glomalin and enzyme activity obtained in this study. Our results showed that CLC treatment increased enzyme activity and MBC but not total extractable glomalin. This may indicate that microbial biomass and enzyme activity are not linked to glomalin, which is specially related to micro-aggregate formation (Six *et al.*, 2004). Instead, glomalin was positively correlated with MAA and SPI.

In this study, the sampling strategy and microbial parameters used revealed useful information regarding the effects of CLC application at the two sites, and this effect seems to be more evident with time (S1y compared to S3y). This is one of the first studies evaluating the effects of aerobically treated manure application on a large set of soil microbial parameters. The application of CLC had significant effects on biological soil properties, increasing microbial biomass, soil enzyme activity and the abundance of microorganisms of different functional groups. Among the analyzed microbial parameters, microbial biomass carbon (MBC) and soil enzyme activity of tested FDA, DHA, AcPh and SPI emerged as reasonable indicators to assess and monitor the effects of CLC application. Among physicochemical variables, N-mineralization potential (NMP) was the best one to discriminate between treatments. The selected soil microbiological properties and NMP were the most sensitive to CLC amendments, but different soil types or sources of organic amendment applications need further research. With no current consensus on the minimal biological data set to assess the impact of a management practice on soil, this research approach provides insight into how microbial communities function and how composition responds to organic fertilization with CLC and its potential relationship with ecosystem services.

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