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Promising indicators for assessment of agroecosystems alteration among natural, reforested and agricultural land use in southern Brazil

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Abstract

Microbiological soil-quality indicators, especially related to C and N cycles, and microbial diversity may be useful tools to determine whether a particular environment responds to an imposed management or reclamation strategy. External influences such as forest clearance and soil management affect biological indicators making them useful to point out whether the land use strategy is sustainable. Accordingly, the aim of this work was to assess the utility of some soil chemical and microbiological properties and 16S rDNA diversity in bacteria domain and their significance as soil-quality indicators in different land use systems in southern Brazil, Paraná State. Nine sites with soil originated from basalt (Rhodic Ferralsol), previously covered with the Atlantic native forest were evaluated: a native forest tract as reference; three sites artificially reforested with native species, but with understory differently managed; secondary forest naturally regenerated from abandoned pasture; artificially reforested with eucalyptus; two wheat-cropped sites at differing vegetative stages; one site in fallow. Twenty-four chemical and microbiological properties and their derivatives were assessed, in addition to molecular diversity of bacteria domain based on denaturating gradient gel electrophoresis (DGGE) analysis. Amongst all variables, the most dissimilar along the sites were total organic C, microbial biomass C and N, and ammonification rate. Total organic C was highest in the native forest, followed by secondary forest, eucalyptus and the artificially reforested sites; the wheat-cropped and fallow sites produced the lowest values. This trend was also observed for ammonification rate, which was closely correlated to organic C. Microbial biomass C and N were also higher in the reforested sites, whereas for microbial N biomass, the eucalyptus site resembled to the wheat-cropped and fallow sites. The DGGE analysis revealed that the fallow, eucalyptus and wheat-cropped sites had less bacterial diversity. All the sites reforested with native species grouped with the native forest, while the eucalyptus, fallow and wheat-cropped sites formed separate clusters. A similar clustering pattern was observed when all chemical and microbiological properties were considered in a grouping analysis. The results for reforestation employing native species tended to be similar to those of the stable native forest, while the use of an exotic species (eucalyptus) tended to be similar to those of the cropped sites. In addition, the fallow site showed general unfavorable trends in microbiological indicators and less bacterial diversity, suggesting that such soil management is not sustainable at least in subtropical areas. In this case, would be preferable provide the soil with vegetal covering that increase the organic C inputs and consequently microbial diversity and activity.
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Keywords: Bacterial molecular diversity; Denaturating gradient gel electrophoresis; Microbial biomass; Soil management; Soil organic C

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1. Introduction

A sustainable ecosystem depends on nutrient fluxes through the trophic levels, which are mainly mediated by microorganisms, the driving force for soil organic matter (SOM) turnover (Singh et al., 1989; Chen et al., 2003). In forests in equilibrium, up to 95% of the N circulates in almost closed plant-microorganism-soil systems (Rosswall, 1976). On the other hand, when forest is cleared and the soil used for agriculture, the system becomes open. This creates a dependence on external inputs of nutrients (Brussaard et al., 2004) in order to balance the output by harvests, leaching and erosion.

Native climax forests are in equilibrium; C and nutrients recycle mainly by means of microbial action and their interactions. After deforestation and soil use, a new equilibrium is reached (Lemenih et al., 2005). At least in tropical and subtropical areas, C inputs in agricultural systems are generally lower than they were in native forests. Thus, the amounts and diversity of C compounds used by microorganisms as energy sources are reduced, affecting biogeochemical cycles in the soil (Badiane et al., 2001). In addition, external inputs such as herbicides may affect the bacterial structure and function in agricultural soil (Seghers et al., 2003). In natural ecosystems, inputs of N to soil from biological and chemical fixation are generally small, so that soil N must be efficiently recycled (Mummey et al., 2002). On the other hand, external inputs of mineral N are necessary in open systems (Brussaard et al., 2004). However, nitrate is easily leached from the soil profile or denitrified. Thus, NH_3 immobilized in the microbial biomass often represents the most important N reservoir, especially in tropical soils (Singh et al., 1989). Furthermore, soil microorganisms are involved in all steps of N cycling. Any environmental change, including soil management, will affect microbial activity and diversity, and consequently N availability for plants.

Soil microbial and biochemical status in both natural and agricultural ecosystems have been used as bioindicators of soil stress or benefits from reclamation efforts (Badiane et al., 2001; Mummey et al., 2002; Dinesh et al., 2003; Andrade, 2004). In addition to chemical and physical properties, soil microbial properties may indicate suitable management and restoration practices towards the sustainability.

In recent years, microbial diversity, activity and resilience have been correlated with the sustainability of crops and/or forests in various ecosystems and soil conditions. This approach is particularly important because many species in several ecosystems are in danger of being lost (Loreau et al., 2001). The use of molecular techniques, such as the denaturating gradient gel electrophoresis (DGGE), can be useful in estimating the soil microbial diversity. This method is based on the melting point of a DNA sequence, which depends in part on the proportion of nucleotides composing the sequence. For bacteria, the DNA sequence used in these studies is the one that codifies for the 16S ribosomal subunit (16S rDNA). Nevertheless, the DGGE technique can result in underestimation of soil microbial diversity. For example, not all species are represented, the dominant species may predominate, and bands from more than one species may be hidden under those of others (Heuer et al., 2001; Müller et al., 2002). However, despite these limitations, DGGE seems to be the most sensitive method till the moment for detecting differences in community diversity (Müller et al., 2002). Moreover, few studies have compared molecular and classical methods for the purpose of gaining better understanding of soil microbial ecology.

The aim of this work was to assess some chemical and biological soil properties related to C and N cycles and the genetic diversity for bacteria domain in soils under natural forest conditions compared with reforested sites and agricultural systems.

2. Materials and methods

2.1. Field sites

The study was performed at the Mata dos Godoy State Park and adjacent cropped areas in Londrina, Paraná State, Brazil (23°27′S, 51°15′W). The climate is classified as Cfa (humid subtropical) according to Köppen. The average annual temperature is 20.9 °C (23.6 °C in January and 16.7 °C in July) and the average annual rainfall is 1615 mm, with most of rain in the spring–summer period (October–March). The areas range of altitude is from 560 to 600 m above sea level.

The sampling sites, nine in total, were selected according to the vegetation cover, soil use and management (Table 1). The parent material is the same in all sites (basalt), resulting to reddish clay soils (Rhodic Ferralsol – FAO, 1994). According to Soil Survey (1999), except site 8, in all the other sites the soil was classified as Typic Eutrudox (slope less than 10%, well drained, deep, horizons A + B with more than 2 m above the C horizon, not rocky, at least 60% and 70% of clay in A and B horizons, respectively, consistency slightly plastic and friable, with fine granular structure. Abundant roots in the upper layers, decreasing with depth). In the site 8, the soil was classified as Typic Rhodudalf (slope about 25%, well drained, horizons A + B with about 0.8 m of depth above the C horizon, rocky parent material on the surface, at least 50% and 75% of clay in horizons A and B, respectively, consistency increasing in plasticity with depth, granular structure, increasingly stony after 40 cm. Abundant roots in this layer, but rarer more deeply). The locations of the sampling sites are given in Fig. 1.

2.2. Sampling procedure

Soil sampling was done at the end of the less rainy season, in August 2003. Samples were collected from the 0 to 10 cm

Table 1
Dominant plant species, previous soil use and actual soil management in each sampling site at the Mata dos Godoy State Park and adjacent cropped areas in Londrina, Paraná State, Brazil

Site	Dominant plant species	Previous soil use	Actual soil management
1. Native forest[a]	*Apidosperma polyneuron* (Apocynaceae) *Euterpe edulis* (Arecaceae) *Croton floribundus* (Euphorbiaceae) *Trichilia clausenii* (Meliaceae) *Cordia trichotoma* (Boraginaceae) *Parapiptadenia rigida* (Fabaceae-Mimosoideae)	No	No
2. Reforested in 1990, understory managed mechanically	*Peltophorum dubium* (Fabaceae-Caesalpinoideae) *Colubrina glandulosa* (Rhamnaceae) *Tabebuia avellanedae* (Bignoniaceae)	Coffee (*Coffea arabica*) until 1975 followed by pasture with Guinea grass (*Panicum maximum*) until 1990	Weeds (Poaceae) cut down mechanically, residues left on surface
3. Wheat under old rotation	Wheat (*Triticum aestivum*) at flowering stage	Coffee until 1980s, followed by annual rotation with soybean (*Glycine max*) or maize (*Zea mays*) in the summer, followed by wheat in the winter	No-tillage crop rotation since 1990
4. Reforested in 1990, understory not managed	Same as site 2, with predominance of Guinea grass in the understory	Coffee until 1975, followed by pasture with Guinea grass until 1990	No
5. Fallow	Scarce weeds (Malvaceae and Poaceae)	Coffee until 1980s, followed by annual cropping of maize in the summer, fallow in the winter. Plant residues ploughed in	Ploughing
6. Reforested in 1990, open canopy due to tree mortality, understory not managed	Same as site 2, with predominance of Guinea grass in the understory	Coffee until 1975, followed by pasture with Guinea grass until 1990	No
7. Wheat under recent rotation	Wheat at maturation stage	Coffee until 1990, abandoned from 1990 to 2002 when the area was cleared again and cropped with soybean in the summer	No-tillage
8. Secondary forest originated from natural seeds dispersion (wind, birds, animals)	*Lonchocarpus muehlenbergianus* (Fabaceae) *Heliocarpus americanus* (Tiliaceae) *Tabernaemontana catharinensis* (Apocynaceae)	Coffee until 1975, followed by pasture with Guinea grass until late 1980s and then abandoned	No
9. Eucalyptus	*Eucalyptus grandis*	Coffee until late 1970s, followed by reforestation with eucalyptus	No

[a] Das-Chagas e Silva and Soares-Silva (2000).

topsoil layer with a 4.5 cm (diameter) stainless steel sampler after removal of the litter. At each representative site, three transects of 15 m × 5 m were selected randomly, in an area not larger than 3000 m^2. Along each transect, 20 sub-samples were collected randomly and pooled to form a composite sample. After mixing, the samples were sieved (<2 mm) and stored at 4 °C at field moisture or air-dried, depending on the analysis to be carried out. For microbiological assessments, the samples were processed up to 72 h after sampling.

2.3. Soil analyses

Water content was determined gravimetrically after oven-dried for 24 h at 105 °C. The water-holding capacity (WHC), determined gravimetrically (McInnes et al., 1994), was similar in all samples, about 40% (w/w). The measurements performed on the air-dried samples (drying for 72 h) were: pH in 0.01 M $CaCl_2$ (1:2.5 soil-to-solution); available P (colorimetrically by the ascorbic acid method after extraction with Mehlich I solution) (Murphy and

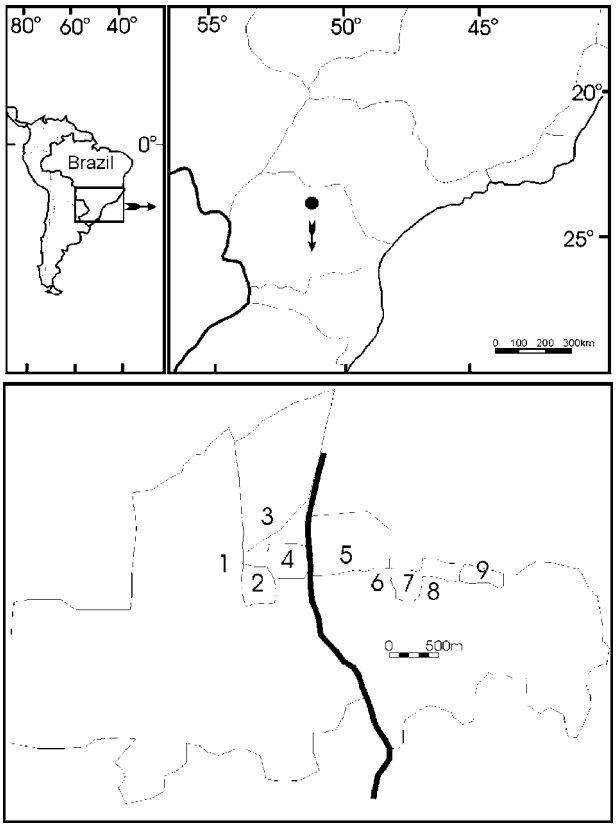

Fig. 1. Location of the sampling sites at the Mata dos Godoy State Park (1, 2, 4, 5, 6 and 8) and adjacent cropped areas (3, 7 and 9), in southern Brazil. (1) Native forest; (2) reforestation, understory managed mechanically; (3) wheat under old rotation; (4) reforestation, understory not managed; (5) fallow; (6) reforestation, understory not managed, open canopy; (7) wheat under recent rotation; (8) secondary forest; (9) eucalyptus. For more details, see Table 1.

Riley, 1962); and organic-C (Yeomans and Bremner, 1988). All the other measurements were performed in field-moist samples. When samples were incubated, the moisture was corrected to 60% (w/w) of the WHC with sterilized distilled water.

Mineral N (NO_3^-–N plus NH_4^+–N) was quantified in 2N KCl extracts by double steam distillation. In the first distillation, the ammonia was determined after MgO addition, following titration of trap solution (2% H_3BO_3 and indicators) with 0.02N H_2SO_4. In the second distillation, the same extract received Devarda's alloy in order to convert the nitrate to ammonia, quantified in the same way in a new trap solution (Keeney and Nelson, 1982).

The ammonification rate ($\mu g\ N\ g^{-1}\ day^{-1}$) was based on mineral N before and after incubation of 100 g soil (dry basis) in a closed vial at 28 °C for 21 days in the dark (Schuster and Schroder, 1990). For nitrification rate (%), an extra sample received $(NH_4)_2SO_4$ solution in order to provide 125 μg of N per gram of soil and incubated in the same conditions. The percentage of NH_4^+–N converted to NO_3^-–N during this period took into account the NH_4^+–N added and released from organic matter mineralization, and the NO_3^-–N before and after incubation (Schuster and Schroder, 1990).

In culture-based assessments, an aliquot of 10 g of field-moist sample was shaken for 30 min in 95 mL of sterile saline (0.85% NaCl). Thereafter, serial dilutions were made with sterile saline up to 10^{-8} (Zuberer, 1994). For enumeration of viable heterotrophic bacteria, sporulating bacteria and total culturable fungi, aliquots of 50 μL from appropriate dilutions were plated on tryptic soy agar (10^{-5}), nutrient agar (10^{-3}) and Martin medium (10^{-3}), respectively, with two replications on Petri dishes. For sporulating bacteria, extra bottles containing the 10^{-3} dilution were incubated in a water-bath at 85 °C for 15 min before plating. In order to inhibit fungal and bacterial growth, the TSA and NA media received 200 mg L^{-1} of Benlate®, while the Martin medium received 100 mg L^{-1} of nalidixic acid, respectively. Plates were incubated at 28 °C, evaluated after 5 days and recounted at 7 days for new colonies and expressed as log of colony forming units (CFU) g^{-1} of soil.

The same serial dilution was used to estimate communities of ammonifiers (Sarathchandra, 1978), nitrifiers (ammonia- and nitrite-oxidizing bacteria) (Schmidt and Belser, 1994) and protozoa (flagellates and ciliates) (Ingham, 1994). Sets of five dilutions (10^{-4}–10^{-8} for ammonifiers, 10^{-2}–10^{-6} for nitrifiers and protozoa) and five tubes per dilution (three for protozoa) were used to estimate each community by the most probable number (MPN) method (Woomer, 1994). The incubation time was 4 days for ammonifiers, 8 weeks for nitrifiers and 5 days for protozoa at 28 °C. Data were expressed as log of MPN g^{-1} of soil.

Dehydrogenase activity (Casida et al., 1964) used 1% triphenyltetrazolium chloride (TTC) as substrate (1:1 soil:solution, w/v). The triphenyltetrazolium formazan (TTF) produced after incubation at 37 °C for 24 h was extracted with 10 mL of methanol after centrifugation. The enzyme activity was quantified spectrophotometrically in the supernatant against a calibration curve constructed with TTF, and expressed in μg TTF g^{-1} of soil.

Microbial biomass C and N were estimated by the fumigation-extraction method (Brookes et al., 1985; Vance et al., 1987). Two portions equivalent to 25 g of oven-dried soil were put in separate beakers. One portion was fumigated for 24 h at 25 °C with ethanol-free chloroform. The fumigated and non-fumigated soils were shaken for 30 min with 100 mL 0.5 M K_2SO_4 at 210 revolutions per minute and filtered (porosity 8 μm). Organic C in the extracts was quantified by back-titration with 33.3 mM $(NH_4)_2Fe(SO_2)\cdot 6H_2O$ after dichromate oxidation in acid medium (Anderson and Ingram, 1993). Microbial biomass C was calculated based on the difference between organic C extracted from fumigated and non-fumigated soil, by using a k_C-value of 0.33. The determinations here were used to calculate the relative proportions of microbial biomass C in relation to soil organic C. Microbial biomass N levels were estimated after N quantification in the extracts used for microbial biomass C, with a k_N-value of 0.68 (Brookes et al., 1985).

Basal soil respiration was taken after 8 days of incubation at 28 °C in the dark. The CO_2 evolved from equivalent 100 g samples of dry soil in hermetically sealed containers was trapped in 1 M NaOH. Back-titration with standardized HCl revealed the remaining NaOH and consequently the CO_2–C evolved. The induced respiration was similarly measured in extra containers after addition of 0.5% glucose and 3 days of incubation. The specific respiration rate (metabolic coefficient, qCO_2) (Anderson and Domsch, 1993) was calculated as the ratio between the CO_2–C from the basal respiration and the microbial biomass C, expressed as ηg CO_2–C μg^{-1} biomass C h^{-1}. All results were expressed on an oven-dried soil basis at 105 °C for 24 h.

For bacterial diversity, soil DNA was extracted using an Ultraclean™ Soil DNA Kit (Mobio Laboratories, USA). The DNA extracted was amplified by polymerase chain reaction (PCR) using the sequences for the 16S rRNA region as described by Kozdrój and van Elsas (2001). Amplification was performed using the following cycles: 2 min at 94 °C; 30 cycles of 1 min at 94 °C, 2 min at 55 °C, 2 min at 72 °C; followed by 10 min at 72 °C. DNA of soil extracts (200–500 ng) was loaded onto 6% (w/v) polyacrylamide gel with a denaturing gradient ranging from 20% to 75%, in a DGGE apparatus (Bio-Rad Dcode) as described elsewhere (Kozdrój and van Elsas, 2001). Gels were photographed and the lanes detected were analyzed with the Bionumerics program (Applied Mathematics, Kortrijk, Belgium), using the UPGMA (unweighted pair-group method, with arithmetic mean) algorithm (Sneath and Sokal, 1973) and the Jaccard coefficient.

2.4. Statistics

The data are presented as arithmetic means of three replications, i.e., three transects for each site. The significance of treatments (sites) was tested by ANOVA using the Duncan's test ($P < 0.05$). Pearson's correlation analysis and significance levels were obtained by means of the procedure CORR (SAS, 1991). All chemical and microbiological properties (24 variables) were used to group the sites in a cluster analysis, using the Complete Linkage and Euclidian Distance method (Everitt, 1993), by means of the Statistica 6.0 software. Before cluster analysis, the data were transformed to $\log(x + 1)$.

3. Results

3.1. Soil humidity and chemical analysis

Water content at sampling varied according to the site and was usually higher in those with more-dense vegetation (1, 4 and 6) and lowest at the fallow (5) (Table 2). With regard to chemical properties, pH varied in the acidic range from 4.9 to 5.6. Soil in sites 3–6 usually had higher pH values as compared to the native forest (1) and to sites 7 and 9. The wheat-cropped sites (3 and 7) had higher soil available P, followed by the native forest and sites 5 and 9. The other sites (2, 4, 6 and 8), all reforested with native trees, had lower P levels. The highest organic C content was found in the soil from native forest, while the soil in sites 8 and 9, secondary forest and eucalyptus, respectively, had intermediate levels. The soil in fallow site had the lowest organic C content. Mineral N (ammonium + nitrate) was in general lower in the soil from reforested sites as compared to site 7, but none differed significantly from the native forest.

3.2. Soil microbial analyses

Some soil microbial properties related to the N cycle in the soil (Table 3) varied with site. The highest ammonification rate occurred in the native forest, followed by the

Table 2
Soil water contents and some chemical properties

Site[a]	Water content (%)	pH ($CaCl_2$ 0.01 M)	P[b] ($\mu g\ g^{-1}$)	Organic C (mg g^{-1})	Mineral N[c] ($\mu g\ g^{-1}$)
1	37.9 a	5.1 cd	9.6 cd	33.7 a	13.9 ab
2	26.0 bcd	5.1 cd	5.7 def	24.5 cd	9.8 b
3	23.0 de	5.5 ab	19.2 a	24.7 cd	9.8 b
4	28.8 bc	5.5 ab	3.1 f	24.6 cd	8.6 b
5	19.8 e	5.5 ab	9.2 cde	20.6 e	13.0 ab
6	30.1 b	5.6 a	4.4 ef	26.2 bcd	8.3 b
7	23.4 cde	4.9 d	16.0 ab	23.7 de	19.3 a
8	22.1 de	5.2 bcd	7.5 cde	28.0 b	9.4 b
9	24.4 cde	5.0 d	11.4 bc	29.5 b	7.0 b
CV[d] %	11.5	4.2	28.6	8.5	35.8

Means sharing the same letter are not statistically different (Duncan $P < 0.05$).

[a] 1. Native forest; 2. Reforestation, understory managed mechanically; 3. Wheat under old rotation; 4. Reforestation, understory not managed; 5. Fallow; 6. Reforestation, understory not managed, open canopy; 7. Wheat under recent rotation; 8. Secondary forest; 9. eucalyptus.

[b] Extracted with Mehlich I solution.

[c] Extracted with KCl 2N.

[d] Coefficient of variation.

Table 3
Soil microbial properties related to nitrogen cycling

Site[a]	Ammonification (μg N g^{-1} day^{-1})	Nitrification (%)	Ammonifiers (log of MPN g^{-1})	Nitrifiers (log of MPN g^{-1})	
				NH_4^+ oxidizers	NO_2^- oxidizers
1	1.4 a	21.1 ab	6.5 a	5.2 a	4.8 a
2	0.8 cd	21.0 ab	6.6 a	4.4 ab	3.9 cd
3	0.5 d	29.2 a	6.1 ab	3.8 b	4.3 abc
4	0.8 cd	29.0 a	6.4 a	4.5 ab	4.0 bcd
5	0.4 d	19.7 ab	5.8 ab	4.4 ab	3.5 d
6	0.6 cd	19.0 ab	6.4 a	4.3 ab	3.7 cd
7	0.5 d	16.2 b	5.5b c	4.1 b	3.3 d
8	1.2 ab	12.8 b	5.8 ab	4.3 ab	4.7 ab
9	0.9 bc	21.2 ab	4.9 c	3.9 b	3.3 d
CV[b] %	27.5	28.1	7.6	11.7	10.5

Means sharing the same letter are not statistically different (Duncan $P < 0.05$).
[a] 1. Native forest; 2. Reforestation, understory managed mechanically; 3. Wheat under old rotation; 4. Reforestation, understory not managed; 5. Fallow; 6. Reforestation, understory not managed, open canopy; 7. Wheat under recent rotation; 8. Secondary forest; 9. eucalyptus.
[b] Coefficient of variation.

naturally restored secondary forest. The soil from other reforested sites had intermediate rates, while the wheat-cropped and fallow areas showed the lowest rates. Nitrification rates were higher in sites 3 and 4 and differed significantly from sites 7 and 8, which scored the lowest values. Nevertheless, no one site differed significantly from the native forest. The highest numbers of ammonifiers were found in native forest and the artificially reforested sites (2, 4 and 6), while the lowest number was found in eucalyptus. Ammonium oxidizers had the highest number in the native forest soil, which greatly differed from the wheat-cropped and eucalyptus soils, while the soils at the other sites were intermediate. The nitrite oxidizers also had the highest values at the native and secondary forests sites, while the lowest numbers were found at sites 5, 7 and 9 (fallow, wheat under recent rotation and eucalyptus, respectively).

Other soil microbiological properties varied with site (Table 4). The lowest values for microbial biomass C were found at the fallow and the two wheat-cropped sites. On the other hand, the highest values for biomass C were found in the native forest, followed by the reforested sites, including eucalyptus. Biomass N, in a similar fashion, was higher at native and secondary forest sites, but did not differ from artificially reforested sites or where the understory was dominated by *Panicum maximum* (4 and 6). The lowest biomasses N were found in sites 5, 7 and 9, fallow, mature wheat and eucalyptus, respectively. The basal respiration was lowest in soils from wheat-cropped sites, differing significantly from the sites 4 and 8 soils, which had the highest respiration rates. Basal respiration in native forest soil was not significantly different from those at all other sites. Induced soil respiration (not shown) did not differ between sites and averaged 17.9 mg CO_2–C g^{-1} per day. In

Table 4
Microbial biomass C and N, basal respiration, dehydrogenage activity, flagellates protozoan and metabolic coefficient (qCO_2)

Site[a]	Microbial biomass (μg g^{-1})		Respiration (mg CO_2–C g^{-1} per day)	Dehydrogenase (μg PNP g^{-1})	Flagellates Protozoan (log of MPN g^{-1})	qCO_2[b] (ηg CO_2–C μg^{-1} biomass C h^{-1})
	C	N				
1	1134 a	25.0 a	2.9 ab	107.0 abc	5.0 bc	29.3 bc
2	882 bc	8.3 bc	3.1 ab	106.3 abc	4.9 bc	40.1 abc
3	765 de	8.7 bc	1.7 b	118.7 ab	5.4 bc	24.0 c
4	828 cd	13.0 ab	4.3 a	126.7 a	7.0 a	57.0 a
5	595 e	0.5 c	2.9 ab	90.7 bc	3.9 c	56.7 a
6	935 ab	14.2 ab	2.8 ab	75.7 c	6.1 ab	34.4 abc
7	667 de	4.4 c	2.0 b	40.7 d	4.5 bc	34.5 abc
8	992 ab	28.3 a	4.2 a	90.1 bc	4.9 bc	48.3 ab
9	889 bc	4.5 c	2.9 ab	77.7 c	3.9 c	35.9 abc
CV[c] (%)	13.6	79.6	36.4	20.5	16.9	31.3

Means sharing the same letter are not statistically different (Duncan $P < 0.05$).
[a] 1. Native forest; 2. reforestation, understory managed mechanically; 3. wheat under old rotation; 4. reforestation, understory not managed; 5. fallow; 6. reforestation, understory not managed, open canopy; 7. wheat under recent rotation; 8. secondary forest; 9. eucalyptus.
[b] Ratio between the CO_2–C evolved in a period per unit of microbial biomass C.
[c] Coefficient of variation.

addition, sites did not affect significantly the ratio between microbial biomass C to soil organic C, which averaged 3.3% although there tended to be lower values at the fallow (5), wheat-cropped (3 and 7) and eucalyptus (9) sites (not shown). Dehydrogenase activity was lowest at site 7 and differed significantly from all the other sites, while the highest activity occurred at site 4, which differed significantly from those at sites 5–9. Among protozoa, flagellates were found in higher numbers in the reforested sites 4 and 6 with understory dominated by *P. maximum*, while the sites under fallow and eucalyptus had the lowest numbers. On the other hand, ciliates (not shown) did not vary with sampling site and the MPN g^{-1} averaged 2×10^3. Finally, the metabolic quotient qCO_2 in the native forest soil was significantly lower than at sites 4 and 5, but did not differ from the other sites.

The total culturable heterotrophic bacteria did not differ among sites and their CFU values averaged 10^9 g^{-1} of soil; the same occurred with sporulating bacteria (10^6 g^{-1}) and total culturable fungi (5×10^4 g^{-1}) (not shown).

3.3. DGGE analysis of 16S rDNA for bacteria domain

As a result of PCR-DGGE analysis (Fig. 2), the lowest bacterial diversity was found in the soil collected from the wheat-cropped sites (3 and 7), and at the sites with eucalyptus (9) and fallow (5), while greater diversity was observed in the native forest soil (1), and at the sites under artificial reforestation with native trees (2, 4 and 6). In the cluster analysis of the PCR-DGGE groups, sites with higher diversity (1, 2, 4 and 6) and site 8 were clustered with a similarity of 65%. PCR-DGGE products of three agricultural sites (wheat at 3 and 7, and fallow at 5) were clustered at a 73% level of similarity, while the eucalyptus site (9) was dissimilar from the other ones. All sites grouped at a final similarity of 50%.

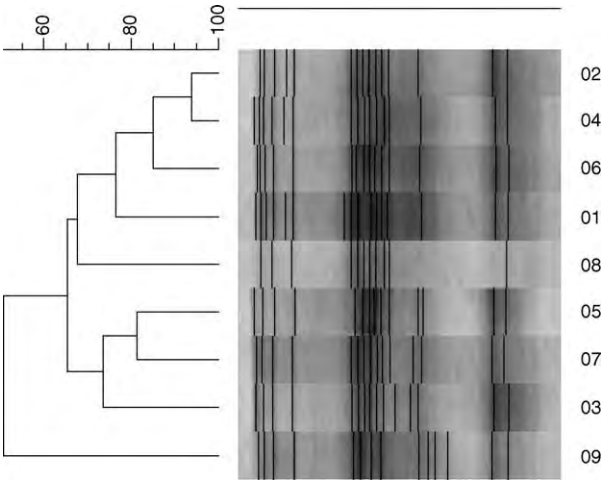

Fig. 2. DGGE fingerprints (right) and cluster analysis (left) from soil DNA (sites 1–9) after amplification with primers for the 16S rDNA region of bacteria domain. Each spot in the fingerprint represents 16S rDNA sequences with different melting point. Clustering was performed using the UPGMA algorithm and the Jaccard coefficient. (1) Native forest; (2) reforestation, understory managed mechanically; (3) wheat under old rotation; (4) reforestation, understory not managed; (5) fallow; (6) reforestation, understory not managed, open canopy; (7) wheat under recent rotation; (8) secondary forest; (9) eucalyptus.

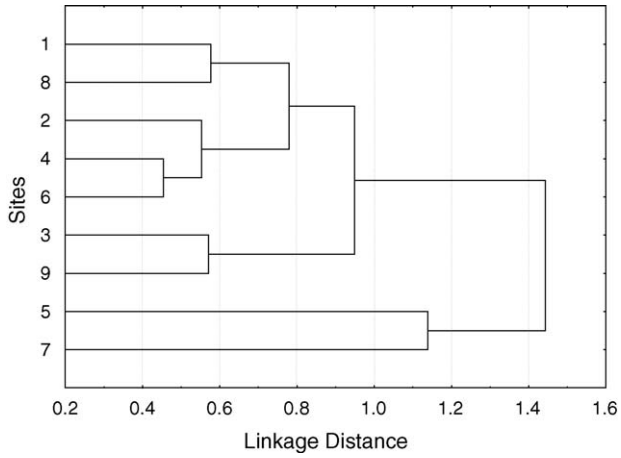

Fig. 3. Dendrogram of similarity based on all variables assessed in this study (water content, pH, heterotrophic culturable bacteria, sporulating bacteria, total culturable fungi, ammonifiers, nitriters, nitraters, flagellate and ciliate protozoa, dehydrogenase activity, available P, basal and induced respirometry, ammonium, nitrate, ammonium + nitrate, ammonification and nitrification rates, microbial N and C biomasses, total organic C, biomass C to total soil C ratio, and metabolic coefficient) by means of Complete Linkage and Euclidian Distance method. (1) Native forest; (2) reforestation, understory managed mechanically; (3) wheat under old rotation; (4) reforestation, understory not managed; (5) fallow; (6) reforestation, understory not managed, open canopy; (7) wheat under recent rotation; (8) secondary forest; (9) eucalyptus.

3.4. Cluster analysis based on soil chemical and microbial data

From cluster analysis, considering all 24 chemical and microbiological characteristics, three main clusters and two sub-clusters were formed (Fig. 3). The first main cluster contained the native forest (1) and all sites reforested with native trees (2, 4, 6 and 8); this cluster comprised two sub-clusters, one containing the native forest (1) and the naturally reforested secondary forest (8), while the other contained the three sites artificially reforested with native trees (2, 4 and 6). The second main cluster grouped the flowering wheat site (3) and the eucalyptus site (9), and the third main cluster grouped the mature wheat-cropped site (7) and the fallow site (5).

4. Discussion

The sites differed significantly in many parameters, attributable to differences in the present vegetation and history of soil use, which affected – quantitatively and qualitatively – the organic matter inputs and, consequently, soil chemical and biological properties (Salamanca et al.,

2002; Dinesh et al., 2003). In general, sites under native forest showed higher microbiological activities, followed by reforested sites, except eucalyptus. These sites usually contrasted with fallow, wheat-cropped, and for some variables, the eucalyptus sites. Fifty years of agricultural use after forest removal showed declines in some soil biological activities and processes mediated by soil microorganisms. Similar patterns have been reported for other tropical soils (Islam and Weil, 2000; Lemenih et al., 2005). However, the restoration of native species showed indications of recovery towards the native forest, used as reference site.

4.1. Soil use and moisture

The soil water contents varied among sites and were probably related to vegetation covering, which in turn affected microbial activity, confirming the results of Jensen et al. (2003) and Yim et al. (2003). Native forest soil had the highest water content, followed by reforested sites 4 and 6, at which *P. maximum* dominated the understory. This plant produces large amounts of biomass and litter that covers the soil and decreases water loss due to surface evaporation. As the evaluation was performed at the end of the less rainy season, most of the *P. maximum* litter was still on the soil surface. Lowest water contents were recorded in the fallow and wheat-cropped sites (3 and 7), all with relatively little litter covering the soil. Although the wheat-cropped sites had been managed without tillage, the previous crop was soybean, which produces much less litter than does *P. maximum*.

Although water content in the soil samples collected from the naturally regenerated secondary forest was as low as at the fallow site, biomass C and N and basal respiration rates were comparable to those of the native forest soil, which can be attributed to organic matter inputs during 20 years of natural restoration after about 10 years of pasture. Although microbial activity at this site may have been limited by dryness before the sampling time, it should be borne in mind that microorganisms could immediately recover biological activity when water becomes available (De Nobili et al., 2001), unless lack of energy sources and nutrients preclude them from doing so (Harris, 2003). Our results here show the importance of soil mulching to reduce loss of water by evaporation and to contribute as nutrient sources for soil microbial activity. In addition, the Pearson's correlation coefficients between biomass C and N and water availability were 0.51^{**} and 0.41^{*}, respectively, which demonstrate this interrelationship.

4.2. Mineral P, N and microbial biomass

At all of the sites reforested with native trees and in the secondary forest site, soil available P was lower than in the native forest, probably due to the depletion by coffee plantation after deforestation. The highest P availability at the wheat-cropped sites was due to fertilization of the annual crops. On the other hand, higher amounts of mineral N were found in the fallow and mature wheat soils, in amounts similar to those in the native forest soil. In this respect, Mummey et al. (2002) suggested that higher mineral N at a site under reclamation means that the N cycle is still ineffective. In our case, higher mineral N at the fallow and the mature wheat soil may have been due to low microbial activities and then less N-immobilization. Inorganic N is potentially subjected to losses by leaching and/or denitrification (Mummey et al., 2002). However, although the native forest samples had similar mineral N contents, the vegetation and microbial biomass in the soil seem able to immobilize N before significant losses occur and recycle it efficiently. Data of biomass C and N came in accordance with this explanation, as the highest values were found in the native forest and the lowest were in the fallow and mature wheat soils.

Despite the higher mineral P and N contents, the wheat-cropped soil showed the lowest microbial C and N biomasses and basal respiration. Carbon and N microbial biomasses were also low in the fallow soil. Microbial growth is known to be dependent on organic C inputs, which were more diverse and plentiful in the reforested and native forest sites. DGGE analysis showed a reduction in bacterial diversity due to clearance and cultivation, what is attributable to decline in available organic matter; corroborating data resulted from a study in a tropical Indian forest (Dinesh et al., 2003).

4.3. Total organic C and microbial processes

As expected, the native forest soil showed the highest amount of organic C and the fallow site showed the lowest. It is known that when such soil is deforested and used for agricultural production, biogeochemical cycling is altered with oxidation of organic C and establishment of new equilibrium in soil C content (Lemenih et al., 2005). Only the secondary forest and eucalyptus sites recovered organic matter, emphasizing that, after disturbance, recovery of soil organic matter is slow. Even in the secondary forest site, not managed for more than 20 years, the organic matter levels were lower than in the native forest soil. Nevertheless, partial recovery of organic C pools suggests that these soils are reacquiring the ability to retain and cycle nutrients (Mummey et al., 2002).

Ammonification rates were lowest at the fallow and wheat-cropped sites, the highest were in the native and secondary forest soils, and intermediate at the eucalyptus and artificially reforested sites. This variable seemed to be highly sensitive to soil use and, therefore, reliable as soil-quality indicator. The lower ammonification rates in wheat-cropped and fallow sites indicate less capacity for the soil to supply N to plants and consequently greater dependence on external inputs (Harris, 2003). Ammonification rate was found closely correlate to soil organic C (0.98^{***}), which is the main source of organic N to be mineralized. In addition, ammonification rate also corre-

lated with total culturable fungi (0.57^{***}), confirming that fungi are more able than bacteria to access recalcitrant N components, such as in soil organic matter (Salamanca et al., 2002). Nitrification rate, on the other hand, did not show a clear trend according to land use.

4.4. Culture-based soil microorganisms

Among the culture-based soil microbial properties, only total fungi showed less CFU in the soil with eucalyptus, a tendency also observed for total bacteria. The quality of the residues (wide C/N ratio) from eucalyptus may be contributory and consistent with the low biomass N found at this site.

The lower population density of ammonifiers in the fallow and wheat-cropped soils is found consistent with the lower ammonification rates. Nevertheless, at the eucalyptus site, in spite of the lowest number of ammonifiers, the ammonification rate was quite high. The NH_4^+ oxidizers were more abundant in the native forest, but the NO_2^- oxidizers also had higher occurrence in the secondary forest, similar to the native forest. This microbial group also correlated positively with microbial biomass N (0.53^{**}) and C (0.50^{**}), suggesting that N cycle in the secondary forest soil is approaching that of the native forest.

4.5. Microbial biomasses C and N

Although significantly correlated ($r = 0.55^{**}$), biomass C was more sensitive to soil use and reforestation system than was basal respiration rate, showing the former to be a more effective indicator. The wheat-cropped and fallow sites had the lowest biomass C, while the highest was found in native forest. After forest clearance and soil use, biomass C fell to about half of that in the native forest soil (e.g., at the fallow and mature wheat sites). After reforestation, irrespective to the system, the biomass C reached higher levels than at the cropped sites, but still lower than in the native forest soil, as also observed for total organic C. In addition, biomass C was more sensitive to land use than organic C. The ratio of biomass C to soil organic C, albeit insignificant statistically, tended to be lower in the fallow and wheat-cropped sites, presumably as a result of lower inputs of organic matter (Joergensen and Scheu, 1999).

As the microbial biomass C represents the living microbial cells in the soil, which is a constituent of total organic C, it becomes sensitive to external influences, such as soil use. Li et al. (2004) attributed reductions in soil microbial biomass C at sites in which herbicides were used (to control the native vegetation, similar to our wheat-cropped sites) to losses in vegetation rather than to toxic effects on microbial biomass; herbicide reduced labile C in root exudates, root decay, and leaf and litter fall leachates. The trend for microbial biomass C was observed also for biomass N. In the latter case, the highest values were found in the native and secondary forest soils. Although eucalyptus growth was as old as that of secondary forest, its soil biomass N did not differ from that of the fallow or the mature wheat-cropped sites, possibly due to wide C/N ratio in the eucalyptus residues. Moreover, at artificially reforested site 2, soil biomass N was similar to that at the wheat-cropped sites, in spite of having been reforested at the same time as the sites replanted with native trees (4 and 6). Nevertheless, site 2 had its understory managed periodically, thus reducing inputs of soil organic matter. These results suggest that not only the period of restoration, but also soil use and organic residue diversity have important effects on formation of microbial biomass N.

The metabolic quotient (qCO_2), the ratio between soil basal respiration to soil microbial biomass C, was significantly higher at two contrasting sites than in the native forest: fallow and the artificially reforested site 4. According to Insam and Domsch (1988), greater values of qCO_2 are found in non-equilibrated environments, but the availability of substrate for microbial activity also plays an important role in this ratio (Joergensen and Castillo, 2001). Dinesh et al. (2003) attributed higher qCO_2 values in forest soils to greater amounts of organic C available for microbial degradation, which also may explain the high qCO_2 at site 4, where the *P. maximum* in the understory increased litter deposition on the soil. Conversely, although the fallow site had little input of organic matter, the qCO_2 was similar to that of the site 4 soil, what is attributable to a most likely change in composition of the microbial community. According to Insam (1990), soils that have recently received rapidly biodegradable organic substrates predominantly contain *r* strategist microbial ecotypes (few species with high growth rates). These organisms evolve more CO_2 per unit of degradable C than *K* strategists (more species, evenness, with lower growth rates), which prevail in soils, like the fallow site, that have not received fresh organic matter. Contrasting microbial community composition between these two sites is apparent, at least for bacterial diversity, from the results of DGGE and grouping analysis.

4.6. Cluster analysis based on molecular bacterial diversity and soil chemical plus microbiological properties

Soil bacterial diversity, as measured by amplification of the 16S rRNA region (Kozdrój and van Elsas, 2001) with universal primers, showed that, in comparison with the native forest, several species were absent from the other sites, indicating less microbial diversity. Bacterial diversity was least affected in the sites where reforestation involved native species. The secondary forest site, although with less diversity, clustered with the artificially reforested and native forest sites. Treatments with similar history of organic matter inputs clustered together, and

indicate that DGGE analysis seems sensitive to changes on soil bacterial diversity.

The genetic diversity for bacteria revealed by DGGE analysis resulted in a clustering pattern very similar to that obtained using microbial and chemical properties, i.e., native forest can be clustered with the other reforested sites, while the wheat-cropped and fallow sites were found belonging to a second cluster. The site with eucalyptus did not cluster with the others in the DGGE-based analysis. In a recent review, Harris (2003) pointed out that microbial activity might be used as a tool to distinguish systems and to estimate the success of efforts to restore degraded soils. In fact, our data – both with molecular and with classical microbiological (and some chemical) analyses – resulted in the grouping of sites that had similar strategies of restoration and management. The fact that reforestation employing native trees, independently of understory management, grouped with the native site, suggests that this strategy tends to return the soil to the pattern of native site. On the other hand, reforestation with eucalyptus, an exotic species, revealed more similarities to one of the wheat-cropped site. In addition, the fallow and the other wheat-cropped site 7 were the most dissimilar sites, as a consequence of soil management.

5. Conclusion

The search for microbiological indices useful as indicators of sustainability in many (agro)ecosystems is a recent endeavor. Although soil organic C has been used for some time as an indirect biological indicator, its response to an interference (soil use or reclamation strategy) was found less sensitive than some microbial parameters. As observed, microbial indices as microbial biomass C and N, and ammonification rates responded readily and reliably to changes in soil use and strategies of reforestation. This make them highly promising soil-quality indicators. In addition, the use of DGGE proved to be reliable for evaluating the impact of reforestation strategy on soil bacterial community, as their cluster pattern was similar to those based on results from classical chemical and microbiological analyses. By means of these two techniques, it is clear that the reforestation strategies containing native species tended to return the soil to its native forest condition. On the contrary, the strategy that employed an exotic species tended to alter soil chemical and microbial characteristics, as did agricultural systems. Fallow, sometimes used as a strategy for soil management, proved to be very negative with regard to soil microbiological and chemical properties, at least under the conditions it has been conducted, indicating that this system runs counter to sustainability, leading to soil degradation. As an option, instead of fallow, areas should be provided with vegetal covering in order to prevent against degradation by erosion and increase microbial activity and diversity. The increase on microbial biomass can also contribute to decrease nutrient losses from soil by leaching.

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