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Microbial Biomass and Community Structure in a Sequence of Soils with Increasing Fertility and Changing Land Use

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ABSTRACT

The microbial biomass and community structure of eight Chinese red soils with different fertility and land use history was investigated. Two community based microbiological measurements, namely, community level physiological profiling (CLPP) using Biolog sole C source utilization tests and phospholipid fatty acid (PLFA) profiles, were used to investigate the microbial ecology of these soils and to determine how land use alters microbial community structure. Microbial biomass-C and total PLFAs were closely correlated to organic carbon and total nitrogen, indicating that these soil microbial measures are potentially good indices of soil fertility in these highly weathered soils. Metabolic quotients and C source utilization were not correlated with organic carbon or microbial biomass. Multivariate analysis of sole carbon source utilization patterns and PLFAs demonstrated that land use history and plant cover type had a significant impact on microbial community structure. PLFAs showed these differences more than CLPP methods. Consequently, PLFA analysis was a better method for assessing broad-spectrum community differences and at the same time attempting to correlate changes with soil fertility. Soils from tea orchards were particularly distinctive in their CLPP. A modified CLPP method, using absorbance readings at 405 nm and different culture media at pH values of 4.7 and 7.0, showed that the discrimination obtained can be influenced by the culture conditions. This method was used to show that the distinctive microbial community structure in tea orchard soils was not, however, due to differences in pH alone.

Introduction

Red soils (equivalent to Ultisols and Oxisols in US soil taxonomy) cover 22% of China and support 43% of the Chinese population [58]. Crop growth on red soils is often constrained by high acidity, poor nutrient status, and a high content of variable-charge minerals and iron oxides. Red soils are highly weathered and are often eroded and lack well-developed topsoil such that their inherent fertility is low and plant nutrients, derived primarily from mineral weath-

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ering, are limited. The turnover of organic matter and nutrient cycling by microorganisms, therefore, plays a key role in establishing and sustaining their fertility.

Soil microorganisms are important in the cycling of almost all the major plant nutrients [46], particularly so in natural and agricultural ecosystems with low inputs. A number of soil microbiological parameters, notably microbial biomass carbon and basal respiration [14, 47], have been suggested as possible indicators of soil quality and have been employed in national and international monitoring programs. Soil microbial biomass can be an important pool of plant nutrients and is often highly correlated with the organic matter content of soils [41]. Consequently, a close relationship has also been reported between soil fertility and microbial biomass [11, 29, 41]. Carbon dioxide evolution, the major product of aerobic catabolic processes in the C cycle, is also commonly measured and indicates the total C turnover. The metabolic quotient, i.e., the ratio of basal respiration to microbial biomass, is inversely related to the efficiency with which the microbial biomass uses the indigenous substrates [1] and can be related to soil development and ecological succession [27].

More recently, microbial diversity (community structure) has also been recommended as a biological indicator of soil quality, although there are several ways to quantify this structure [32]. Phospholipid fatty acids (PLFAs) are specific components of cell membranes that are only found in intact (viable) cells. A range of various PLFAs can be extracted from soil that are indicative of major microbial groups, e.g., eukaryotes, Gram-positive and Gram-negative bacteria, and actinomycetes [53]. Therefore, changes in the PLFA profile represent changes in the total soil microbial community, and it has been used to compare different land use systems and crop management [8] as well as stress [16, 42]. Community level physiological profiles (CLPPs), assessed using the Biolog system [18], are a means of investigating the physiological diversity present in soils, because they reflect how the microbial communities can potentially utilize a range of carbon substrates. Differences in utilization patterns are interpreted as differences in the major active members of the microbial community. The assay is based on measuring oxidative catabolism of the substrates to generate patterns of potential sole C source utilization. This method [18] uses multiwell plates that can test 95 different substrates simultaneously. It has also been widely used to examine communities from different environments [18], different vegetation [23], and pollutant stress [3, 30]. The use of Biolog sole C-source test plates for testing oligotrophic soil communities has several potential limitations, not least because the method primarily selects for a small proportion of the total community largely made up of fast-growing copiotrophic bacteria [45]. In addition, the C-sources usually tested are not necessarily those found in soil and are present in high concentration [13], the triphenyl-tetrazolium chloride (TTC) indicator dye can be toxic [15], and the pH of the medium is buffered at 6.5. The buffered pH is of concern in our study because most red soils have much lower pH values. Although both PLFA and CLPP have been used to measure community responses, few studies have compared both techniques together [3, 7, 8, 12, 17, 25].

Most studies on nutrient cycles in red soils have focused on the organic matter and total biomass in relation to soil fertility status [56, 33, 24], but there have been no studies of microbial community structure or ecology of red soils. In the research reported in this paper we measured a range of microbiological parameters in a sequence of eight red soils that have undergone land improvement and change in land use. The soils were selected from the same climatic region and were derived largely from the same soil parent material so as to remove edaphic and climatic influences. We used the PLFA and CLPP techniques to further understand the microbial ecology of this important soil resource and to test the hypothesis that their community structure would be primarily determined by their different land uses. In addition, because the Biolog test plates were buffered near to neutrality, we also set out to develop and test a modified sole-carbon-source utilization approach for specifically investigating the effect of low pH. This was then also used to test the hypothesis that CLPPs are conditioned by the culture conditions (pH) of the test medium.

Materials and Methods

Soils

The soils used were taken from the surface layer (0–20 cm) of Chinese red soils in Longyou county, Zhejiang Province, in southwest China. Mean annual rainfall in this area is about 1,450 mm and mean temperature is 17°C. All soils were developed on quaternary red earth except for soil no. 2, which was derived from red sandstone. The soils were Ultisols with kaolinite, chlorite, and Fe, and Al oxides as the dominant clay minerals. The soils were selected to cover a wide spectrum of soil fertility, i.e., from the noncultivated and severely eroded to highly fertile soils with high organic matter and total N and P. The land use history, geographical location, and some physicochemical properties of the soils are shown in Table 1. Soil samples were taken at random using soil cores (25 mm diameter) to a depth of 20 cm. Three replicate samples com-

Table 1. Land use history, geographical location, and average basic physicochemical properties of the tested soils

Soil no.	Land use	Latitude/longitude	pH (H_2O)	Organic C (g kg^{-1})	Total N (g kg^{-1})	Total P (g kg^{-1})	Extractable Al (mg g^{-1})	Extractable Fe (mg g^{-1})	Clay (%)
1	Eroded wasteland	29°2′ N/119°10′E	5.42	2.34	0.28	0.17	7.59	38.41	35.5
2	Upland vegetable—3yr (*Brassica pekinensis Rupr.*)	29°5′ N/119°8′E	6.31	4.81	0.43	0.28	0.82	6.18	9.2
3	Citrus orchard—4yr (*Citrus unshiu Marc.*)	29°3′ N/119°9′E	4.68	4.08	0.42	0.25	5.26	31.56	40.9
4	Citrus orchard—8yr (*Citrus unshiu Marc.*)	29°4′ N/119°12′E	5.53	14.54	1.73	0.42	5.04	27.57	35.6
5	Citrus orchard—12yr (*Citrus unshiu Marc.*)	29°2′ N/119°00′E	5.59	16.46	1.82	0.57	4.26	31.83	32.5
6	Paddy—15yr (*Oryza sativa L.*)	29°2′ N/119°11′E	5.11	16.00	1.41	0.51	3.66	22.29	29.1
7	Tea orchard—30yr (*Camellia sinensis O. Ktze.*)	29°2′ N/119°12′E	4.34	26.33	2.04	0.52	4.43	25.67	32.4
8	Forest—38yr (*Pinus massoniana Lamb.*)	29°4′ N/119°10′E	4.57	34.48	2.26	0.77	3.56	22.27	36.9
$LSD_{0.05}$			0.10	1.34	0.13	0.04	0.41	2.33	2.8

prising 20 pooled soil cores were taken at each site. Field moist soils were sieved <2 mm and large pieces of plant material and soil animals were removed before use. The soils were conditioned at 45% of their water-holding capacity for 7 days at 25°C prior to microbial biomass and respiration measurements.

Soil Chemical Analyses

Soil pH (soil:H_2O ratio = 1:2) was measured using a pH meter with a glass electrode. The soil organic C and total N and P were determined by dichromate oxidation, Kjeldahl digestion, and H_2SO_4–H_2O_2 digestion methods, respectively [36, 9, 49]. Al and Fe were extracted by the dithionite–citrate bicarbonate method and determined by ICP-OES [35].

Microbial Biomass and Basal Respiration

The fumigation–extraction method [50] was used to determine soil microbial biomass-C of the soil samples. The content of K_2SO_4-extracted C from the $CHCl_3$-treated and untreated soils was determined by an automated TOC Analyser (Shimazu, TOC-500) and a K_{ec} of 0.45 was used to convert the measured flush of C to biomass-C [24]. Basal respiration (CO_2 evolution) was measured in 100-cm^3 soil jars by using gas chromatography to measure the headspace CO_2 that accumulated over 6 n at 25°C from 20 g fresh soil.

Community Level Physiological Profiles (CLPP) by Sole Carbon Source Utilization Tests

CLPPs were determined by direct incubation of fresh soil extracts in 96-well multiwell plates containing different C sources in individual wells to determine changes in relative and absolute rates of utilisation of individual substrates. Fresh soil (10 g) was added to 100 ml of distilled water in a 250 ml flask and shaken on a wrist action shaker at full speed for 10 min. Tenfold serial dilutions were made and the 10^{-3} dilution was used to inoculate the Biolog plates. The dilution was centrifuged at 500 g for 10 min to separate the soil, and 150 μl of supernatant was inoculated into each well of a "GN" type plate (Biolog Inc., Hayward, CA) and an "MT" type plate prepared with additional carbon sources [13]. In all, 125 C sources were tested using Biolog GN plates and customized Biolog MT plates in which the wells contained 30 additional, ecologically relevant C sources [13]. Plates were incubated at 25°C for 7 days, color development was measured as absorbance (A) using an automated plate reader (VMAX, Molecular Devices, Crawley, UK) at 590 nm, and the data were collected using Microlog 3.5 software (Biolog Inc.). Plates were read twice daily and ANOVA of the average well color development (AWCD) over time was used to select comparable time points to avoid confounding effects of inoculum density differences between treatments in the multivariate analysis [19]. The average well color development (AWCD) for all carbon sources was calculated as a measure of total activity. Since the 10^{-3} dilution of the tea orchard soil (No. 7) had low AWCD, three different inoculum dilutions (10^{-2}, 5×10^{-2} and 10^{-3}) were also tested.

Modified CLLP for Acid Soils

To investigate the effect of pH on sole C source utilization tests a modified procedure was used. Ninety-six well multiwell plates (Sero-Wells, Bibby Sterilin, Stone, Staffordshire, UK) were prepared using a base characterization mineral medium [31]. The plates had no triphenyl-tetrazolium chloride (TTC) but had two different pH characterization media at pH 4.7 and 7.0. Twenty-one

Table 2. List of common root exudate compounds used as carbon sources in customized sole C source tests at two different pH values

Sugars	**Carboxylic acids**
Glucose	Fumaric acid
Arabinose	Oxaloacetic acid
Fructose	Citric acid
Sucrose	Succinic acid
Amino acids	**Phenolic acids**
Glutamic acid	*p*-OH benzoic acid
Aspartic acid	Protocatechuic acid
Glycine acid	Ferulic acid
Arginine	Caffeic acid
Lysine	
Alanine	**Long-chain aliphatic acids**
	Oleic acid
	Palmitic acid
	Stearic acid

carbon sources were chosen to represent the main types of compounds exuded by plant roots [13], namely, sugars, amino acids, carboxylic acids, phenolic acids, and long-chain aliphatic acids (Table 2). The characterization medium (1.25 gL^{-1} ammonium phosphate monobasic, 0.25 g L^{-1} potassium chloride, and 0.25 g L^{-1} magnesium sulfate was adjusted to the chosen pH with 1.0 M KOH before use. The test plates were made by dispensing the C source solutions (0.3 mg per well) and 150 µl of the mineral salts medium into each well and then leaving them to dry under sterile air flow. The 21 C-sources and 3 blanks (with no C source added) were replicated 4 times in a single 96-well multiwell plate. Substrate utilization and microbial growth were monitored by measuring absorbance at 405 nm.

Plate Counts of Culturable Bacteria

Numbers of colony forming units (cfu) of bacteria were estimated by the dilution plate method. The same samples used in the carbon utilization profiles were serially diluted and suspensions (0.1 ml) spread in triplicate on to the 0.1 strength Tryptone Soya agar (Oxoid). The plates were incubated at 25°C and colonies counted after 7 days.

PLFA analysis

Lipid extraction and PLFA (phospholipid fatty acid) analyses were performed [16] using the modified Bligh and Dyer method [4]. Briefly, 2.0 g (freeze-dried sample) was extracted with a chloroform–methanol–citrate butter mixture (1:2:0.8), and the phospholipids were separated from other lipids on a silicic acid column. The phospholipids were subjected to a mild-alkali methanolysis and the resulting fatty acid methyl esters were separated by gas chromatography. Individual fatty acids were designated in terms of total number of carbon atoms: number of double bonds, followed by the position of the double bond from the methyl end of the molecule. The prefixes a and i indicate anteiso and iso branching, respectively, br indicates unknown branching, and cy indicates a cyclopropane fatty acid. The PLFA 18:2ω 6, 9 was taken to indicate predominantly fungal biomass [16]. Ratios of Gram-positive to Gram-negative bacteria were calculated by taking the sum of the predominant Gram-positive PLFAs 16:0(10Me), 17:0(10Me), 18:0(10Me), i15:0, a15:0, i16:0, i17:0, and a17:0 divided by the sum of the predominant Gram-negative bacterial PLFAs 16:1ω5, 16:1ω7 t, 16:1ω9, cy17:0, 18:1ω5, 18:1ω7 and cy 19:0 [43, 54].

Statistics

All ANOVA, regression, and multivariate analyses were conducted using Genstat 5.3 (NAG Ltd., Oxford, UK). Means, least significant differences of 5% level were calculated by a one way ANOVA. The PLFA data were expressed as mol% for multivariate analyses and as nmol g^{-1} and log transformed for ANOVA. For multivariate analysis of the CLPP data the absorbance values at equivalent AWCD from different times of incubation were compared and were also transformed by dividing by the AWCD to avoid bias between samples with different inoculum density [20]. For one soil (No. 7) different inoculum densities were also tested because of large differences in AWCD. The absorbance data were analyzed by canonical variate analysis (CVA), after first reducing the dimensionality by principal component analysis and by comparison of mean inter group Mahalanobis distances with simulated confidence limits (SCL) [13]. SCL for eight groups (soils) with three replicates were 2.71 and 3.43 at the 95 and 99% confidence limit, respectively.

Results

Microbial Biomass, Respiratory Activity, and Colony Forming Units

Microbial biomass-C (C_{mic}) in the soils ranged from 29 to 466 µg g^{-1}, accounting for, 1.88% of total organic C (C_{org}) (Table 3). The highly fertile soil (soil 8) contained 16 times more C_{mic} than the noncultivated eroded soil (soil 1) which had a very low biomass in comparison with all the other soils. Soil C_{mic} was strongly correlated with organic C ($P < 0.001$, $r = 0.95$) and total N ($r = 0.92$). The C_{mic}: C_{org} ratio varied widely and in the red sandstone soil (soil 2) was much higher than in other soils. This effect may be attributable to the more intensive management of this soil for vegetable production, but may also reflect underlying soil differences due to the different soil parent material. Basal respiration was five times higher in the forest soil (Soil 8) than in the low organic matter noncultivated soil (Soil 1), and was significantly correlated with both C_{mic} and C_{org} in all soils. The metabolic quotient was highest in the eroded soil (Table 1) and lowest in the tea orchard, paddy field, and 8-year old citrus orchard soils. There was no significant correlation

Table 3. Microbial biomass-C (C_{mic}), colony-forming units (cfu) of bacteria, respiration rate, and total PLFA of eight Chinese red soils

Soil no.	Microbial biomass-C ($\mu g\ g^{-1}$)	Numbers of bacteria ($\times 10^4$ cfu g^{-1})	Respiration rate (CO_2-C $\mu g\ g^{-1}\ h^{-1}$)	Metabolic quotient (h^{-1})	Fungal PLFA 18:2 ω6,9 (nmol g^{-1})	Total PLFA (nmol g^{-1})
1	29.2	390	0.117	0.0040	0.18	2.37
2	152.2	1005	0.212	0.0014	0.19	5.35
3	108.4	599	0.183	0.0017	0.11	4.53
4	235.2	851	0.209	0.0009	0.44	10.11
5	264.4	1188	0.373	0.0014	0.93	24.88
6	301.8	1061	0.254	0.0008	0.58	22.66
7	400.2	5013	0.325	0.0008	1.04	29.86
8	465.6	2292	0.564	0.0012	1.23	42.21
$LSD_{0.05}$	20.1	280	0.034	0.0006	0.51	2.73

between the metabolic quotient and increasing soil fertility (organic matter).

The numbers of colony forming units (CFU) in the soils counted on tryptone soya agar plates increased from the lowest numbers (3.9×10^6) in the noncultivated soil (soil 1) to the highest numbers (5.0×10^7) in the tea orchard soil (soil 7) (Table 3). CFUs were positively correlated with soil microbial biomass. The tea orchard soil (soil 7) had relatively high numbers of bacteria, predominantly growing as small (<1 mm diameter), white, opaque "pinhead"-type colonies. Gram staining showed that they were Gram-negative rods. Attempts to identify them using the Biolog GN identification system failed because there were too few positive reactions. No other significant differences in colony morphotype were observed between the soils.

Community Level Physiological Profiles

The average utilization (AWCD) of the carbon sources for the eight soil samples using the Biolog GN and MT plates generally followed the same pattern with incubation time (data not shown). However, the AWCD of the microbial communities from the tea orchard soil (soil 7) was significantly less ($P < 0.001$) than all other soils (Table 4). Even when 5- and 10-fold higher inoculum densities were used to inoculate the Biolog plates, the AWCD was only increased by 5 and 10% and was still significantly lower than the other soils (data not shown). The soil effects were similar for different groups of carbon sources (Table 4). Generally the basic amino acids had the highest utilisation in all the soils except the tea orchard soil (soil 7). The microbial communities from the tea orchard soil always had the lowest utilization rate irrespective of different carbon source groups, but the differences were smallest with acidic amino acids, carboxylic acids, and sugars and greatest for oligosaccharides and basic amino acids (Table 4). The utilization of other groups varied only slightly for different soils.

Canonical variate analysis, using all 125 carbon sources, gave good discrimination between several of the soils with a mean Mahalanobis distance = 11.1 (SCL = 3.5 at 5% level), indicating that they had significantly different patterns of potential carbon utilization and different microbial communities (Fig. 1). In particular there was clear discrimination between samples from the tea orchard soil, which had higher ordinate values on canonical variate (CV) 1 (explaining 60.0% of the variance), when compared with the other soils. This discrimination was still evident when 5- and 10-fold higher inoculum densities were tested at equivalent AWCD values (Fig. 1). The eroded soil and 4-year old citrus orchard was also distinct from other soils on CV1. Some soils were also discriminated on CV2 (explaining 20.1% data variance). The soil under vegetables (soil 2) had the highest ordinates. Two of the older orchard soils (soils 4 and 5) were clustered together while the other younger orchard soil (soil 3) was quite different and was closer to the eroded soil (soil 1). The paddy soil (soil 6) and forest soils were also discriminated in this region. Correlation and analysis of the loadings of the most influential carbon sources on CV1 indicated that cellobiose, rhamnose, chlorogenic acid, and xylitol were positively correlated and L-serine, D-serine, glycerol, and maltose were negatively correlated with CV1. The C sources with highest loadings on CV2 were fumaric acid, which was negatively correlated, as were glucuronic acid and glucose 1,6-phosphate. Cellobiose, succinamic acid, glycogen, and L-glutamic acid were positively correlated with CV2.

In the modified CLPP method employing no TTC, the average A_{405} was higher in the pH 7.0 plates than in the pH 4.7 plates for all soils, except the tea orchard soils (Table 5).

Table 4. Average well color development (AWCD) after 168 h for all 125 C sources tested and for different C source groups for eight Chinese red soils

Carbon source group	Soil no.								
	1	2	3	4	5	6	7	8	$LSD_{0.05}$
All 125 C-sources	0.46	0.65	0.59	0.50	0.66	0.69	0.30	0.60	0.03
Sugars	0.51	0.70	0.75	0.55	0.75	0.74	0.42	0.62	0.04
Oligo-sugars	0.38	0.58	0.41	0.40	0.72	0.50	0.16	0.59	0.04
Alcohols	0.43	0.61	0.67	0.48	0.60	0.73	0.37	0.65	0.07
Carboxylic acids	0.47	0.59	0.59	0.48	0.60	0.67	0.37	0.58	0.04
Acidic amino acids	0.65	0.92	0.67	0.64	0.86	0.89	0.42	0.58	0.06
Basic amino acids	0.68	1.10	0.96	0.75	0.99	1.15	0.34	0.81	0.08
Neutral amino acids	0.47	0.87	0.55	0.63	0.77	0.90	0.25	0.69	0.04
N-heterocyclics	0.31	0.71	0.55	0.43	0.66	0.60	0.18	0.74	0.07
Phenolic acids	0.41	0.34	0.61	0.42	0.60	0.70	0.24	0.45	0.04
Long-chain aliphatics	0.44	0.51	0.47	0.37	0.52	0.65	0.21	0.45	0.04

Expressing the differences in pH response as a ratio showed that it was lowest in the tea orchard soil, with a value of 1.0, followed by the youngest of the citrus orchard soils (soil 3). The largest ratio was found in the red sandy soil (soil 2) with the highest soil pH. Moreover, ANOVA showed that the youngest citrus orchard soil (soil 3) exhibited a higher utilization of carbon sources than two other citrus orchard soils (soils 4 and 5) in the pH 4.7 plates, but no difference was found in the pH 7.0 plates (Table 5).

Canonical variate analysis of the A_{405} data (Fig. 2) showed that using pH 4.7 plates gave greater discrimination between the soils, mean Mahalanobis distance = 9.1, than the use of pH 7.0 plates with a mean Mahalanobis distance = 6.1 (Fig. 3). Discrimination of the soils at pH 4.7 was found on CVs 1, 2, 3, and 4 (Figs. 2a and 2b) explaining 62, 23, 10, and 4% of the variation, respectively. The tea orchard soil was again found to be quite distinct with low ordinate values on both CV1 and CV2, but was much closer to other soils, especially the vegetable soil and the 8- and 12-year-old citrus orchard soils. Also distinct was the 4-year-old citrus orchard soil, with high ordinates on CV2 and low ordinates on CV1. The paddy soil and forest soils were clustered together with high ordinate values on CV1 (Fig. 2a).

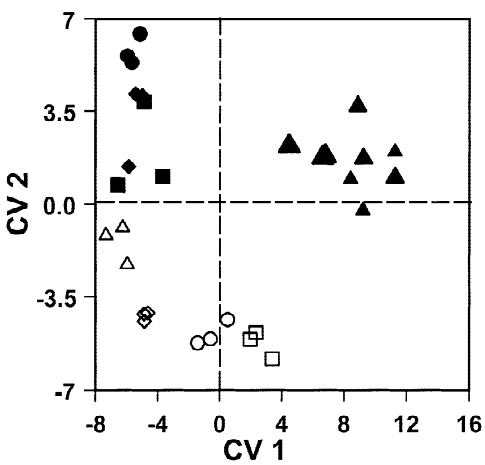

Fig. 1. Plot of ordination of canonical variates (CV) CV 1 against CV 2 generated by canonical variate analysis of sole carbon source tests after 168 h in Biolog and MT plates showing discrimination between different Chinese red soils: eroded soil (○), upland vegetable (●), citrus orchard—4yr (□), citrus orchard—8yr (■), citrus orchard—12yr (◆), paddy soil (△), tea orchard (▲), forest (◇). For soil No. 7 small, medium, and large symbol size (▲, ▲, ▲) refer to 10^{-2}, 5×10^{-3}, and 10^{-3} dilutions, respectively.

In the pH 7.0 plates, there was again similar highly significant discrimination of the tea orchard soil on CV1 (Fig. 3) as found with the Biolog CLPP. Most of the variation was explained on CV1 (80%) and CV2 (13%). Also, the upland vegetable soil (no. 2) was significantly discriminated on CV2 with low ordinate values compared to the other soils. There were, however, few other significant discrimination among the soil samples on CV1 or CV2 (Fig. 3). Analysis of the loadings of original carbon sources on CV1 showed that the most influential carbon sources were fumaric, caffeic, oleic, and stearic acids.

In order to compare modified plates with the use of Biolog plates, the analysis of the data using Biolog plates was also repeated but only for the 21 carbon sources also tested in the new plates. This analysis of the Biolog test plate data showed a similar pattern to that found with 125 C sources, with the tea orchard soil (soil 7) being most distinct (data not shown) as found with all 125 carbon sources (Fig. 1). The overall distance between soils was, however, much less

Table 5. Mean absorbance (A_{405}) for the eight red soils over 168h for sole C source test at two pH values

	Soil no.								
	1	2	3	4	5	6	7	8	$LSD_{0.05}$
pH 4.7	0.034	0.022	0.080	0.058	0.062	0.051	0.032	0.080	0.009
pH 7.0	0.087	0.096	0.123	0.128	0.133	0.144	0.032	0.130	0.013
Ratio	2.6	4.4	1.5	2.2	2.1	2.8	1.0	1.6	

with a mean Mahalanobis distance = 4.75 using only these 21 C sources. This was still significant at the 5% level and was close to that found with the modified plates at pH 7.0 (Fig. 3).

Phospholipid Fatty Acid Analysis

All the soils contained a variety of PLFAs composed of saturated, unsaturated, methyl-branched, and cyclopropane fatty acids (Fig. 4). Thirty-five PLFAs with chain lengths from C12 to C20 were identified and varied significantly in their relative abundance between soils (Fig. 4). The total amount of PLFA (Table 3) in the soils was significantly correlated with organic matter ($P < 0.001$, $r = 0.97$) and C_{mic} ($r = 0.93$). More fungal PLFA, 18:2ω 6,9, was present in the high organic matter and low pH soils planted with forest trees (soil 8) and tea orchard orchards (soil 7) than in the other soils (Table 3). The three soils under citrus trees (soils 3, 4, and 5) for 4, 8, and 12 years, respectively, had progressively more fungal PLFA (Table 3). The characteristic PLFAs for bacteria, i15:0, a15:0, 15:0, i16:0, 16:0, i17:0, a17:0, cy17:0, 18:1ω9, and cy19:0, were all strongly correlated with soil organic matter and microbial biomass-C (data not shown). The ratio of Gram-positive to Gram-negative bacteria was significantly higher in the eroded soil (soil 1) than the other soils and was also high in the tea orchard soil (soil 7) and 4-year-old citrus orchard soil compared to the other soils (Fig. 5).

Canonical variate analysis of the PLFA data showed the

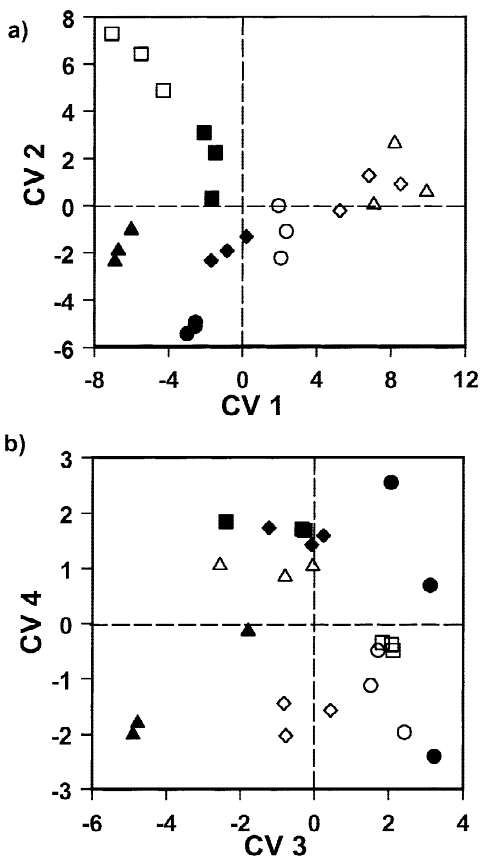

Fig. 2. Plot of ordination of canonical variates (CV), (a) CV1 against CV2 and (b) CV 3 against CV 4, generated by canonical variate analysis of A_{405} using modified sole carbon source test plates showing discrimination at pH 4.7 and between eight different soils: eroded soil (○), upland vegetable (●), citrus orchard—4yr (●), citrus orchard—8yr (■), citrus orchard—12yr (♦), paddy soil (△), tea orchard (▲), forest (◇).

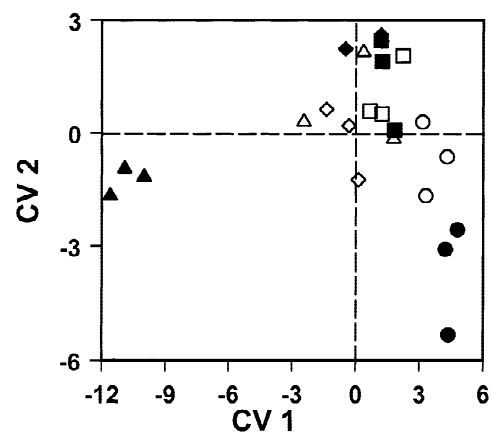

Fig. 3. Plot of ordination of canonical variates (CV) CV 1 against CV 2 generated by canonical variate analysis of A_{405} using modified sole carbon source test plates showing discrimination at pH 7 and between eight different Chinese red soils: eroded soil (○), upland vegetable (●), citrus orchard—4yr (□), citrus orchard—8yr (■), citrus orchard—12yr (◆), paddy soil (△), tea orchard (▲), forest (◇).

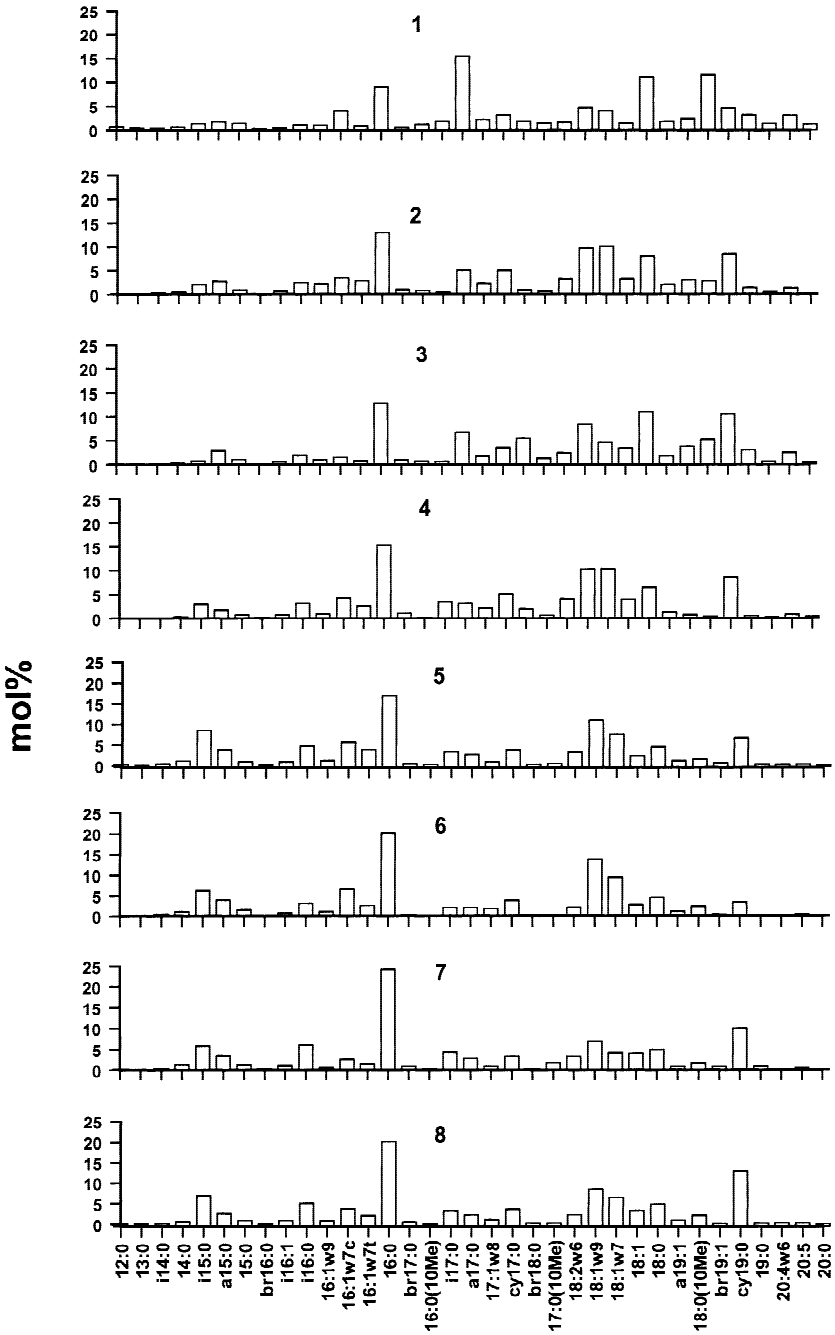

Fig. 4. Mol% PLFAs in eight different Chinese red soils: eroded soil (1), upland vegetable (2), citrus orchard—4yr (3), citrus orchard—8yr (4), citrus orchard—12yr (5), paddy soil (6), tea orchard (7), forest (8).

soils were also clearly discriminated by their PLFA profile (Fig. 6) with a mean Mahalanobis distance of 9.8, which was highly significant ($P < 0.001$, SCL = 3.5). Canonical variate 1 explained 57% of the variation and discriminated the eroded soil (soil 1) and the youngest citrus orchard soil (soil 3). CV2 explained 42% of the variation in the data and discriminated the forest and tea orchard soils (soils 8 and 7, respectively) from the other soils. Soil microbial biomass and basal respiration were not significantly correlated with these CVs. The other citrus orchards soils, the paddy soil, and the vegetable soil were also significantly different from one another, but were ordinated more closely together. On CV1, the young citrus orchard soil (soil 3) and the eroded soil were most distinct from the other soils. Some PLFAs (16:0, cy19:0, and i15:0) were enriched in these soils, but they had a lower relative abundance of 18:1ω7 and 18:1ω9. However, some branched PLFAs (br16:0, br17:0) as well as i17:0, a17:0, and 10Me17:0, all of which are commonly found in Gram-positive bacteria [43], were found in high relative abundance in the tea orchard soil (Fig. 4).

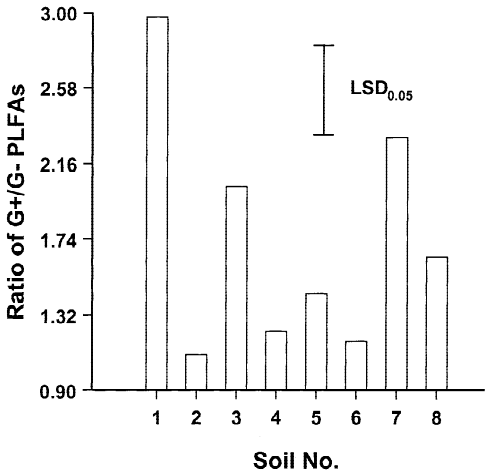

Fig. 5. Ratio of Gram-positive to Gram-negative PLFAs in eight different Chinese red soils: eroded soil (1), upland vegetable (2), citrus orchard—4yr (3), citrus orchard—8yr (4), citrus orchard—12yr (5), paddy soil (6), tea orchard (7), forest (8).

Discussion

Effect of Culture pH on CLPP Method

The modified method for assessing CLPP showed that the highly significant discrimination of the tea orchard soil found using Biolog plates was, in part, due to the pH of the growth media. The discrimination of the tea orchard soils was much less when tested with the modified plates at pH 4.7 compared to pH 7.0 (Figs. 2 and 3). Conversely the relative discrimination of the vegetable soil, which had a neutral pH, was altered when tested at pH 4.7 (Fig. 2a) compared to the tests at more neutral pH values (Figs. 1 and 3). Thus, our hypothesis that the discrimination obtained is conditioned by the culture conditions appears to be true. Clearly, using media buffered at different pH values to the soil may result in artefacts if such factors are not taken into account.

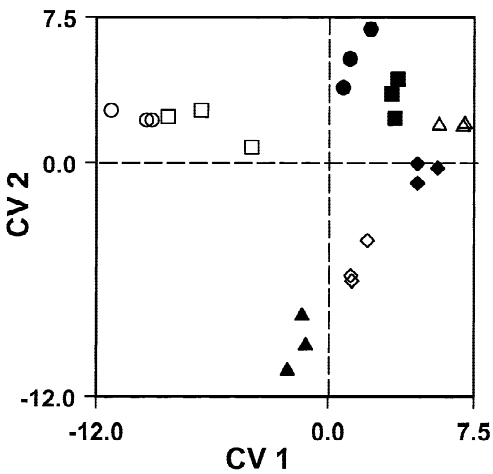

Fig. 6. Plot of ordination of canonical variates (CV) CV 1 against CV 2 generated by canonical variate analysis of PLFA pattern between eight different Chinese red soils: eroded soil (○), upland vegetable (●), citrus orchard–4yr (□), citrus orchard–8yr (■), citrus orchard–12yr (◆), paddy soil (△), tea orchard (▲), forest (◇).

It has previously been argued that the media pH of Biolog plates would have little effect on differences in pH between soils [34]. Bacterial growth and activity are generally higher at pH 7, and so the higher AWCD found in the plates at pH 7 was expected. There was, however, in our study no increase in AWCD at the higher pH in the tea orchard soil when the modified CLPP method was used. This is quite unusual, as most bacteria have wide pH tolerance, and suggests that the response of the tea orchard soil seen in the Biolog plates and in two different pH plates was not entirely due to the difference in soil pH. However, we found that the forest soil, which also had a low soil pH, did show an increase in average A_{405} at pH 7 compared to pH 4.7 (Table 4) that is consistent with a wide pH tolerance [34]. Microorganisms from tea orchard soils are known to be acid tolerant [37], and it might be argued that the neutral pH of the Biolog plates would have inhibited their growth.

The conventional Biolog plates, buffered at pH 6.5, are quite different from the normal acid environment of red soils. Consequently, microorganisms adapted to low pH conditions may either respond in an unrepresentative way or not respond at all in the near neutral pH environment, and this may mask differences between acid red soils. This may be the reason why the modified plates buffered at pH 4.7 showed greater discrimination for the red soils. In general it may be better to select a pH characterization medium that is closer to the pH of the soils under study when using such sole C source tests.

In several other studies using CLPP methods, organic acids have high loadings correlated with the main canonical variates and soil pH [13, 22, 30]. Grayston and Campbell [22] observed that the major influences on the C utilization profiles of microbial communities taken from the rhizosphere of hybrid larch and Sitka spruce was previous crop history, but this was manifested as a difference in soil pH. The effect of soil pH on potential C source utilisation also showed an interesting trend when utilization was compared against soil pH for all soils (Fig. 7). Sole C source utilization increased with increasing soil pH up to pH values of 5.1, and then declined before rising again (Fig. 7). This pattern was

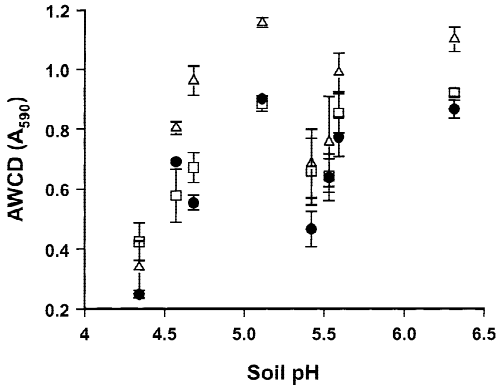

Fig. 7. Scatter plot of soil pH against potential C source utilization measured as A_{590} for different types of C source groups, namely, carboxylic acids (○), acidic amino acids (△), and basic amino acids(■).

observed for all groups of C sources tested, but was more marked for the basic and neutral amino acids than for the acidic amino acids (Fig. 7). The adsorption of amino acids by soil, and thereby their availability as substrates to microorganisms, is extremely sensitive to pH [48]. Below their isoelectric point (i.e., pH 5–6), amino acids exist in the cationic form and are progressively more absorbed to minerals as the pH falls. This is consistent with the pattern we observed up to pH 5.1 for our soils (Fig. 7). Above pH values between 5 and 6, the zwitterion and anionic forms are dominant and would be more bioavailable. Consequently differences observed in sole C source tests might reflect differences in availability rather than differences in the total amounts present in the soil or exuded by plant roots. In red soils, with their high iron oxide content, low organic matter content, and low pH, this type of organo-mineral interaction may dominate the availability of carbon sources to microorganisms.

Modified CLPP for Low pH Soils

The modified method for assessing CLPP by sole C-source tests was novel in two ways. First, two different pHs were used, and the results clearly showed that this has a large effect on the discriminatory power of this method when comparing soils with different pH. The approach may be particularly appropriate for looking at pH tolerance if several different pH values are to be compared and would be analogous to the approach of assessing pollution-induced community tolerance (PICT) proposed in relation to other types of stress imposed on soil communities [44].

The other main difference was the omission of the TTC indicator dye. The Biolog sole carbon source test plates were originally designed to aid the rapid identification of pure cultures of bacteria [5]. The composition of mineral media, C source profiles, use of TTC indicator dye, and high inoculum densities were all designed to give rapid detection of the oxidation of C sources, often within 4–24 h. The adaptation of Biolog plates for the discrimination of soil communities [18], however, involves incubation over several days, and color development is a consequence of growth on the C sources [55]. When used in this way it can be reasoned that there is no need for the TTC dye, which may in fact be toxic to some organisms [15], and as we have shown, absorbance readings of turbidity alone can give comparable results. As an indicator of dehydrogenase activity, with an optimum pH near neutral, the TTC indicator dye will not work at low pH values. In our experiments we used absorbance at 405 nm to monitor growth on the C sources and achieved comparable results with the Biolog multiwell plates to measure CLPP of these eight soils. A direct comparison of the new method using absorbance alone against Biolog with TTC is not, however, entirely unequivocal because different wavelengths were used and there were slight differences in pH and nutrients. Although similar patterns with respect to differences between the soils were obtained using both methods, the plates at pH 7.0 had lower sensitivity (Mahalanobis distances) and fewer soils were discriminated. Further evidence that absorbance readings alone without TTC dye can work was found when we compared Biolog SFN plates, which have no TTC dye, to Biolog GN plates for CLPP. We found there was little difference in the overall discrimination of different soils using the different plate types (unpublished data, C.D. Campbell).

The advantages of the modified procedure described here are that in preparing the plates there is greater flexibility to make the culturable conditions closer to the soil conditions under investigation, and also that more ecologically relevant C sources can be selected. In addition, because fewer C sources are used, more replicates can be tested with greater economy and statistical efficiency [13].

Structure of Microbial Communities in Chinese Red Soils

In this study we selected soils from the same climatic region and that had similar mineralogy [24] so that main influence on community structure was the land use history. All the test soils were derived from the quaternary red earth and had a

high clay content and low pH value, except for the vegetable soil (soil 2), which was derived from red sandstone and had a relatively high pH. The soils varied in fertility because of the difference in the period of cultivation, soil management, and crop type (Table 1). Some key soil fertility indexes, i.e., organic carbon and total N and P, increased significantly from the eroded and noncultivated wasteland soil (soil 1) to the soil that had been under forest for 38 years (soil 8). All the soils studied were eroded wastelands with sparse plant cover prior to their land use at the time of sampling. Consequently, the noncultivated soil (soil 1) is typical of unmanaged soils in the red soil area and can be considered as a starting point for these soils as they undergo agricultural improvement or afforestation. Although this initial noncultivated base soil had a low microbial biomass, the PLFA analysis showed that all the major microbial groups were present so that it had at least a broad genetic potential. Given the right management this soil would in all probability develop a high microbial diversity and a community structure selected by the crop cover.

Red soils used for growing rice are periodically waterlogged and might have been expected to be quite different in microbial community structure from the other land uses [7]. The PLFA analysis (Fig. 6), however, showed that the paddy field soils were only slightly different and that the PLFAs i16:1, i16:0, and a17:0, usually associated with anaerobic bacteria, were not present in any higher amounts in the paddy field soil compared to other soils. This contrasts with other studies that found PLFAs were highly sensitive to flooding and that showed consistent patterns in PLFA responses when measured seasonally [7]. The PLFAs i17:0 and a17:0 were positively correlated with high soil moisture content [7]. Consistent with the PLFA analysis in our study, the CLPP showed that while the paddy field soils had different communities, the differences were not large. Although Biolog CLPPs, which measure aerobic respiratory activities, may not be suited to anaerobic soils, they have also been shown to be significantly affected by flooding [6]. The small differences found in our study might possibly be explained by the duration of flooding, which can be significant [7].

The three orchard soils showed a progressive increase in fungal PLFAs with increasing age of the orchard (Table 3). This might have been expected because of the increase in acidity and organic matter that would favor fungal growth [21]. Both the PLFA and CLPP methods showed that the two older orchard soils (soils 4 and 5) were, however, clustered together more closely than the other younger orchard soil (soil 3), which was quite different and closer to the eroded soil (soil 1) (Fig. 1, 2a, 3, 6). This conclusion is also supported by the observation that soil 3 had a high ratio of Gram-positive to Gram-negative species, similar to the eroded soil, as shown by the PLFA analysis (Fig. 5). It is probable that the youngest orchard soil was still evolving a new microbial community but had changed little from its starting point (the eroded soil) in the first 4 years of growing citrus trees, but then had reached a new steady state after 8 to 12 years. The switch from a Gram-positive dominated population to more Gram-negative species may be indicative of progressive change from oligotrophic to more copiotrophic conditions.

The most distinct land use effect was, however, the cultivation of tea bushes, as shown by both the CLPP and the PLFAs. It was interesting that the forest soil (soil 8) and tea orchard soil (soil 7) had similar acid pH values (Table 1) but were still discriminated in both types of CLPP (Figs. 1 and 2). This suggests that the communities in the tea and forest soils were different for reasons other than just pH. The tea orchard soil was found to be very distinctive using the CLPP approach with Biolog plates and the modified plates at both pH values tested. The fact that the AWCD was low in all the CLPP methods in itself suggests that there were unusual physiological types present in the tea orchard soil. Even if these results were at least partly affected by the culture conditions, the PLFA analysis still suggested that the tea orchard soil had a distinct microbial community with a higher Gram-positive to Gram-negative ratio.

The total viable counts showed that the majority of culturable cells formed "pinhead"-type colonies that are often a characteristic of slow-growing organisms. This was confirmed by subculturing these colonies and monitoring colony development. Gram staining showed that they were Gram-negative rods. In addition, attempts to identify them using the Biolog identification system using high inoculum densities failed, as there were too few positive reactions. The Biolog system assesses the metabolic diversity of the culturable, primarily fast-growing bacteria [45]. Fungi and slow-growing bacteria may therefore have minimal influence on the CLPP. This was probably true for the tea orchard soil we tested that had high numbers of slow-growing bacteria. The reason for the distinctive discrimination of the tea orchard soil shown by the CLPP may therefore have been the presence of slow-growing bacteria inherently unable to grow in the Biolog plates because of the cultural conditions, which normally select for fast-growing organisms [45]. Other ex-

planations such as selective toxicity of the TTC or selective pH sensitivity are unlikely, because the modified CLPP method without TTC and tested at two different pH values gave similar responses.

The roots of tea bushes have been found to be suppressive to microorganisms [38] supporting lower microbial numbers than found in the bulk soil, such that it has been suggested that inhibitory root exudates are produced [38]. Tea bushes are grown in dense rows and have high root length densities so that nearly all the soil would be affected by such exudates. In addition, the tea bush leaf litter can also contain antimicrobial substances and the low pH can result in Al toxicity [38]. Pandey and Palni [39], using plate count methods, suggested *Bacillus* species, which exhibited a high antifungal activity [40], were the most dominant bacterial species in the tea rhizosphere because they appeared to be closely associated with tea bush roots. We found several Gram-positive PLFAs were present in higher relative abundance in the tea orchard soil than in the other soils and also that the tea orchard soil had a higher ratio of Gram-positive to Gram-negative PLFAs than most of the soils, except the eroded soil and 4-year-old citrus soil. Our data therefore support these earlier studies [39], but we also found that the slow-growing bacteria, found as isolates on TSA plates in large numbers, were mostly Gram-negative species. Nevertheless, the PLFA method does not exhibit the bias of culturable techniques and so may be a better reflection of the microbial composition of tea orchard soils.

The tea orchard soil had high microbial biomass, large numbers of bacteria, and high amounts of total PLFA, but microorganisms from this soil exhibited the slowest utilization of carbon sources and grew as small colonies on the agar plates. The metabolic quotient was lowest in the tea orchard soil, suggesting that growth was accomplished at low expenditure of energy and/or that the microorganisms had a low metabolic rate [29]. This would support the conclusion that the microbial community structure in the tea orchard soil had high numbers of low-activity species and their significance and unusual ecology warrants further investigation.

Comparison of CLPP and PLFA Analysis

Sole-carbon-source utilization tests have previously proved to be a satisfactory method of characterizing microbial communities, based on their metabolic profiles [18]. However, AWCDs in the Biolog and MT plates were not correlated with either the number of bacteria or microbial biomass. It is often assumed that the number of viable and active bacteria in the sample is strongly correlated with the AWCD [20, 23]. The exception to this in our study was the tea orchard soil. For this soil widely different inoculum densities gave similar AWCDs in the Biolog plates. It appears that CLPPs using sole carbon source tests can provide us with information about differences in community structure, but they are clearly not always related to either the numbers of microorganisms or their biomass.

Both the PLFA and CLPP profiles showed that the microbial communities were different in soils with different land use history. In this study the CLPP method using Biolog plates showed more discrimination of the soils (Mahalanobis distance = 11.7) than the PLFA method (Mahalanobis distance = 9.8). This initially may appear to be in contrast to other studies that found that the PLFA method was more discriminating than CLPP methods [3, 17]. However the large Mahalanobis distances found with the CLPP Biolog technique were primarily due to the unusual response of the tea orchard soil and the wide pH differences between the soils. At pH 4.7 the CLPP method was less discriminating (Mahalanobis distance = 6.8) than PLFA analysis, and this is probably a truer reflection of the two methods' respective ability to compare different soils. The PLFA method has the advantage not only of being unbiased by cultural conditions, but also of taking into account the contribution of fungi and slow-growing bacteria [53, 3] that current CLPP methods probably miss [45].

Relationship of Microbial Parameters to Soil Fertility

Soil microbial biomass C was closely related to soil fertility (as defined by chemical analysis), as has been found for other soils [10, 29] and also in highly weathered red soils [24]. These effects are largely due to the close correlation between C_{org} and C_{mic}, but a good relationship between crop yield and soil microbial biomass has also been reported from other field experiments [10, 26, 29]. Basal respiration can serve as an indicator of total C turnover and reflect the availability of soil organic matter. Determination of total PLFA is also a useful approach for quantitative analysis of soil microorganisms. Zelles et al. [57] reported a significant correlation between ester-linked PLFA and microbial biomass C. As expected, in the present study, microbial biomass C was highly correlated with organic C and total N, and also with basal respiration and total PLFA. Of the individual PLFAs it was, however, the bacterial components that were most strongly correlated with microbial biomass and fertility levels. Soil microbial biomass and basal respiration were not

significantly correlated with the CVs from the CLPP analysis, and there was no correlation between AWCD and microbial biomass or soil C and N content. This supports other studies that found no systematic change in CLPP associated with increasing soil fertility [8].

The metabolic quotient did not respond in a consistent manner across the gradient of organic matter and fertility. Increases in microbial metabolic quotients (qCO_2) are generally thought to be indicative of stress [2], such as heavy metal contamination [11] and ecosystem disturbance [28, 51, 17], though its value in the assessment of ecosystems in this way has been questioned [52]. In this respect our results would also suggest this parameter should be interpreted carefully with respect to soil conditions and land cover. These results showed that the total PLFAs and microbial biomass C may serve as important indicators of soil quality and be closely related to soil fertility status.

Conclusions

Microbial community structure differed widely in Chinese red soils with different land use history, but this did not vary in a consistent manner with other conventional chemical and biological indices of soil fertility. PLFAs showed these differences without the bias associated with the culturable methods, and the total PLFA was correlated with organic matter content and fertility. Consequently, PLFA analysis was the best method for assessing broad-spectrum community differences and at the same time attempting to correlate changes with soil chemical indices of fertility. The CLPP methods employed did, however, reveal interesting physiological differences between the soils, but these were not related to either the size of microbial biomass or other soil fertility indices, and careful consideration of culture conditions must be made to interpret any differences found. Results from both methods demonstrated that land use history had a significant impact on microbial community structure and that it is a major determinant of soil microbial community structure [8].

Acknowledgments

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