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ORIGINAL ARTICLE

Assessment of the bacterial community structure in a Brazilian clay soil treated with atrazine

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Abstract In the present paper, the bacterial communities in two soils, one from an agricultural sugarcane cropped field and the other from an unperturbed soil with similar geopedological characteristics, were characterized using the Fluorescence In Situ Hybridization (FISH) method. FISH consists of in situ identification of bacteria using fluorescent labeled 16S rRNA targeted oligonucleotide probes visualizable under epifluorescence microscope. In the cultivated soil, in line with agricultural practice, the pre-emergence herbicide atrazine had been regularly applied each year at a concentration of 5 L/ha. The Shannon Diversity and Evenness Indices were also calculated using the phylogenetic data obtained from the FISH analysis. Although, at the sampling time (6 months after soil atrazine treatment), no residual herbicide concentration was found, the overall bacterial community results show a lower diversity and evenness in the agricultural soil than in the unperturbed one, demonstrating how microbiological indicators are sensitive to anthropogenic disturbance. In the natural soil, the dominant groups were α-Proteobacteria, β-Proteobacteria, and γ-Proteobacteria (representing more than 50 % of the bacteria), but in the agricultural soil, their abundance decreased significantly and represented just 31 % of the bacteria domain.

Keywords Triazine herbicides · Fluorescence in situ hybridization · Soil bacterial diversity

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Introduction

One of the main impacts on soil ecosystems is due to the spread of pesticides in global agricultural practices. For example, the herbicide atrazine has been definitively banned in EU owing to its toxicological characteristics (U.S. EPA 2006); however, it is being intensively applied in Brazil on cultures of sugar cane, maize, sorghum and coffee. Atrazine is commonly found as a water contaminant, and residual concentrations can be found both in soil and water for a long time after its application for agricultural purposes. Triazine persistence is directly dependent on microbial degradation (Grenni et al. 2009a, b; 2012). Microbial communities play key roles in natural ecosystem functioning, such as primary production, organic matter decomposition, nutrient cycling, and natural attenuation from contaminants, and thus contribute to soil and water purification processes, providing essential ecosystem services. The maintenance of overall ecosystem services is linked to that of bacterial diversity and functioning. Several microorganisms able to remove atrazine from soil by degrading it have been enriched and isolated (Ralebitso et al. 2002; Singh et al. 2004; Piutti et al. 2003; Rhine et al. 2003; Vaishampayan et al. 2007; Getenga et al. 2009). However, the occurrence of bacterial populations able to remove atrazine does not exclude that chronic contamination can negatively affect the most sensitive natural populations and consequently decrease bacterial diversity and to some extent ecosystem functioning. The maintaining of biodiversity is particularly important in Brazilian soil ecosystems, which contain a wide and well-known diversity of flora and fauna, although knowledge about the microbial communities is quite scarce (Faoro et al. 2010). Pollution and intensive cultivation of agricultural land may influence soil quality and productivity, but little is known of their effects on soil microbial communities, and the consequent impacts on soil functioning. Diverse communities are believed by macro-ecologists to promote ecosystem stability, productivity, and sustainability

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and this relationship has also been found in the case of soil bacterial diversity (Girvan et al. 2005).

It is known that few microorganisms from natural populations (less than 1 % of those in soil) are capable of growth on culture media (Amann et al. 1995), and DNA extraction from and PCR with soil samples is often hampered by possible interference from clay and humic acids in PCR reactions. Moreover, this technique is unable to distinguish between active and inactive cells in the bacterial community. The Fluorescence In Situ Hybridization (FISH) method overcomes all these problems and makes it possible to analyze in situ microbial communities using specific oligonucleotide probes labeled with a fluorochrome. The target of the probe is the rRNA, which occurs in a large number of copies and is characterized by structural and functional conservatism in all bacteria (Moter and Göbel 2000; Baker et al. 2003). The method detects the specific sequences of rRNA in single cells, which correspond to the classification of a bacterial population at different phylogenetic levels (from phylum to species). Only viable and highly active cells have a sufficient number of ribosomes for in situ hybridization to be applied successfully with a specific probe. This method can also, therefore, be an indicator of the physiological state and viability of cells (Lew et al. 2010). However, because the detection of stained cells was hampered by background particles, FISH was not applied to soil until a successful method of separating cells from particles was designed (Barra Caracciolo et al. 2005a, b; 2010).

The main objective of this study was to identify both the structure of the bacterial community of an agricultural soil which had been used for sugarcane cultivation for 6 years and that of an unperturbed soil (natural soil). We propose the use of FISH for improving knowledge about soil microbial diversity, in particular of soils located in areas that are important reserves of biodiversity and, therefore, particularly interesting for their ecosystem complexity and services.

Materials and methods

Soil sampling and characterization

Bulk soil samples (10–20 cm depth) were collected from an agriculture field cultivated with sugarcane for 6 years, where atrazine had been regularly applied each year at a concentration of 5 L/ha, located close to the city of Assis Chateaubriand, Paraná. The sampling was performed 6 months after the last herbicide pre-emergence application. Other samples were collected from a natural soil (virgin forest) which had never received any anthropogenic pressure, located in Cascavel, Paraná.

Samples were collected with sterile spatulas and then placed in sterile polypropylene bags (15×20 cm) and sent to the laboratory in a portable cooler box. Some sub-samples were processed for microbiological analysis and others for geochemical characterization.

Chemical analysis

Some sub-samples of both agricultural and natural soils were analyzed to assess the organic carbon (OC) content, by using an elemental carbon analyzer. For determining the occurrence of atrazine in the agricultural soil, 25-g subsamples in triplicate were extracted with 25 ml methanol in an ultrasonic bath for 5 min, followed by filtration and concentration in vacuum (del Valle and Nelson 1994). Atrazine concentration was determined by HPLC using a C18 (150×4.6 mm) column, UV detector at 230 nm, eluent methanol:water (50:50 v/v) at a flow rate of 1 ml/min, temperature 35 °C, and injection volume 20 μl. Moreover, inorganic elements K, P, Cu, Zn, Fe, and Mn were assessed using the Mehlich-1 extraction method, and Al, Ca, and Mg were extracted with a 1 M KCl solution, using standard methods described in detail in Embrapa (2009).

Microbiological analysis

Bacterial abundances were measured using the epifluorescence direct count method, reported in detail in Barra Caracciolo et al. (2005a, b), using 4′,6′-diamidino-2-phenylindole (DAPI) as the DNA fluorescent agent. In order to investigate the bacterial community structure of the two soils, the Fluorescence In Situ Hybridization (FISH) method was performed on samples from both the agriculture field and from the natural site. Before each sampling, plants, roots, and decomposing organic matter were removed from the soil samples.

For each soil, six sub-samples (1 g each) were fixed (1:1) with a solution composed of phosphate-buffered saline: 130 mM NaCl; 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; 2 % formaldehyde; 0.5 % Tween 20; and 100 mM sodium pyrophosphate. A density gradient centrifugation was carried out in order to separate and extract the detachable bacteria from the soil particles by high-speed centrifugation with the non-ionic density gradient medium, as described in detail in Barra Caracciolo et al. (2005a, b). Each sub-sample was then filtered on a 0.2-μm polycarbonate membrane. The filters were stored at −20 °C until further processing.

Fluorescence In Situ Hybridization (FISH) of the harvested cells, counterstained with DAPI, was performed using fluorescent probes for the identification, under the epifluorescence microscope, of the major bacterial divisions commonly found in soil (Barra Caracciolo et al. 2005a, b; 2010), such as α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Planctomycetes, Gram-positive low G+C-content bacteria and *Nitrospira*. Moreover, some bacterial groups, such as nitrite-oxidizing bacteria (within *α*-Proteobacteria) and

ammonia-oxidizing bacteria (within β-Proteobacteria), were investigated at more specific hierchical levels in order to look for bacteria involved in the N cycle. Species in the *Nitrobacter* genus plays an important role in the nitrogen cycle by oxidizing nitrite into nitrate in soil. The *Nitrosomonas* genus is also found in soils and is important in the nitrogen cycle as it increases the availability of nitrogen to plants. Moreover, some *Nitrosomonas* species possess the enzyme, urease, which catalyzes the conversion of the urea molecule to two ammonia molecules and one carbon dioxide molecule. As the final step of the degradation of atrazine comprises transforming urea into CO_2, the occurrence of this bacterial group in the soil treated with atrazine is expected (Barra Caracciolo et al. 2005b). For this purpose, the following Cy3-labeled oligonucleotide probes were applied: EUB338, EUB338 II, EUB338 III (for bacteria), and inside this domain ALF1b, BET42a, GAM42a, PLA46, together with PLA886, HGC69a, NSR1156, Nso190, and NIT3. Further details of these probes are available at http://www.microbial-ecology.net/probebase (Loy et al. 2007). The protocols for both FISH and bacterial abundance by DAPI counts are described in detail in previous works (Barra Caracciolo et al. 2005a, b; Grenni et al. 2009a, b).

The averages of the cells binding the bacterial probes were calculated as a percentage of the total DAPI-positive cells from 10 to 20 randomly selected fields on each filter section (corresponding to 500–1,000 stained cells). The slides were mounted with a drop of Vectashield Mounting Medium and the preparation examined with an Olympus BX60 epifluorescence microscope at 1,000× magnification. The microscopic fields were photographed and stained cells were counted with the help of UTHSCA (University of Texas Health Science Center, San Antonio, TX, USA) Image Tool software. Statistical analysis of the overall bacterial group data was done using an analysis of variance (ANOVA), with significant differences at the $p<0.05$ level. The PC Program used was SISVAR 5.0.

Results and discussion

Geochemical characteristics of the soils

The main geochemical characteristics of the two soils are described in Table 1. Both soils were classified as dark red dystrophic clay Latosoil on the basis of the Brazilian System of Soil Classification (SiBCS) (Embrapa 2006) and clay on the basis of USDA soil taxonomy. This kind of soil is always acid, deep, old, well-drained and slightly airy, with a low silt content, low content of easily weathered materials, and saturation below 50 %. It has a homogeneous granular structure and has a constant level of aluminum and iron oxides.

Table 1 Main geochemical characteristics in the natural and agriculture soils

	Natural soil	Agricultural soil
Soil layer (cm)	0–20	0–20
Clay	63	75
Silt	19	15
Sand	18	10
Texture class (USDA)	Clay	Clay
pH	4.30	4.20
OC (g/kg)	9.74	11.30
N (g/kg)	4.87	5.65
P (mg/kg)	1.00	1.00
K^+ (cmol/kg)	0.09	0.12
Ca^+ (cmol/kg)	0.32	0.74
Mg^{2+} (cmol/kg)	0.08	0.30
$H^+ {}^+Al^{3+}$ (cmol/kg)	7.76(−)	8.36(−)
Al^{3+} (cmol/kg)	0.77	1.12
Cu (mg/kg)	13.20	10.00
Zn (mg/kg)	0.80	0.80.
Fe (mg/kg)	33.00	77.00
Mn (mg/kg)	10.00	16.00

Residual concentrations of atrazine were not found in the soils analyzed, showing that the herbicide was degraded in the soil 6 months after its application.

Microbiological analysis

The bacterial numbers, obtained by DAPI counts, were lower in the agricultural soil (4.07E+09 cell/g soil) than in the natural soil (7.23E+09 cell/g soil).

The percentages of bacteria detected in the agricultural soil were significantly lower than in the natural soil for all probes applied (ALF1b, BET42a, GAM42a, PLA46/886, HGC69a, NSR1156, Nso190, NIT3) ($p>0.05$). In Fig. 1, as an example of visualization under the microscope, a photo of *Bacteria* and *α-Proteobacteria* detected from a natural soil sample is reported.

FISH analysis made it possible to identify all the active bacteria occurring in the soil samples (Bouvier and Del Giorgio 2003; Barra Caracciolo et al. 2010). The cells (47.80 % in agricultural soil and 37.55 % in natural soil, respectively) which were not identified by FISH were therefore presumably not active and/or belonging to the Archaea domain, which was not searched for in this analysis.

The number (no. bacterial cells/g soil) of bacteria for each taxa searched for was obtained by multiplying the total cell abundance (determined by DAPI direct counts) and the percentage of cells detected by each specific probe (Fig. 2).

The most abundant and dominant groups in the natural soil were α-Proteobacteria, β-Proteobacteria and γ-Proteobacteria.

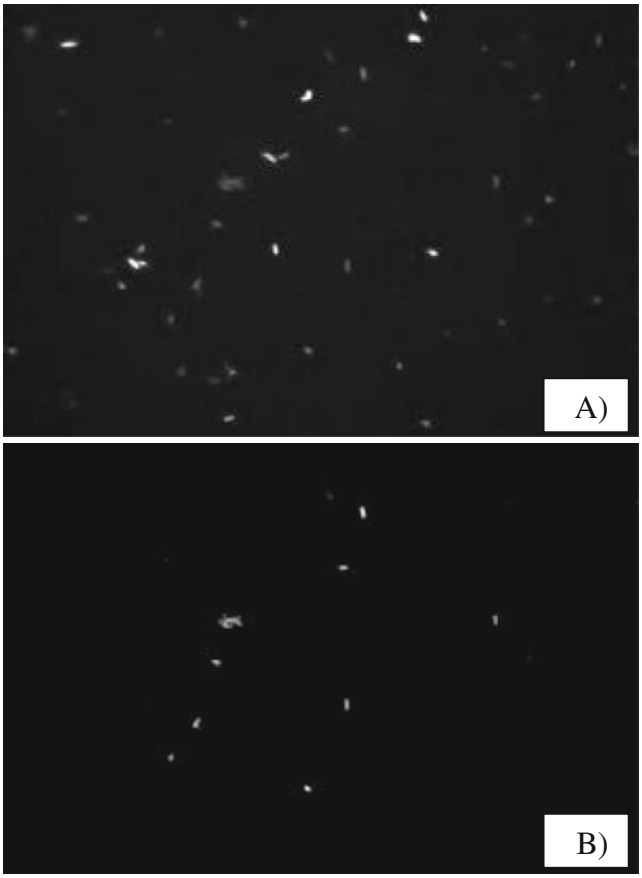

Fig. 1 Images of FISH under the epifluorescence microscope of a natural soil sample after application of: **a** EUB mix oligonucleotide probes for *Bacteria*; **b** the specific ALF1b probe for α-Proteobacteria

In line with these results, *Nitrobacter* spp., which are nitrite-oxidizing bacteria (included in α-Proteobacteria) and β-ammonia-oxidizing bacteria were significantly higher than in the agricultural soil, showing the occurrence of a greater number of bacteria actively involved in specific N cycle transformation.

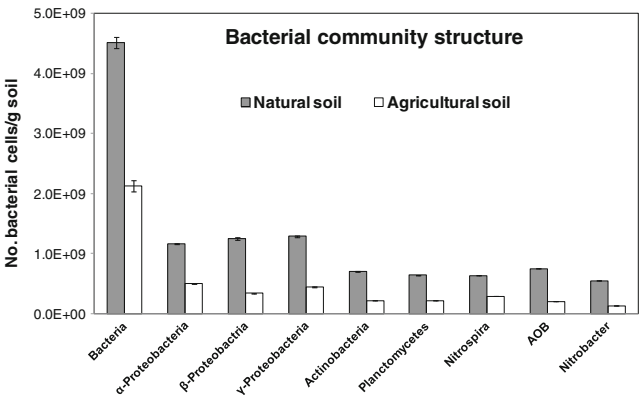

Fig. 2 Bacterial community structure detected by FISH in the natural and agriculture soils. *AOB* Ammonia-Oxidizing bacteria (β-Proteobacteria); *Nitrobacter* nitrite-oxidizing bacteria (α-Proteobacteria)

Proteobacteria are dominant in natural soils (Faoro et al. 2010) and in good quality state soils (Barra Caracciolo et al. 2010; Mocali et al. 2012), since this group includes many bacterial species involved in various ecosystem functions (e.g., C and N cycles). The fact that in the agricultural soil the Proteobacteria dominance was not so evident and that the bacterial numbers were generally lower although the organic carbon content, which generally promotes microbial activity (Grenni et al. 2009a, b; Chen et al. 2003), was higher, confirms that the agricultural soil had a lower quality state than the natural soil from a microbial community point of view.

In order to represent the results obtained in terms of bacterial community diversity, the Shannon Diversity (H_s) and Evenness indices were applied to the two soils. The results are shown in Table 2.

As can be seen in Table 2, the bacterial diversity in the agricultural soil is lower than that in the natural soil in terms of both diversity and evenness. These results are in line with the findings of Torsvik et al. (1996) and Girvan et al. (2005), who stated that environmental stress and agricultural management can reduce bacterial diversity.

It is known that bacterial diversity can be influenced by abiotic factors, such as pH, soil particle sizes, and contaminant occurrence. Because the two soils studied had a similar pH value and both were classified as clay, we might ascribe the lower diversity and viability of the agricultural soil compared to the natural soil to the anthropogenic disturbance, including the chronic application of the herbicide atrazine. Faoro et al. (2010) report that physicochemical characteristics have specific contributions to soil biodiversity, which can be influenced by factors such as altitude, Ca^{2+}/Mg^{2+} ratio, and Al^{3+} and phosphorus content. These conclusions are in line with those found in the present work, since a higher Ca^{2+}/Mg^{2+} ratio was associated with a higher level of biodiversity, while a higher level of Al^{3+} was associated with a lower level of biodiversity. However, we cannot exclude that an additional factor such as Fe content, which was higher in the agricultural soil than the natural soil, could have also influenced the bacterial community diversity to some extent.

Table 2 Bacterial diversity (H_s, Shannon Diversity and Evenness) in the natural and agriculture soils

	Natural soil	Agricultural soil
No. of bacterial taxa found (S)	6	6
No. bacteria/g soil	7.23E+09	4.07E+09
$H_{max}=\log S$	0.78	0.78
$H_s=-\sum_{i=1}^{6} p_i \log p_i$	0.65	0.43
$Evenness=H/H_{max}$	0.83	0.56

In conclusion, the FISH method is shown to be a very sensitive tool not only for assessing the structure of the bacterial community of a particular soil directly in their natural environment but also for giving us information about its activity with regard to the ecosystem quality state.

We propose the use of microbiological indicators for assessing soil quality state, because they are sensitive not only to a present but also to a previous contamination and/or anthropogenic disturbance.

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