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Soil attributes and microbial diversity on 28 years of continuous and interrupted for 12 months of pig slurry application

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ABSTRACT

The pig slurry (PS) application as an organic fertilizer in Brazilian soils occurs for many years and the effects of this application are known, but in this study our objective was to evaluate the microbiological attributes and microbial diversity of the soil after the interruption of the PS application. The PS rates were 0, 30 and 60 m^3 ha^{-1} yr^{-1} and in 2016 the plots were divided and the PS application was stopped in half of the plot, generating the factorial scheme 2 × 2, being two PS rates (30 and 60 m^3 ha^{-1} yr^{-1}) and two application conditions (continuous or interrupted). Two soil samples were taken at 5 and 12 mo after interruption. The PS interruption reduced the microbial biomass C (MBC) by 5% and 14% after 5 and 12 mo, respectively. Microbial biomass N (MBN) was reduced too by 32% and 29% due to the PS interruption. The soil basal respiration (SBR) decreased when PS application was interrupted. The metabolic quotient (qCO_2) did not change with the PS interruption but was high in the 60 m^3 ha^{-1} yr^{-1}. The bacteria and archaea genetic profiles showed similarity above 69% between continuous and interrupted applications. The bacterial diversity and richness index were higher than those found for fungal and archaeal communities. In contrast, the interruption had a strong effect on the soil fungi, which had diversity reduced due to the increase of the species dominance. The PS interruption up to 12 mo has an effect on the biomass, diversity, and activity of the microorganisms.

Key words: Animal manure, microbial biomass, microbial diversity, microbiological activity.

INTRODUCTION

In the world pork market, Brazil is in fourth place as the largest producer, totaling in 2018 3.9 million tons of meat, with the southern region of Brazil responsible for 68% of the total pigs slaughtered in the country (ABPA, 2019). Pig production is predominantly performed in a confinement system, which makes the liquid form of manure the most representative, called pig slurry (PS) (Balota et al., 2014). It is estimated that the PS average volume produced by a pig in the growing and finishing phase is 4.5 L d^{-1} (3.5 L feces and urine and 1 L water for cleaning stalls) (Oliveira et al., 2007), thus, as 44.2 million pig were slaughtered in 2018 (IBGE, 2019), it is estimated that the volume of PS generated was around 198 million liters per day.

One of the alternatives for PS recycling is the use as organic fertilizer as it can provide essential nutrients to plants, increase the soil organic matter (SOM) content (Plaza et al., 2004; Couto et al., 2013) and enable greater activity and diversity of microorganisms (Marschner et al., 2003; Balota et al., 2014; Li et al., 2015; Navroski et al., 2019). Authors have observed that the use of organic fertilizers increased the diversity of microorganisms compared to mineral fertilizers or without fertilizer (Zhen et al., 2014; Li et al., 2015; Faissal et al., 2017).

Nevertheless, the transporting cost of PS to distant areas is high what restricts its use and makes its application in large quantities and for long period mostly in the same area (Oliveira et al., 2007), becoming thus an environmental risk factor, as it modifies the chemical, physical and biological soil properties, due to salt accumulation, heavy metals and the possibility of introducing pathogenic organisms.

Studies have shown that microorganisms, as well as plants and animals, develop adaptations to survive in the environmental conditions in which they are exposed (Kavamura et al., 2013). In soil, the intensity of management, cultivation of different plants, and fertilizers can cause disturbances and, consequently, select certain microbial groups (Andreote and Cardoso, 2016) that have the ability to remain in the environment even with change. In this case, there is a reduction in diversity that compromises its functionality.

Knowing that frequent PS applications can cause disturbances in the soil, one alternative would be to reduce the number of annual applications or the applied rate. However, the interruption of PS has yet to be studied. In this context, the hypothesis of the study is that the interruption of PS application modifies the microbiota on a structural and functional scale, which can be verified through the variety of attributes such as microbial biomass C and N (MBC and MBN), soil basal respiration (SBR), and 16S and 18S rDNA gene diversity. Therefore, the objective was to evaluate the effect of interruption of PS application in an area with 28 yr of manure on microbiological attributes and genetic diversity of the microbial community.

MATERIALS AND METHODS

The experimental area was established in 1988 at Experimental Station of the Paraná Institute of Agronomy (IAPAR), located in Palotina (24°17' S, 53°50' W), Paraná State, Brazil. The experimental area consisted of plots with 9.0 × 5.0 m (45 m^2), arranged in a randomized block design with three replicates. The area was managed under no-tillage system (NT) with soybean (*Glycine max* [L.] Merr.) or maize (*Zea mays* L.) rotation in the summer crop and with wheat (*Triticum sativum* Lam.) or oat (*Avena sativa* L.) in the winter crop. In 2016, the plots were divided (except the control), and PS application in half the plot was interrupted, generating the 2 × 2 factorial arrangement, with two PS rates (30 and 60 m^3 ha^{-1} yr^{-1}) and two application conditions (continuous or interrupted). The last PS application in the whole plot was performed in May 2016, thenceforth the PS addition was stopped on half plot to perform soil sampling, which occurred in the summer pre-cultivation (SPC) in October 2016 and the other in the winter pre-cultivation (WPC) in May 2017, corresponding to 5 and 12 mo after the interruption of the PS application.

The soil was classified as a typical dystroferric red latosol (dos Santos et al., 2018) (Oxisol, according to the USDA Soil Taxonomy; USDA, 1999) with 695.4 g kg^{-1} clay, 134.2 g kg^{-1} silt and 170.4 g kg^{-1} sand content. The samples were composed of three soil subsamples in each of the three replicates from the treatment and collected at the 0.0-0.1, 0.1-0.2 and 0.2-0.4 m depths. For more information about experimental design, soil sampling and soil chemical attributes, see Navroski et al. (2019).

Microbiological analyses

The soil samples were homogenized, sieved through 4.0 mm, and stored at 7 ºC until evaluation. The humidity determination was performed by the gravimetric method. To determine the soil microbial biomass C (MBC) and microbial biomass N (MBN) we used the fumigation-extraction method (Jenkinson and Powlson, 1976) and for C and N extraction we used the procedure proposed by Vance et al. (1987). We used correction factor KC = 0.33 to calculate MBC (Sparling and West, 1988) and KN = 0.54 to calculate MBN (Brookes et al., 1985).

Soil basal respiration (SBR) was determined by the method described by Jenkinson and Powlson (1976) and the metabolic quotient (qCO_2) was obtained by the ratio between SBR per unit of soil MBC at incubation time, and the microbial quotient (qMIC) was calculated by the relationship between MBC and total organic C (TOC).

Soil DNA extraction and bacteria, archaea and fungi amplification

The total DNA extraction was performed on samples from depth 0.0-0.1 m using a soil DNA isolation kit (PowerMax, MO BIO Laboratories, QIAGEN, Venlo, The Netherlands) according to manufacturer's instructions.

The amplification of the V6 region encoding the bacteria's 16S DNAr gene was performed by 968F-GC and 1401R primers (Felske et al., 1996). The reactions were performed for a final volume of 50 µL containing 2.5 mM $MgCl_2$, 5 µL 10X buffer, 0.2 mM each dNTP, 0.4 mM each primer, 0.5 µL formamide, 5 DNA Taq polymerase, 4 µL total DNA (7 to 15 ng) and ultrapure water. Amplification conditions were: 1 initial denaturation cycle at 94 °C for 4 min; 35 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min and final extension of 72 °C for 10 min; and 4 °C for maintenance.

For amplification of the Archea's 16S DNAr gene fragment, the ARCH21F and ARCH958R primers were used (Moyer et al., 1998) and, later on in the second PCR (Nested), the ARCH340F-GC and ARCH519R primers were used (Ovreas et al., 1997). Both reactions were performed using 1.8 mM $MgCl_2$, 5 µL 10 × 0.2 mM buffer from each dNTP, 0.2 mM from each primer, 2.5 DNA Taq polymerase units, 0.02 µL bovine serum albumin (BSA, 5 mg mL^{-1}), 3 µL total DNA (7 to 15 ng) and ultrapure water. The conditions for amplification were: 1 initial denaturation cycle for 5 min at 95 °C; 30 cycles of 95 °C for 30 s, 53 °C for 30 s, 72 °C for 1 min and 1 final extension cycle for 6 min at 72 °C; and 4 °C for maintenance.

The DNA fragments (16S gene) from bacteria and archaea were concentrated to final volume of 13 µL in Concentrator plus (Eppendorf, Hamburg, Germany).

The bacteria and archaea amplification products (~ 400 ng) were subjected to denaturing gradient gel electrophoresis (DGGE) analysis (Model equipment: DGGEK-2401, CBS Scientific Company, San Diego, California, USA). We used a 6.0% polyacrylamide gel (acrylamide:bisacrylamide ratio, 37.5:1.0) with denaturing gradient from 25% to 60% to bacteria and 8% polyacrylamide gel with denaturing gradient of 20% to 65% to archaea.

To the first PCR for 18S rDNA amplification (fungi) we used 0.2 mM EF4 (Anderson et al., 2003) and ITS4 (White et al., 1990) primers, 1.2 µL 10X buffer, 0.45 µL $MgCl_2$, 0.25 mM each dNTP, 2.5 units Taq polymerase DNA, 3 µL total DNA (7 to 15 ng) and ultrapure water. The reaction was conducted under the following condition: 94 °C for 5 min (1 cycle); 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and 30 s (34 cycles); 72 °C for 5 min (1 cycle); and maintenance temperature of 4 °C until removal. For the second PCR (nested), 3.0 µL first reaction product was mixed with 0.4 mM each dNTP, 0.75 mM $MgCl_2$, 5.0 µL 10X buffer; 0.2 mM ITS1 primer (Gardes and Bruns, 1993) and 0.2 mM ITS2 primer (White et al., 1990), 2.5 units Taq polymerase DNA and ultrapure water. The reaction conditions were: 1 initial denaturation cycle at 94 °C for 5 min; 34 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C 30 s and 1 final extension cycle at 72 °C for 5 min. The maintenance temperature until removal was 4 °C. The second PCR products of fungi were subjected to DGGE analysis (Model equipment: DGGEK-2401, CBS Scientific Company, San Diego, California, USA) on 8% polyacrylamide gel (acrylamide:bisacrylamide ratio, 37.5:1.0) using a denaturing gradient of 35% to 60%.

Statistical analysis and DGGE profiles

Data were tested for adequacy of residues to a normal distribution by Shapiro-Wilk test and homoscedasticity by Bartlett test. The variables that showed no normality or homoscedasticity were transformed by the Box-Cox method and retested. Data were subjected to ANOVA and, when significant, rate effects and continuous and interrupted application were analyzed by Tukey test ($P \leq 0.05$). Control treatment and continuous and interrupted rates were compared by Dunnet's test ($P \leq 0.05$). Statistical analyses were processed using R software version 3.3.2 (The R Foundation for Statistical Computing, Vienna, Austria).

DGGE profiles were analyzed by the BioNumerics software version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). Positioning in the dendrogram was performed using the algorithm unweighted pair-group method with arithmetic mean (UPGMA) and the Jaccard coefficient with a tolerance index of 1%. With the DGGE band profiles, the Menhinick richness (S), Shannon diversity (H') and Simpson dominance (D) indices were calculated according to the equations:

$$S = \frac{hi}{\sqrt{H}} \qquad H' = \sum_{i=1}^{n} \frac{hi}{H} ln \frac{hi}{H} \qquad D = \frac{\sum_{i=1}^{n} hi\,(hi-1)}{H\,(H-1)}$$

where n is the number of bands in the DGGE profile; h is the intensity (i) of individual bands, and H is the total intensity of all bands in the profile.

RESULTS AND DISCUSSION

Microbiological attributes

Interruption of PS application after 5 and 12 mo influenced soil microbiological attributes. The interaction between factors was significant for none of the attributes, but there was an effect of the isolated factors. At depth 0.0-0.1 m, the MBC of the interrupted application presented a reduction of 5% in SPC sampling and 14% in WPC sampling in relation to the continuous application (Table 1), showing that the soil microbiota responds to the lack of PS.

The soil microbial biomass under interrupted application was limited to using only nutrients and compounds that were in the soil from previous applications, so the MBC was lower. In the continuous application, the microbial biomass was not limited because it received PS and with it a labile C charge and other easily assimilable elements. As shown by Notaro et al. (2018), inputs and PS are used as a substrate by soil microorganisms. In addition, PS itself transfers a microorganism bulk to the soil that can result in increased MBC, as observed by Plaza et al. (2004).

Lalande et al. (2000) reported that the labile C of PS added to the soil surface is available to indigenous microorganisms as a source of energy and nutrients, so it is assumed that they develop without limitations. In contrast, zymogen microorganisms show growth spike in response to soil residue addition (Moreira and Siqueira, 2002). Therefore, as soil sampling was performed after 10 d of application, C biomass was higher in the continuous application.

In SPC sampling, the MBC also differed with the rates, showing up higher in 60 m^3 ha^{-1} yr^{-1} (673.2 mg kg^{-1}) and lower in 30 m^3 ha^{-1} yr^{-1} (631.4 mg kg^{-1}) (Table 1).

At 0.1-0.2 m depth, the interrupted application showed a reduction on MBN of approximately 32% in SPC sampling and 29% in WPC sampling in relation to the continuous application. This fact is also attributed to the higher nutrient input from PS, as discussed above on MBC content. Balota et al. (2012) concluded that microbial biomass was enriched with N and P due to increasing rates of PS.

Table 1. Microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) on a Typic Oxisol under pig slurry (PS) continuous application for 28 yr and interrupted for 5 mo (sampling in the summer pre-cultivation) and 12 mo (sampling in the winter pre-cultivation).

Rate (R)	Summer pre-cultivation sampling						Winter pre-cultivation sampling					
	MBC			MBN			MBC			MBN		
	mg kg^{-1}						mg kg^{-1}					
	0.0-0.1 m											
	Application (A)			Application (A)			Application (A)			Application (A)		
	Int.	Cont.	$\bar{x}$ (R)	Int.	Cont.	$\bar{x}$ (R)	Int.	Cont.	$\bar{x}$ (R)	Int.	Cont.	$\bar{x}$ (R)
30 m^3 ha^{-1} yr^{-1}	604.7*	658.1*	631.4b	49.9	57.3	53.6a	515.6*	636.6*	576.1a	37.0	52.3	44.7a
60 m^3 ha^{-1} yr^{-1}	668.7*	677.8*	673.2a	64.8	59.8	62.3a	581.2*	637.2*	609.2a	40.8	51.6	46.2a
$\bar{x}$ (A)	636.7B	668.0A		57.4A	58.5A		548.4B	636.9A		38.9A	51.9A	
0 m^3 ha^{-1} yr^{-1}	360.4			40.2			304.8			34.5		
CV, %	2.6			30.2			5.2			27.6		
	0.1-0.2 m											
30 m^3 ha^{-1} yr^{-1}	518.8*	535.1*	526.9a	24.3	34.7	29.5a	450.7*	456.2*	453.5a	24.9	38.9	31.9a
60 m^3 ha^{-1} yr^{-1}	533.9*	548.5*	541.2a	29.8	44.6*	37.2a	448.0*	455.1*	451.5a	30.1	38.9	34.5a
$\bar{x}$ (A)	526.4A	541.8A		27.1B	39.6A		449.3A	455.6A		27.5B	38.9A	
0 m^3 ha^{-1} yr^{-1}	349.7			24.6			294.4			33.9		
CV, %	7.1			19.4			9.1			19.8		
	0.2-0.4 m											
30 m^3 ha^{-1} yr^{-1}	369.3	371.5	370.4a	22.0	24.4	23.2a	336.9*	398.0*	367.5a	18.1	22.5	20.3a
60 m^3 ha^{-1} yr^{-1}	384.3	397.9*	391.1a	41.3	24.1	32.7a	338.3*	400.2*	369.2a	18.9	24.4	21.6a
$\bar{x}$ (A)	376.8A	384.7A		31.6A	24.3A		337.6B	399.1A		18.5A	23.4A	
0 m^3 ha^{-1} yr^{-1}	293.8			22.8			219.6			17.5		
CV, %	7.5			55.5			2.6			23.6		

Lowercase letters in the column compare rates (30 and 60 m^3 ha^{-1} yr^{-1}) and uppercase letters in the column compare application. Equal letters do not differ significantly by the Tukey test ($P \leq 0.05$).
*Significant difference from the control treatment in relation to continuous and interrupted rates by Dunnet's test ($P \leq 0.05$).
Cont.: Continuous; Int.: interrupted; CV: coefficient of variation.

When the areas with continuous and interrupted applications were compared with the control treatment, there was difference in the three depths and for the two samplings. These differences were found in MBC content, which was smaller in control and higher in continuous or interrupted of 30 and 60 m^3 ha^{-1} yr^{-1} (Table 1). This occurs in response to the availability of nutrients supplied to the microbial community by PS. The addition over 28 yr of easily degradable organic C sources, such as PS, stimulates biological activity compared to plots that do not receive the organic material application (Marschner et al., 2003).

In this study, the TOC content was significantly equal for all treatments (Table 2). This could demonstrate the importance of using faster indicators such as MBC and MBN since the PS interruption occurred up to 12 mo and according to Plaza et al. (2007), changes in TOC may take decades to appear because most of the organic matter is nonliving and relatively stable.

The continuous and interrupted rates also did not differ regarding qMIC in the two samplings (Table 2), which shows that even after interrupting the PS application for 12 mo, the amount of soil organic matter (SOM) remained at a steady level.

The RBS was altered as a function of the interruption and applied rates. At 5 mo after discontinuation (SPC sampling), SBR was reduced (depth 0.1-0.2 m) from 10.5 mg C-CO_2 kg^{-1} d^{-1} to 8.6 mg C-CO_2 kg^{-1} d^{-1} due to locking in the PS applying (Table 3). As for the rates, the SBR was higher at the rate 60 m^3 ha^{-1} yr^{-1} in the layer 0.0-0.2 m, while in the 0.2-0.4 m depth, the SBR was significantly equal for both factors.

In WPS sampling, the differences for SBR were verified only in layer 0.0-0.1 m, following the same tendency of the previous sampling. The SBR decreased from 13.1 to 8.4 mg C-CO_2 kg^{-1} d^{-1} due to discontinuation of PS, showed up higher at 60 m^3 ha^{-1} yr^{-1} compared to 30 m^3 ha^{-1} yr^{-1} (Table 3). This can be attributed to the fact that the organic bulk is higher in the continuous application of 60 m^3 ha^{-1} yr^{-1} that provides C easily degradable by the microbial biomass that releases more CO_2 in the biodegradation process.

Table 2. Total organic carbon (TOC) and microbial quotient (*q*MIC) on a Typic Oxisol under pig slurry (PS) continuous application for 28 yr and interrupted for 5 mo (sampling in the summer pre-cultivation) and 12 mo (sampling in the winter pre-cultivation).

	Summer pre-cultivation sampling						Winter pre-cultivation sampling					
Rate (R)	TOC			qMIC			TOC			qMIC		
m^3 ha^{-1} yr^{-1}	g dm^3			%			g dm^3			%		
	0.0-0.1 m											
	Application (A)			Application (A)			Application (A)			Application (A)		
	Int.	Cont.	$\bar{x}$ (R)	Int.	Cont.	$\bar{x}$ (R)	Int.	Cont.	$\bar{x}$ (R)	Int.	Cont.	$\bar{x}$ (R)
30	18.7	18.0	18.4a	3.2	3.6	3.4a	18.7	20.9	19.8a	2.8	3.1	2.9a
60	18.5	18.0	18.3a	3.6	3.8	3.7a	19.7	20.0	19.8a	3.0	3.2	3.1a
$\bar{x}$ (A)	18.6A	18.0A		3.4A	3.7A		19.2A	20.4A		2.9A	3.1A	
0	17.6			2.1			19.3			1.6		
CV, %	9.8			23.5			6.1			8.1		
	0.1-0.2 m											
30	13.1	13.4	13.2a	4.0	4.0	4.0a	12.5	14.0	13.2a	3.7	3.3	3.5a
60	12.3	11.9	12.1a	4.4*	4.6*	4.5a	13.5	12.7	13.1a	3.3	3.6	3.4a
$\bar{x}$ (A)	12.7A	12.6A		4.2A	4.3A		13.0A	13.4A		3.5A	3.4A	
0	11.9			3.0			12.7			2.3		
CV, %	4.3			8.5			5.9			10.2		
	0.2-0.4 m											
30	9.2	10.1	9.7a	4.0	3.7	3.9a	7.8	9.3	8.5a	4.4*	4.3*	4.3a
60	9.8	8.2	9.0a	4.1	4.8*	4.5a	8.7	8.2	8.4a	4.1*	4.9*	4.5a
$\bar{x}$ (A)	9.5A	9.2A		4.0A	4.3A		8.3A	8.7A		4.2A	4.6A	
0	9.3			3.2			8.7			2.7		
CV, %	13.2			13.4			21.9			18.3		

Lowercase letters in the column compare rates (30 and 60 m^3 ha^{-1} yr^{-1}) and uppercase letters in the column compare application. Equal letters do not differ significantly by the Tukey test ($P \leq 0.05$).
*Significant difference from the control treatment in relation to continuous and interrupted rates by Dunnet's test ($P \leq 0.05$).
Cont.: Continuous; Int.: interrupted; CV: coefficient of variation.

Table 3. Soil basal respiration (SBR) and metabolic quotient (qCO_2) on a Typic Oxisol under pig slurry (PS) continuous application for 28 yr and interrupted for 5 mo (sampling in the summer pre-cultivation) and 12 mo (sampling in the winter pre-cultivation).

	Summer pre-cultivation sampling						Winter pre-cultivation sampling					
Rate (R)	SBR			qCO_2			SBR			qCO_2		
$m^3\ ha^{-1}\ yr^{-1}$	—— mg C-CO_2 kg^{-1} d^{-1} ——			—mg C-CO_2 g^{-1} MBC h^{-1}—			—— mg C-CO_2 kg^{-1} d^{-1} ——			—mg C-CO_2 g^{-1} MBC h^{-1}—		
						0.0-0.1 m						
	Application (A)			Application (A)			Application (A)			Application (A)		
	Int.	Cont.	$\bar{x}$ (R)	Int.	Cont.	$\bar{x}$ (R)	Int.	Cont.	$\bar{x}$ (R)	Int.	Cont.	$\bar{x}$ (R)
30	14.6	22.2	18.4b	1.0*	1.4*	1.2b	7.3	11.3*	9.3b	0.6	0.7	0.6a
60	25.1*	26.5*	25.8a	1.6	1.6	1.6a	9.5	14.9*	12.2a	0.7	1.0	0.8a
$\bar{x}$ (A)	19.9A	24.3A		1.3A	1.5A		8.4B	13.1A		0.6A	0.8A	
0	16.5			1.9			4.8			0.7		
CV, %	15.5			14.9			13.1			27.1		
						0.1-0.2 m						
30	5.3*	6.8*	6.1b	0.4*	0.5*	0.5b	3.7	3.3	3.5a	0.3	0.3	0.3a
60	11.9	14.1	13.0a	0.9*	1.1*	1.0a	3.9	3.4	3.7a	0.4	0.3	0.3a
$\bar{x}$ (A)	8.6B	10.5A		0.7A	0.8A		3.8A	3.4A		0.3A	0.3A	
0	13.9			1.7			3.0			0.4		
CV, %	9.8			15.6			21.8			23.9		
						0.2-0.4 m						
30	3.8*	5.0*	4.4a	0.4*	0.6*	0.5a	2.2	1.7*	1.9a	0.3*	0.2*	0.2a
60	4.1*	5.1*	4.6a	0.4*	0.5*	0.4a	2.1	1.9*	2.0a	0.3*	0.2*	0.2a
$\bar{x}$ (A)	3.9A	5.0A		0.4A	0.6A		2.1A	1.8A		0.3A	0.2A	
0	9.4			1.4			3.3			0.6		
CV, %	21.4			23.6			28.3			28.7		

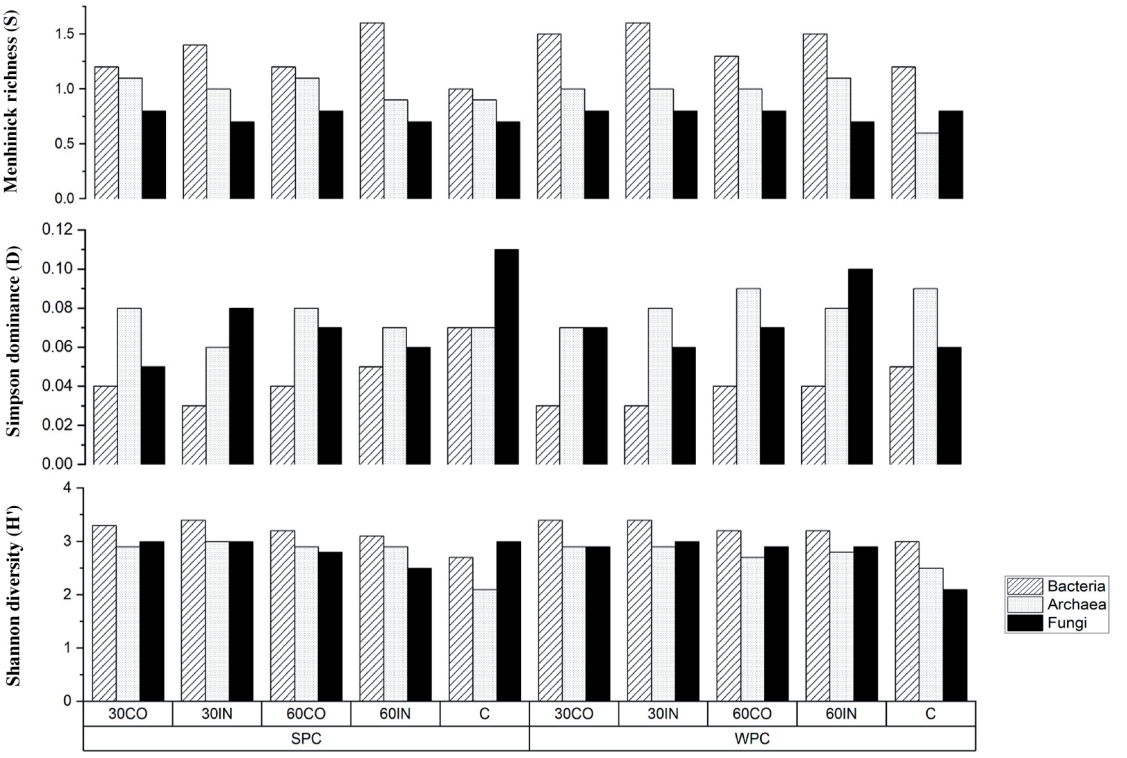
Lowercase letters in the column compare rates (30 and 60 $m^3\ ha^{-1}\ yr^{-1}$) and uppercase letters in the column compare application. Equal letters do not differ significantly by the Tukey test ($P \leq 0.05$).
*Significant difference from the control treatment in relation to continuous and interrupted rates by Dunnet's test ($P \leq 0.05$).
Cont.: Continuous; Int.: interrupted; CV: coefficient of variation.

According to Couto et al. (2013), the high labile C levels and low C:N ratio of PS stimulate soil microbial activity. While in the interrupted application, the soil had not received PS for 5 and 12 mo, in the continuous application, the soil received PS 10 d before sampling. According to Rochette et al. (2000), at least half of total CO_2 emissions occur during the first week after the addition of manure to the soil. Plaza et al. (2007) evaluated SBR up to 120 d after PS application and observed that CO_2 release peaks occurred in the first 14 d after PS application and stabilized after 30 d.

The qCO_2 showed difference only in the SPC sampling as a function of rate factor and ranged from 0.2 to 1.6 mg C-CO_2 g^{-1} MBC h^{-1} at depth 0.0-0.1 m and 0.5 to 1.0 mg C-CO_2 g^{-1} MBC h^{-1} at depth 0.1-0.2 m, at both depths was higher at 60 $m^3\ ha^{-1}\ yr^{-1}$ PS rate and lower at 30 $m^3\ ha^{-1}\ yr^{-1}$ following the same tendency as MBC and SBR (Tables 1 and 3). The control treatment at some depths presented higher SBR (Table 3), which indicates high microbiological activity. However, when analyzing the data together, it is noted that the low MBC and high qCO_2 indicate a lack of available substrate for growth and maintenance of microorganisms.

Plaza et al. (2004) and Balota et al. (2014) observed higher qCO_2 values the lowest PS rates and discussed this fact due to abiotic soil conditions such as pH, differences in substrate accessibility by microorganisms and change in microbial community composition (Xu et al., 2017). In this study, the bacterial richness index (S) was higher than fungi (Figure 1), which may increase qCO_2 due to the predominance of bacterial populations. This is corroborated by Dilly and Munch (1998) when noting that the reduction of qCO_2 indicates that the proportion of r-strategists, that prefer easily degradable organic compounds, decreases in relation to the population of K-strategists, that are believed to utilize humic material and the fatty residues of cuticular waxes and cutins as substrates. Or even that, the fungal biomass is larger than the bacterial biomass, due to a higher substrate efficiency by fungi compared to bacteria (Ho et al., 2017).

Figure 1. Menhinick richness (S), Simpson dominance (D) and Shannon diversity (H ‘) index of bacteria, archaea and fungi in soil influenced by pig slurry (PS) continuous application for 28 yr and interrupted for 5 mo (sampling in the summer pre-cultivation, SPC) and 12 mo (sampling in the winter pre-cultivation, WPC).



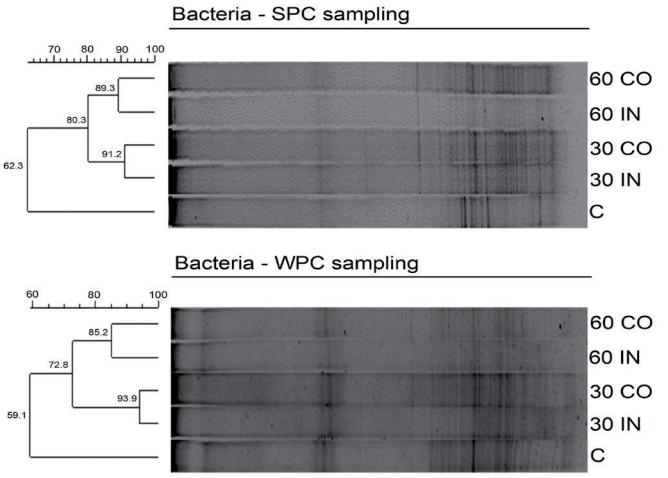
C: Control, 0 m^3 ha^{-1} yr^{-1}; 30IN: 30 m^3 ha^{-1} yr^{-1} rate with interrupted application; 30CO: 30 m^3 ha^{-1} yr^{-1} rate with continuous application; 60IN: 60 m^3 ha^{-1} yr^{-1} rate with interrupted application; 60CO: 60 m^3 ha^{-1} yr^{-1} rate with continuous application.

Genetic profiles of microbial community by DGGE

DGGE profiles revealed a high similarity between continuous and interrupted applications. For bacteria, it was observed that the interruption of PS had an effect on the profile of this group (Figure 2). In the SPC sampling, the similarity between 60 m^3 ha^{-1} yr^{-1} continuous and 60 m^3 ha^{-1} yr^{-1} interrupted treatments was 89% and between 30 m^3 ha^{-1} yr^{-1} continuous and 30 m^3 ha^{-1} yr^{-1} interrupted was 91%, and of these in relation to control (C) it was 62% (Figure 2).

In the WPC sampling, the similarity was 85% for treatment 60 m^3 ha^{-1} yr^{-1} continuous and 60 m^3 ha^{-1} yr^{-1} interrupted and 93% for 30 m^3 ha^{-1} yr^{-1} continuous and 30 m^3 ha^{-1} yr^{-1} interrupted, and of these in relation to the control, the similarity was 59% (Figure 2). Despite the fact that the soil did not receive PS for 12 mo, there was a high similarity between continuous and interrupted applications within rates, this is because the C and nutrients source used by the microorganisms was the same; therefore, stimulating the microorganisms equally because there is no diversity of substrates and according to the study developed by Souza et al. (2012) the soil bacterial communities structure is affected by cover vegetation due to the release of specific forms of C representing energy sources.

Figure 2. DGGE profiles of the bacterial community of a Typic Oxisol under pig slutty (PS) continuous application for 28 yr and interrupted for 5 and 12 mo.

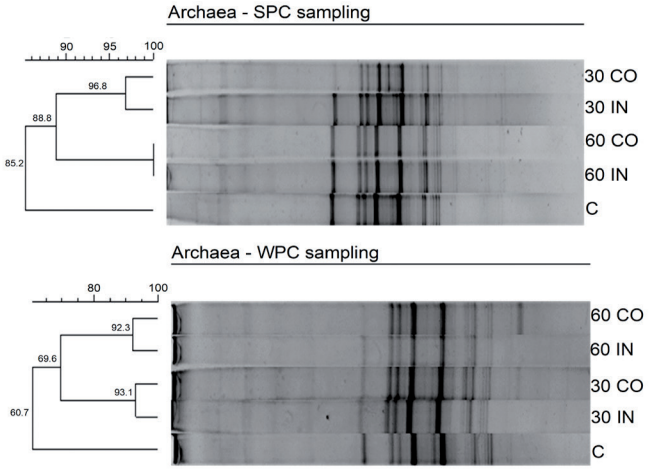


C: Control; 30IN: 30 m^3 ha^{-1} yr^{-1} rate with interrupted application; 30CO: 30 m^3 ha^{-1} yr^{-1} with continuous application; 60IN: 60 m^3 ha^{-1} yr^{-1} with interrupted application; 60CO: 60 m^3 ha^{-1} yr^{-1} and with continuous application; SPC: sampling in the summer pre-cultivation; WPC: sampling in the winter pre-cultivation.

The control (C) was 62% similar to the other treatments in the SPC and 59% in the WPC (Figure 2). As the control never received PS, its microbiota was limited to using only the C of cultural residues that are deposited in the soil after harvest as an energy source, thus having less diversity of substrates.

The archaeal community profile was similar to observed for bacteria. The similarity between 30 m^3 ha^{-1} yr^{-1} continuous and 30 m^3 ha^{-1} yr^{-1} interrupted treatments was 96% in SPC and 93% in WPC (Figure 3) and for 60 m^3 ha^{-1} yr^{-1} continuous and 60 m^3 ha^{-1} yr^{-1} interrupted treatments was 100% in SPC and 92% in WPC (Figure 3). The control was similar in 85% in SPC and 60% in WPC with other treatments that received PS (Figure 3).

Figure 3. DGGE profiles of the Archaea community of a Typic Oxisol under pig slurry (PS) continuous application for 28 yr and interrupted for 5 and 12 mo.



C: Control; 30IN: 30 m^3 ha^{-1} yr^{-1} rate with interrupted application; 30CO: 30 m^3 ha^{-1} yr^{-1} with continuous application; 60IN: 60 m^3 ha^{-1} yr^{-1} with interrupted application; 60CO: 60 m^3 ha^{-1} yr^{-1} with continuous application; SPC: sampling in the summer pre-cultivation; WPC: sampling in the winter pre-cultivation.

The fungi profile showed less similarity between treatments compared to bacteria and archaea (Figure 4). Unlike what occurred for the 16S profiles, for fungi, the PS interruption in SPC caused some effects. When establishing a cut at 70% similarity for forming clusters, three groups were formed in SPC (Figure 4). A group was formed by 60 m^3 ha^{-1} yr^{-1} rate, continuous or interrupted, and by 30 m^3 ha^{-1} yr^{-1} rate continuous, showing a similarity of 85%. Another two groups were formed by 30 m^3 ha^{-1} yr^{-1} rate interrupted and the control, which were separated individually. This shows that the PS rate influenced the separation because the control and 15IN did not receive or received the least amount of PS, respectively.

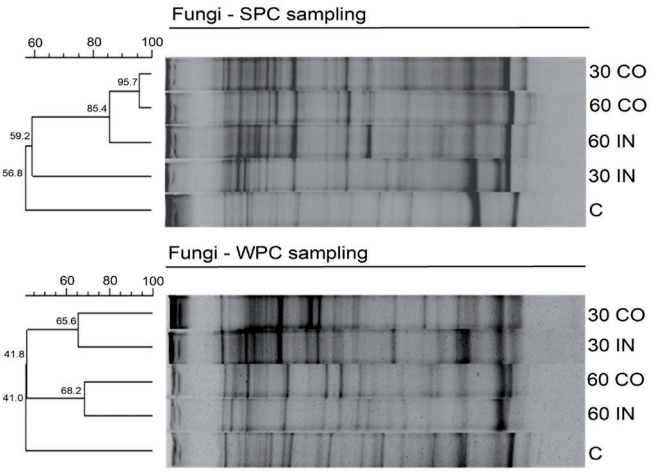
In the WPC sampling, the fungi community profile was not the same as that found in SPC. The 30 m^3 ha^{-1} yr^{-1} continuous and interrupted treatments showed 65% similarity, and for 60 m^3 ha^{-1} yr^{-1} continuous and interrupted treatments, the similarity was 68%. Compared with the control, the similarity of these treatments was 41% (Figure 4). That is, based on the cut in 70% similarity, all treatments are isolated, showing that the fungi communities have a very different dynamic than archaea and bacteria. The difference between fungi profiles of SPC sampling and WPC can be explained by the vegetal cultural residues remaining the winter and summer harvests that may have modulated an uneven environmental condition that led to different responses and between SPC and WPC for the fungi group. Furthermore, the soil chemical and biological composition of PS can be very variable.

The control treatment (C) without PS, and the 30 m^3 ha^{-1} yr^{-1} interrupted treatment had the lowest PS amount, so the fungi community profile was modified due the PS interruption up to 5 mo because in these plots there may have been a species selection that led to the prevalence of K-strategists when substrate availability is lower, corroborating Moreira and Siqueira (2002). This result is confirmed by the dominance index (D), which was higher in the control treatment (C) and 30 m^3 ha^{-1} yr^{-1} interrupted treatment in SPC sampling, in contrast to the diversity that was lower (Figure 1).

The differences in the SOM composition and the substrate availability may be one of the reasons for the differences in the microbial community structure, as generally readily decomposable compounds such as organic acids and carbohydrates are probably used mainly by soil bacteria, while fungi decompose the more recalcitrant and less soluble materials (Marschner et al., 2003).

Marschner et al. (2003), when evaluating five organic and inorganic fertilizers for 31 yr, observed that the treatments had effects on the biomass and soil bacterial community structure, but there was no effect on the fungal community structure.

Figure 4. DGGE profiles of the fungal community of a Typic Oxisol under pig slurry (PS) continuous application for 28 yr and interrupted for 5 and 12 mo.



C: Control; 30IN: 30 m^3 ha^{-1} yr^{-1} with interrupted application; 30CO: 30 m^3 ha^{-1} yr^{-1} with continuous application; 60IN: 60 m^3 ha^{-1} yr^{-1} with interrupted application; 60CO: 60 m^3 ha^{-1} yr^{-1} with continuous application; SPC: sampling in the summer pre-cultivation; WPC: sampling in the winter pre-cultivation.

Considering the Shannon diversity index (H'), it was possible to observe that the microbial diversity was greater when PS was applied than in the control without application (Figure 1). This result corroborates Faissal et al. (2017), which showed that Shannon index was significantly higher in the soil fertilized with manure from poultry and cattle than in the reference soil (without fertilization) and with the study of Zhen et al. (2014), when concluding that the animal manure application can improve the genetic structure of the microbial community and the diversity in soils. Li et al. (2015) observed that both bacterial abundance and diversity were greater in mineral fertilizer + swine manure treatment than in non-fertilized soil treatment.

It was observed that PS application provided an increase in the microbial genetic diversity up to 30 m^3 ha^{-1} yr^{-1}, whereas the diversity decreased in 60 m^3 ha^{-1} yr^{-1} rate. It is possible that this occurred due to a selection of anaerobic microorganisms in detriment to aerobic ones. PS presents a high biochemical oxygen demand, and when applied to the soil, it tends to reduce the partial pressure of O_2 in the soil atmosphere, favoring anaerobic microorganisms. Associate to this, the high amount of rain during the application period until the soil sampling (65 mm in SPC sampling and 69 mm in WPC sampling) further reduced the available O_2 in the soil. Lalande et al. (2000) comment that high PS rates can create temporary anaerobic zones in the soil.

CONCLUSIONS

The pig slurry (PS) interruption up to 5 and 12 mo after 28 yr of continuous application causes a decrease in microbial biomass C and N and a reduction in the microbiological activity measured by basal soil respiration.

The bacterial and archaeal community structures are less affected by the PS interruption than the fungal community, which had its diversity reduced due to the increased dominance of some species.

The PS application increase microorganism's diversity, especially at the 30 m^3 ha^{-1} yr^{-1} rate, even after 12 mo of PS interruption and the less diversity was found in the control treatment that never received PS.

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