RESEARCH ARTICLE

Microcalorimetric assessment of microbial activity in long-term fertilization experimental soils of Southern China

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Abstract

Microcalorimetry, plate count and PCR–denaturing gradient gel electrophoresis (DGGE) were employed to investigate microbial diversity and activity in soils from the Red Soil Experimental Station of the Chinese Academy of Agricultural Sciences, Hunan Province, China, where a wheat–corn rotation with 12 fertilization treatments was established in 1990. Fertilization greatly increased microbial biomass carbon (C) and nitrogen (N) (C_{mic} and N_{mic}) as well as the activities of phosphatase, urease, invertase, protease, catalase and dehydrogenase. Manure alone (M) enhanced the number of denitrifying and aerobic bacteria by 54.4% and 20.5%, respectively, whereas fallow (H) increased the number of aerobic cellulose decomposing bacteria by 31.4%. Fallow and soils amended with mineral fertilizers plus pig manure or straw increased both the DGGE band patterns and the Shannon index compared with mineral fertilizers or the control. Mineral treatments with lower bacterial numbers enhanced the values of the peak time (t_{max}) more than did organic treatments. The peak height (P_{max}) was positively correlated ($P < 0.01$), with soil enzymes, C_{mic} and N_{mic}, and the number of microorganisms, whereas the peak time (t_{max}) was negatively connected ($P < 0.01$) with these parameters. The microbial growth rate constant (k) was linked to bacteria ($P < 0.01$), actinomycetes ($P < 0.05$) and catalase ($P < 0.05$). The total heat evolution (Q) had no relationships with any soil microbial properties (except for catalase). We propose that P_{max} and t_{max} could be used as indices of soil microbial activity, while the values of k and Q are poor indicators.

Introduction

There is increasing concern about the impacts of long-term agricultural practices on soil quality and health. Numerous investigators have studied these practices and their links with soil biochemical and microbial properties. It has been reported that cultivation may reduce soil biological activity through the decrease of macroaggregates, whereas crop rotations compared with monoculture improve the microbial biomass and the activities of soil enzymes by suppressing the deleterious rhizobacteria (Dick, 1992). Long-term fertilization may also exert an influence on soil quality (Li *et al.*, 2008b). Organic fertilizers usually increase soil microbial biomass, carbon dioxide evolution and enzyme activities, whereas inorganic fertilizers have relatively less effect on these soil properties (Chu *et al.*, 2007). The influence of long-term application of chemical fertilizers and organic matter inputs, such as farmyard manure, green manure or straw, either alone or in combination with mineral fertilizers, on soil biological health is an important area of investigation for assessing soil sustainability. This area is of particular significance for the sustainable management of degraded Red soils (Ultisols and Oxisols according to USDA Soil Taxonomy) derived from Quaternary Red Clay and characterized by low pH and deficiencies in available nutrients such as phosphorus (P) and nitrogen (N). These types of soils are widespread in the tropical regions in Southern China. So far, little is known about the effects of

long-term fertilization on the microbial properties of Red soils and how these properties can be used as indices of their sustainable productivity and health.

Soil microorganisms are vital to agroecosystem health through their roles in residue decomposition, nutrient cycling and their associations with other organisms (Ge *et al.*, 2008). In agricultural soils, microorganisms are known to impact profoundly the status of soil fertility, especially the availability of soil nutrients (Li *et al.*, 2008a). The most suitable biological and biochemical properties for estimating soil quality are those related to the cycling of biogenic elements and to the transformation of organic matter (Trasar-Cepeda *et al.*, 2008). Soil microbial properties such as microbial biomass, population, activity and enzyme activities have a strong correlation with soil health and have been considered its biological indices (Li *et al.*, 2008b). However, due to the complex dynamics of soil ecosystems, no one parameter is sufficient to study biological processes in soils. Moreover, understanding the microbial community in the soil environment has proven to be a challenging task because of the extremely high abundance and enormous diversity of microorganisms in soils. To our knowledge, the relationships between fertilization and the shifts of soil microbial communities have still not been unanimously determined and it is necessary to examine them through long-term field experiments.

During the development of microbial activity stimulated by the presence of nutrients, a flow of thermal effect is generated, and can be monitored by microcalorimetry. Microcalorimetry has been successfully employed to study the microbial growth and metabolism in soils as it permits the continuous monitoring of the activity of living process *in situ* for a prolonged period without disturbing the system (Núñez-Regueira *et al.*, 2002; Barros & Feijoo, 2007).

This highly sensitive tool has been applied for estimating the influence of agricultural practices on soil microbial activity (Laor *et al.*, 2004; Zheng *et al.*, 2007). The connections between soil microbial properties and microcalorimetric parameters could elucidate which microcalorimetric parameter best indicates microbial activity in soils. Microcalorimetry and the recent development of culture-independent molecular techniques can provide us with an in-depth understanding of the soil biological processes. It would be very useful to study these questions; publications lack the sensitivity of calorimetric indices to detect microbial and metabolic activity changes caused by long-term fertilization. This is important as any new indicator must be sensitive to the activity and biomass changes related to soil properties (Barros *et al.*, 2007). We benefited from this simple and accurate method as well as plate count, soil DNA extraction, PCR and denaturing gradient gel electrophoresis (DGGE) to examine the soil microbial activities, community structure and diversity at a long-term fertilization experiment in an upland derived from Quaternary Red Clay of Southern Hunan Province, China. An attempt was made to analyze the connections between soil microbial properties and microcalorimetric parameters to establish the sensitivity of the latter indices to different types of fertilization.

Materials and methods

Experimental site

A long-term fertilization experiment with double-cropping wheat and corn was begun in September 1990 at the Red Soil Experimental Station of the Chinese Academy of Agricultural Sciences, Southern Hunan Province (26.8°N, 111.9°E, 120-m altitude). The soil originated from the Quaternary Red Clay and was classified as Ultisol. Under average climate conditions, the area receives 1290 mm of annual precipitation, about 70–80% of which occurs from April to October. The mean annual temperature, annual evaporation, annual frost-free days and sunshine hours are 18 °C, 1470 mm, 300 days and 1610 h, respectively. Before the experiment, the field had been under wheat–corn rotation for several years.

Experimental design

Twelve plots (27×10 m) with different fertilization treatments were established and included control without fertilizer (CK), fallow (H), combinations of fertilizer N, P and potassium (K): N, NP, NK, PK, NPK, NPK plus straw (NPKS), NPK plus pig manure (NPKM), NPKM plus wheat–soybean–sweet potato between lines (NPKMR), 1.5 times NPKM (1.5NPKM) and pig manure alone (M). The N, P and K fertilizers for corn and wheat were provided as urea at 456 and 195 kg N hm^{-2}; P as single superphosphate at 699 and 300 kg P hm^{-2}; and K as KCl at 140 and 60 kg K hm^{-2}, respectively. Chemical fertilizer N was applied yearly as top dressing (40%) and base dressing (60%); chemical P and K fertilizers and manure were fertilized only as base dressings. Thirty percent of total N was used as chemical N and 70% as manure for the NPKM treatment; the same amount of chemical N was applied for other treatments. Thirty percent of the total amount of individual fertilizer applied each year was used for wheat and 70% for corn.

Soil sampling and analyses

Soil samples were collected each November using an auger to a depth of 0–20 cm at nine randomly selected points in each plot and mixed to form a bulk sample. Samples were sieved (< 2 mm) and kept in polyethylene bags at 4 °C.

Basic soil chemical properties

All soil chemical properties were determined conventionally. The soils in 2007 had a pH (H_2O, 1 : 2.5 solid/liquid ratio) of

4.3–6.7, a carbon (C)/N ratio of 6.9–11.8 and soil organic C (SOC) of 7.1–13.6 g kg^{-1}. Soil N ranged from 0.9 to 1.3 g kg^{-1}, total P from 0.4 to 1.8 g kg^{-1} and total K from 12.0 to 14.6 g kg^{-1}. The available N varied from 52.9 to 130 mg kg^{-1}, available P from 3.8 to 286.8 mg kg^{-1} and available K from 90.8 to 285 mg kg^{-1}.

Microbial biomass

Microbial biomass C (C_{mic}) and N (N_{mic}) were determined using the 48-h fumigation–extraction method (Vance *et al.*, 1987). Fumigated and nonfumigated soil samples were extracted by 0.5 M K_2SO_4. Total organic C and total N in the extract were analyzed using an automatic carbon analyzer (TOC5000, Shimadzu, Kyoto) and the Kjeldahl method, respectively. The extractable C and N in the fumigated and nonfumigated soils were assumed to be released from lysed soil microorganisms. The released N and C were converted to N_{mic} and C_{mic} using extraction factors of 0.54 and 0.45, respectively (Brookes *et al.*, 1985; Wu *et al.*, 1990).

Enzyme activities

Activities of dehydrogenase, neutral phosphatase, urease, casein protease and invertase were determined by the methods of Casida *et al.* (1964), Tabatabai & Bremner (1969), Nannipieri *et al.* (1980), Gil-Sotres *et al.* (1992) and Schinner & von Mersi (1990), respectively. As substrates, 2,3-5-triphenyltetrazolium chloride (Tris-HCl buffer, pH 7.6), *p*-nitrophenylphosphate (citrate buffer, pH 7.0), 10% urea (Tris-HCl buffer, pH 7.6), 2% sodium caseinate (Tris-HCl buffer, pH 7.6) and 8% saccharose (phosphate buffer, pH 5.5) were used. The activities of catalase were determined by measuring the oxygen absorbed by $KMnO_4$ after addition of hydrogen peroxide to the sample (Garcia-Gil *et al.*, 2000). All determinations were performed in triplicate.

Soil microbial community characterization

The total numbers of cultivable bacteria, fungi and actinomycetes were determined as CFU on agar plates by dilution plate methods. Ten grams of fresh soil was homogenized in 90 mL of 0.1% (w/v) sodium pyrophosphate (pH 7) and 10-fold serial dilutions were performed. For the enumeration of bacteria, fungi and actinomycetes, 0.1-mL aliquots were spread onto beef extract peptone medium, Czapek's medium and Gause's No. 1 synthetic medium, respectively (Xu & Zheng, 1986). The plates were incubated at 37 °C. The colonies on replicate plates with 30–300 colonies were counted and the mean values determined.

Viable counts were conducted for aerobic bacteria (aerobic cellulose decomposing and aerobic N fixing) as well as denitrifiers. Aerobic bacteria were enumerated by plate counts using a diluted soil extract agar (100 mL soil extract, 15 g agar and 900 mL distilled water). Triplicate plates were incubated at 20 °C for 1 week. Initially, spread plates were inoculated with a dilution series that was wetted for 5 min with 2% saline solution. Denitrifiers' densities were assessed by non-substrate-specific most probable number (MPN) methods (Tiedje, 1982). Screw-top tubes were filled with 10 mL nitrate broth solution (prepared by mixing 8.0 g nutrient broth and 0.5 g potassium nitrate per liter water). Five tubes per dilution were employed. After 14 days of incubation at 28 °C, 0.5 mL of medium was pipetted to test for NO_3^- and NO_2^- by adding six drops of diphenylamine reagent. A colorless response (no NO_3^- or NO_2^-) was considered evidence of denitrification. The number of denitrifiers was then calculated using an MPN table (Cochran, 1950).

Soil DNA extraction was undertaken as follows: 1 g soil was mixed with the extraction buffer (100 mM Tris, 100 mM EDTA and 1.5 M NaCl; pH 8.0) containing lysozyme in the centrifuge tubes. The samples were shaken at 180 r.p.m. and 37 °C for 2 h. Then, 220 μL SDS (20%) was added to the tubes and the samples were incubated at 65 °C for 1 h. After centrifugation at 4250 ***g***, the supernatants were collected. The aqueous phase was extracted with a solution of phenol–chloroform–isoamyl alcohol (25 : 24 : 1). Isopropanol was then added to precipitate the DNA and the samples were centrifuged at 13 000 ***g*** and the DNA pellets were suspended in 50 μL TE (pH 8.0).

For detailed downstream characterization of microbial communities, a PCR-dependent method was used in automated thermal cyclers to obtain 16S rRNA genes from the extracted soil DNA. The presence of PCR products was confirmed by electrophoresis on 1.5% agarose gels stained with ethidium bromide.

The DGGE method was used for visualizing the major members of the microbial community. For this purpose, PCR-amplified 16S rRNA gene fragments were community fingerprinted using a DCode TM Universal Mutation Detection System (Bio-Rad). The conditions for separation were as follows: running at 80 V for 16 h in a 10% polyacrylamide gel with the denaturing gradient of 45–65% (*apr*) or 25–50% (*npr*) at 60 °C. Excised bands of DGGE gels were washed twice with 1 mL sterilized distilled water in a 1.5-mL tube. A portion of the gel piece was used as for the direct template for PCR to recover DNA fragments. The conditions for recovering *apr* and *npr* genes were the same as for the original PCR, except that the forward primer had no GC clamp attached.

To take into account both the richness and the evenness of diversity, the Shannon index (H') was used in the form of

$$H' = -\Sigma(n_i/N)\log(n_i/N) = -\Sigma p_i(\log p_i)$$

where n_i is the importance value for each species, N is the total of importance values and p_i is the importance probability for each species (n_i/N).

Microbial activity measured by microcalorimetry

A TAM III thermal activity monitor (Thermometric AB, Sweden) was used for all heat-effect measurements. Soil samples were incubated at 25 °C for 24 h and their moisture maintained at 35% (water-holding capacity) to maximize microbial activity (Prado & Airoldi, 2001). All determinations were performed in 4-mL stainless-steel ampoules at 25 °C. Before experimenting, the ampoules were sterilized by rinsing in 75% ethanol and sterile deionized water for 10 min and dried under a laminar flow hood. One gram of soil was placed into the sterile ampoule and 0.2 mL of a solution containing 1.5 mg glucose and 1.5 mg ammonium sulfate was added immediately. The ampoules were introduced into the multichannel of the microcalorimeter. They were lowered to a preheating position for 15 min and then to the measuring position. Once the baseline was stable, data and growth power–time curves were monitored and recorded by a computer until the signal was back to baseline again. Each measurement lasted for about 48 h. All the experiments were performed in triplicate.

The total heat output, Q, was obtained through the integration of each curve. The value of peak height (P_{max}) and corresponding time (t_{max}) of each curve were picked through the TAM assistant software kit (Thermometric AB). P_{max} and t_{max} quantitatively reflect how the bacterial growth cycle and activity are altered by different environmental conditions (Rong *et al.*, 2007). The microbial growth rate constant (k) determined by microcalorimetry is based on the assumption that the heat evolved from metabolism in the vegetative stage is proportional to the rate of cell division (Boling *et al.*, 1973). This parameter was calculated by fitting a logarithmic growth model based on data of the power time curve in the logarithmic growth stage. Thus, if the cell number is n_0 at time 0, and n_t at time t,

$$n_t = n_0 \exp(kt) \quad (1)$$

where k is the growth rate constant. If the power output of each cell is w, then

$$n_t w = n_0 w \exp(kt) \quad (2)$$

If the heat output power is P_0 at time 0 and Pt at time t, then

$$P_0 = n_0 w$$

and

$$Pt = n_t w$$

giving

$$Pt = P_0 \exp(kt) \text{ or } \ln Pt = \ln P_0 + kt \quad (3)$$

The growth power–time curves of the log phase correspond to Eqn. (3). So, using the data ln Pt and t taken from the curves to fit a linear equation, the thermokinetic equation for the soil microbial activity and the correlation coefficients can be obtained.

Statistical analysis

DGGE data were analyzed using the Dice correlation coefficient and the unweighted-pair-group method with arithmetic averages. Three replicates of each experimental measurement were done to show the results as the average and SD. The quantitative microcalorimetric parameters and correlations among them and soil properties were analyzed with ANOVA and significance was expressed at $P < 0.01$.

Results

Microbial activity and response to fertilization as measured by the microcalorimetric method

Figure 1 shows that all recorded power–time curves presented a typical process of microbial metabolic activity. The values of microcalorimetric parameters (P_{max}, t_{max}, Q and k) were higher with organic treatments (M, NPKM, NPKMR, NPKS and 1.5NPKM), except for t_{max}, than with mineral fertilizers (PK, NK, NP, NPK and N). The total heat evolution (Q), obtained by integration of the curves, reached higher values in manure and NPKM, followed by the fallow (H), N, NPKS and 1.5NPKM. The highest values of both P_{max} and k were observed in NPKMR. The growth rate (k) values enhanced with increasing bacterial quantity (Fig. 2b). Substantial correlations were observed between k and the numbers of bacteria and actinomycetes ($P < 0.01$ and $P < 0.05$, respectively), whereas no correlation was found between Q and soil microbial properties, except for catalase.

In this work, a more pronounced increment of microcalorimetric parameters was observed with organic treatments than with mineral fertilizers. The values of the peak height and peak time were opposite (positive and negative, respectively), but both parameters were substantially correlated with soil microbial properties.

Soil microbial biomass and its links with microcalorimetric parameters

The content of C_{mic} ranged from 58.4 mg kg^{-1} in the control (CK) to 792.5 mg kg^{-1} in the fallow (H), whereas that of N_{mic} in the corresponding treatments varied from 21.8 to 86.7 mg kg^{-1} (Table 1). These trends displayed increasing values of C_{mic} and N_{mic} from mineral treatments to organic ones with the highest values in H. The lowest value of C_{mic} was recorded in NK, whereas that of N_{mic} was found in CK.

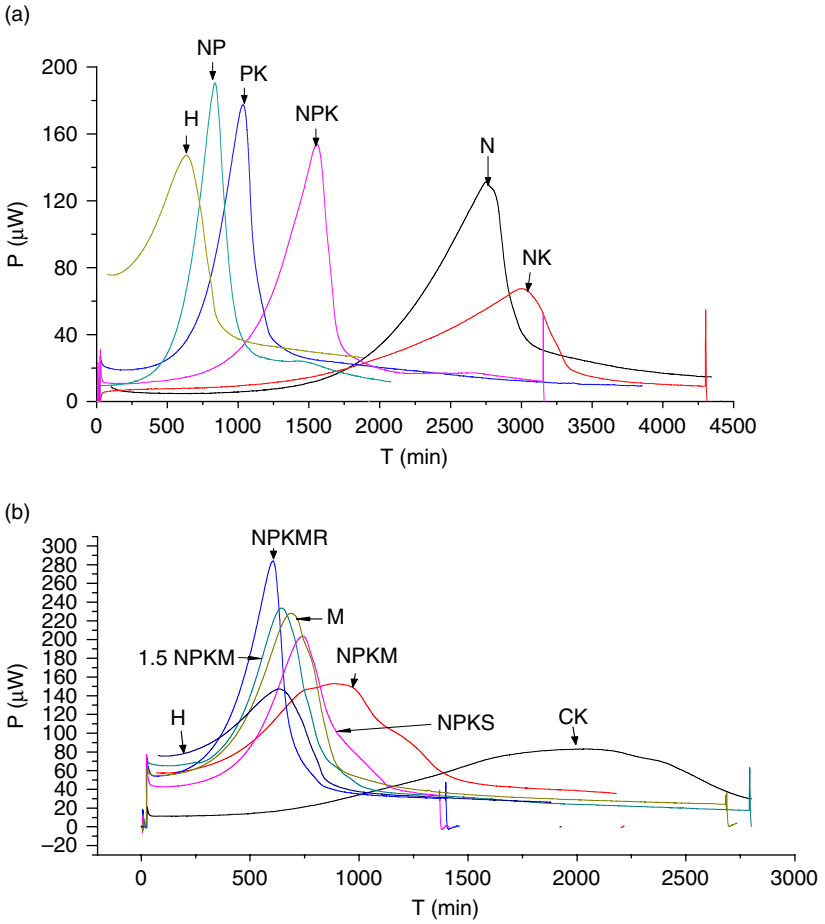

Fig. 1. Power–time curves recorded microcalorimetrically from 12 treatments amended with glucose and ammonium sulfate and organized in two groups of graphs. The first group of graphs (mineral fertilizers) consists of seven treatments: no fertilizer (CK); fallow (H); only nitrogen fertilizer (N); and different combinations of mineral fertilizers, NP, NK, PK and NPK. The second group is composed of organic treatments (M, NPKM, 1.5NPKM, NPKMR and NPKS). CK and H are common to both the two groups. In these power–time curves, thermal power (μW) is plotted against time (min). Integration of these curves provides values of the total heat evolved the process. The evolution of peak height (P_{max}) is the power at the maximum of the peak, and peak time (t_{max}) is the time to reach the maximum of the peak.

Of all treatments, the content of C_{mic} was essentially different at $P < 0.01$, except for NK and N. The N_{mic} also showed significant differences among treatments at $P < 0.01$. Table 4 displays significant correlations of the peak height (P_{max}) and peak time (t_{max}) with both microbial biomass C and N. Conversely, the total heat (Q) and the microbial growth rate constant (k) showed no striking correlations with soil microbial biomass.

Soil enzyme activities and their relationships with microcalorimetric parameters

Enzyme activities varied markedly among the treatments. Invertase exhibited larger variations (maximum divided by minimum = 219.4), followed by urease (133), dehydrogenase (20.5), protease (7.76), phosphatase (7.11) and catalase (4.27). The highest enzyme activities were found with the fallow (H) and NPKM treatments, except for catalase, whereas lower activities were recorded with the CK and N treatments. P_{max} and t_{max} were substantially correlated with all enzymes assayed ($P < 0.01$), whereas Q and k were only linked with catalase ($P < 0.05$). These results indicate important relationships between soil enzymes and microcalorimetric parameters.

Soil microbial communities and their correlations with microcalorimetric parameters

Great variations in the numbers of microorganisms and bacterial species were observed (Table 3). The number of microorganisms and species was reduced with mineral fertilizers as compared with organic treatments. The stepwise regression analysis showed that bacteria and actinomycetes significantly correlated with SOC ($R^2 = 0.697$ and 0.728, respectively), whereas the number of cultivable fungi was noticeably linked with the C/N ratio ($R^2 = 0.636$, Table 2). Bacteria, fungi and actinomycetes showed important correlations with P_{max} and t_{max} ($P < 0.01$). The microbial growth rate constant (k) was also correlated with the numbers of these microorganisms ($P < 0.05$). Unlike other microcalorimetric parameters, no significant correlations were found between Q and the microbial populations.

Greater numbers of denitrifying and aerobic N-fixing bacteria were counted in manure alone, whereas the highest

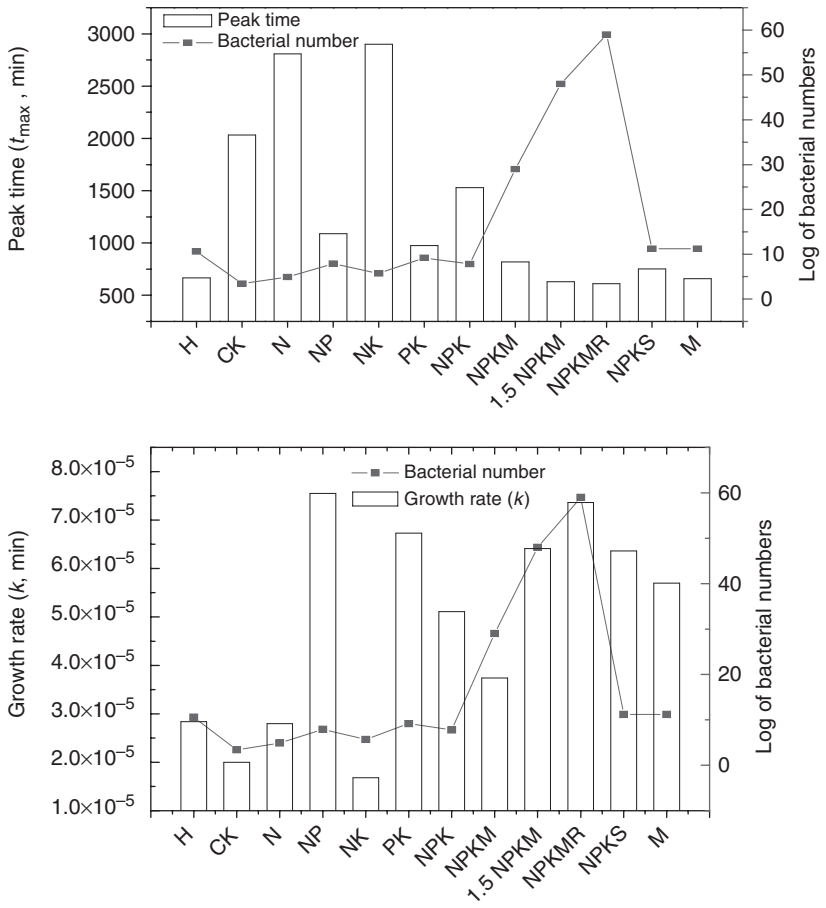

Fig. 2. Relationships between bacterial number and both the peak time and growth rate in the treatments of upland soils (0–20-cm layer).

Table 1. Microbial biomass and richness in different treatments of upland Red soil (0–20-cm layer)*

Treatment	C_{mic}	N_{mic}	C_{org}	C_{mic}/C_{org}	C_{mic}/N_{mic}	Shannon index	95% confidence interval
CK	58.45 (J)	21.83 (FG)	7.410 (E)	7.890 (DE)	2.693 (EF)	3.362	(3.358, 3.366)
NK	43.67 (K)	24.82 (FG)	8.840 (D)	4.936 (E)	1.797 (F)	2.993	(2.990, 2.996)
NPK	283.9 (G)	38.02 (D)	8.450 (C)	33.66 (C)	7.530 (C)	2.389	(2.382, 2.396)
PK	161.3 (H)	33.80 (DE)	7.930 (H)	20.58 (D)	4.787 (D)	2.831	(2.827, 2.834)
NP	97.57 (I)	28.52 (EF)	8.530 (EF)	11.47 (DE)	3.425 (DE)	2.833	(2.831, 2.834)
NPKM	535.8 (D)	58.26 (C)	13.13 (FG)	40.83 (BC)	9.350 (A)	3.043	(3.041, 3.045)
NPKMR	593.9 (C)	77.91 (B)	14.57 (G)	51.51 (B)	7.623 (BC)	3.058	(3.051, 3.064)
1.5NPKM	529.6 (E)	76.55 (B)	13.65 (FG)	38.94 (BC)	7.923 (ABC)	3.43	(3.427, 3.433)
NPKS	403.8 (F)	54.20 (C)	8.840 (B)	46.02 (BC)	7.453 (C)	3.431	(3.428, 3.434)
M	655.7 (B)	78.36 (B)	12.74 (A)	51.74 (B)	8.397 (ABC)	2.485	(2.483, 2.486)
N	47.48 (K)	19.89 (G)	7.150 (B)	6.667 (E)	2.488 (EF)	3.043	(3.041, 3.045)
H	792.5 (A)	86.71 (A)	11.56 (EF)	68.71(A)	9.243 (AB)	3.254	(3.250, 3.257)

*C_{mic} and N_{mic} are microbial biomass carbon and nitrogen; C_{org} is soil organic carbon.
Different letters in the same column indicate a significant difference at $P < 0.01$.

number of aerobic cellulose-decomposing bacteria was observed in the fallow (H). No differences were found for the denitrifying bacteria between mineral fertilizers and the control, except for NPK and NP, whereas for aerobic N-fixing bacteria, the differences were substantial. Almost all bacterial species revealed significant differences between organic treatments and mineral fertilizers. Furthermore, for most of the bacterial species, the differences were remarkable among organic treatments, whereas a 50–50 trend of difference was observed among mineral fertilizers. Aerobic

N-fixing bacteria were substantially correlated with both denitrifying and aerobic cellulose-decomposing bacteria. Denitrifying bacteria were more related to P, whereas aerobic cellulose-decomposing and N-fixing bacteria showed important links to the C/N ratio and pH, respectively. Moreover, aerobic cellulose-decomposing and aerobic N-fixing bacteria had also strong relationships with the C/N ratio and soil pH.

Table 2. The variables that were found by stepwise regression analysis to be correlated with microbial properties or indicators in the soils from the long-term experiment

Dependents	Variables related	R^2
C_{mic}	C/N	0.821**
N_{mic}	C/N, TK	0.911**
Bacteria	C_{org}	0.697**
Fungi	C/N	0.636**
Actinomycetes	C_{org}	0.728**
Denitrifying bacteria	TP, AP	0.955**
Aerobic cellulose-decomposing bacteria	C/N, AP	0.816**
Aerobic N-fixing bacteria	pH, AP	0.848**
Shannon index	C_{mic}/N_{mic}	0.401**

**, significant at 1% level.

The DGGE band patterns (data not shown) revealed differences of microbial community structures in mineral treatments from those of organic ones. The greatest similarity in bacterial community structure occurred in the mineral treatments. Band variation was also observed among the fertilizer treatments. For example, NPKM, 1.5NPKM, NPKS and H showed increasing bands compared with the other treatments, and the same increment was observed for the values of the Shannon index (Table 1).

Discussion

Microcalorimetric parameters as indices of soil microbial activity

To our knowledge, studies of the peak height (P_{max}) and peak time (t_{max}) as indicators of soil microbial properties are relatively scarce compared with those concerning the total heat evolution (Q) and the microbial growth rate constant (k). In this work, P_{max} and t_{max} showed positive connections ($P < 0.01$) to microbial biomass C and N (Table 4). P_{max} demonstrates higher links ($P < 0.01$) with phosphatase (0.648) and invertase (0.645), whereas lower

Table 3. Different cultivable microorganisms in the treatments of upland Red soils (0–20-cm layer)

Treatment	Denitrifying bacteria ($\times 10^4$ CFU g^{-1})	Aerobic cellulose-decomposing bacteria ($\times 10^3$ CFU g^{-1})	Aerobic N-fixing bacteria ($\times 10^4$ CFU g^{-1})	Bacteria ($\times 10^6$ CFU g^{-1})	Fungi ($\times 10^3$ CFU g^{-1})	Actinomycetes ($\times 10^5$ CFU g^{-1})
CK	4.500 (F)	2.800 (FG)	2.600 (D)	3.400 (H)	1.400 (I)	9.000 (GH)
NK	4.500 (F)	0.200 (H)	0.290 (F)	5.700 (G)	2.800 (HI)	10.00 (G)
NPK	45.00 (C)	4.500 (E)	1.300 (E)	7.800 (F)	12.70(F)	3.200 (IJ)
PK	1.500 (F)	1.800 (G)	10.00 (B)	9.200 (EF)	14.15 (F)	69.00 (F)
NP	15.00 (E)	0.400 (H)	1.300 (E)	7.900 (F)	4.100 (GHI)	1.900 (J)
NPKM	110.0 (B)	12.00 (D)	7.500 (C)	29.00 (C)	43.00 (D)	94.00 (E)
NPKMR	45.00 (C)	12.00 (D)	9.500 (B)	59.00 (A)	159.80 (A)	390.0 (A)
1.5NPKM	110.0 (B)	28.00 (B)	10.00 (B)	48.00 (B)	37.00 (E)	300.0 (B)
NPKS	15.00 (E)	3.500 (EF)	0.800 (EF)	11.20 (D)	4.900 (GH)	7.200 (H)
M	450.0 (A)	14.00 (C)	13.00 (A)	11.20 (D)	123.0 (B)	111.0 (D)
N	0.950 (F)	0.250 (H)	0.420 (F)	4.900 (GH)	6.900 (G)	94.60 (I)
H	25.00 (D)	32.00 (A)	6.800 (C)	10.60 (DE)	98.33 (C)	164.0 (C)

Different letters in the same column indicate significant difference at $P < 0.01$.

Table 4. Correlative coefficient between microcalorimetric parameters, soil microbial properties and soil microbial community composition of the upland Red soil (0–20-cm layer)

	Enzyme activities						Microbial biomass			No. of CFUs		
	Phosphatase	Urease	Invertase	Protease	Catalase	DHA	C	N	C_{org}	Bacteria	Fungi	Actinomycetes
P_{max}	0.648**	0.548**	0.645**	0.588**	0.539**	0.520**	0.736**	0.746**	0.679**	0.688**	0.661**	0.715**
Q	NS	NS	NS	ns	−0.351*	NS	NS	NS	NS	NS	NS	NS
t_{max}	−0.709**	−0.608**	−0.657**	−0.614**	−0.569**	−0.571**	−0.779**	−0.774**	−0.641**	−0.520**	−0.555**	−0.563**
k	NS	NS	NS	NS	0.404*	NS	NS	NS	NS	0.455**	NS	0.388*

*, **Mean significant at 5% and 1% levels, respectively; NS, nonsignificant ($r_{0.05} = 0.329$, $r_{0.01} = 0.424$, $n = 36$).

relationships were found between this parameter and both dehydrogenase (0.520) and catalase (0.539). P_{max} was also correlated positively ($P < 0.01$) with the number of actinomycetes, bacteria and fungi in soils. It is interesting to note that t_{max} showed significant negative relationships ($P < 0.01$) with the activities of phosphatase (-0.709), invertase (-0.657), dehydrogenase (-0.575) and catalase (-0.569) as well as with microbial biomass C and N (-0.779 and -0.774), the number of culturable bacteria (-0.520), fungi (-0.555) and actinomycetes (-0.563). These findings suggest that an intense soil enzyme activity may enhance the microbial activity in soils and consequently could shorten the t_{max}. This feature is well illustrated on Fig. 1, where organic treatments reduced the values of t_{max} compared with those of mineral fertilizers. The growth rate constant, k, was found to be correlated more with bacteria (0.455 at $P < 0.01$) than with actinomycetes (0.388 at $P < 0.05$) and had no links with fungi. Moreover, k was only linked with the activity of soil catalase (0.404 at $P < 0.05$). This indicates that k can be considered a poor index of soil microbial activity. From different areas in Wuhan (China), a lower correlation was observed between k and the number of bacteria in crop lands, but this connection was found to be higher in uncultivated and nursery lands (Zheng *et al.*, 2007). Observations on the influence of temperature, moisture content, pH and C/N ratios on microbial growth in different soils in Galicia (Spain) showed that microbial growth rate constant (k) is only an apparent value (Núñez-Regueira *et al.*, 2002). These investigators argued that k does not give information about the biochemical activity of the individual microorganisms. However, they showed that, as heat evolution is proportional to the amount of glucose degraded, k can reasonably be considered the specific degradation rate of glucose and may be used as an index to express how fast the material is decomposed by microbial action. The absence of any connection between Q and soil microbial properties except for catalase (-0.351 at $P < 0.05$) implies that Q is not a good indicator of soil microbial properties. It has also been reported that Q was not correlated with soil biomass C and the number of microorganisms because the higher dissipation of the heat per unit of cell is linked to a less efficient metabolism (Barros *et al.*, 2007). Results of microbial biomass C compared with those of calorimetry in three different areas of Sao Paulo (Brazil) did not show the contribution of the total interactive effect of active microbiota in soils. This suggests that soil biomass C only denotes the total amount of C in the soil and does not necessarily reflect activities (Critter *et al.*, 2002). Therefore, we propose that P_{max} and t_{max} could be used as the indices of soil microbial activity, whereas the values of the microbial growth rate constant, k and the total heat evolution, Q are poor indicators.

In the present study, the changes of the microcalorimetric parameters may depend on their sensitivity to the influence of fertilization on soil microbial properties. As compared with mineral fertilizer treatments, the enhancement of the values of P_{max}, k and Q (Fig. 2a and b and data not shown, respectively) in organic treatments can be partially explained by the larger number of microorganisms in the soil (Table 3). The increment of microcalorimetric parameters is also due to a possible stimulation of microbial growth and activity by the organic materials added to mineral fertilizers (here pig manure and straw), which are nutrient and energy sources for microorganisms. A strong increase of heat evolution (Q) was observed upon wetting of predried compost in Israel, probably due to more C and available energy sources provided by the dead biomass and changes in organic matter of compost, respectively (Laor *et al.*, 2004).

Impact of fertilization on soil biochemical and microbial properties

Our results show that manure alone mostly enhanced the number of denitrifying and aerobic N-fixing bacteria by 54.4% and 20.5%, respectively, whereas the fallow increased the number of aerobic cellulose-decomposing bacteria by 31.4% (data not shown). This suggests that manure treatment and fallow probably have higher denitrification potential and basal respiration rates compared with other treatments. It may also demonstrate that in contrast to microbial activity, the type of organic amendment has an important impact on the soil community composition. A comparison of the impacts of 7-year applications of composted pig manure and ammonium nitrate on the structure and activity of the denitrifying community in Rennes, France, showed higher denitrifying activity rate in composted pig manure than in ammonium nitrate fertilizers (Dambreville *et al.*, 2006). The study further revealed that aerobic N-fixing and aerobic cellulose-decomposing bacteria were highly dependent on soil pH and C/N ratio, respectively ($R^2 = 0.848$, $P < 0.01$ and $R^2 = 0.816$, $P < 0.01$), whereas denitrifying bacteria were more related to both total and available soil P ($R^2 = 0.955$, $P < 0.01$). Among factors affecting the composition and activity of bacterial species in soils, pH and organic C content are determinant. In a study conducted near Zurich, Switzerland, about the structure and activity of the nitrate-reducing community, the pH effect was ranked at the second position after the season in the canonical correspondence analysis, accounting for 18% of the variance (Deiglmayr *et al.*, 2004). However, in this work we found that denitrifying bacteria were related to total soil and available P rather than soil pH. This is probably due to the poor availability of P in many Red soils and the competition between plants and soil microorganisms for this limited nutrient. Examining the

effects on soil microbial composition and diversity of long-term organic and chemical fertilizer regimes in a Chinese upland Red soil, Jiangxi Province, He *et al.* (2008) found that phosphorus fertilizer could be considered a key factor in the control of microbial CFUs and diversity in this soil.

In this study, the fallow (H) and soils amended with mineral fertilizers plus pig manure or straw highly stimulated soil microbial properties, whereas the application of inorganic fertilizers reduced the Shannon indices. These results confirm that organic amendments generally enrich the soil bacterial community, promote diversity and keep a more even distribution of bacterial species within the community (Ge *et al.*, 2008). The organic treatments increased both the DGGE band patterns and the richness of bacterial community when compared with plots amended with mineral fertilizers or with the control. It was reported that horse manure significantly altered the structural composition of the bacterial community in Black soil (Mollisol) collected from Harbin (Northeastern China), whereas mineral fertilizers (N, P, K and NPK) increased the diversity of this community (Wei *et al.*, 2008). Conversely, our data showed that inorganic fertilizers reduced the Shannon index as compared with organic treatments. These inconsistencies are perhaps because of differences in soil types and especially in the lower values of the pH and organic matter content of Red soils as compared with Black soils. Fertilizer, soil type, the content of SOC, soil pH, the C/N ratio and P content were the main drivers shaping the microbial communities in this study.

Conclusions

The results reported permit the conclusion that microcalorimetry is a good method to assess the biomass, the number and activity of microorganisms in soils. The study demonstrates that P_{max} and t_{max} quantitatively reflect the influence of fertilization on soil microbial properties much better than do Q and k. Therefore, we propose that P_{max} and t_{max} can be used as indices of microbial activity in soils. This also can be the basis for further studies on the thermodynamics and kinetics of soil enzymes through a combination of the microcalorimetric method and other analytical techniques, focusing on accurate design and careful interpretation. These further studies are fundamental in improving our knowledge of soil microbial ecology.

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