Manure substitution of mineral fertilizers increased functional stability through changing structure and physiology of microbial communities

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Abstract:

Soil function, such as decomposition of organic materials, is of crucial importance to sustain soil fertility and may be enhanced through soil management. We hypothesized that manure amendment would increase soil functional stability more effectively than mineral fertilization when soil nutrients were not limited. By using a 22-yr field experiment, the objectives were 1) to determine the effects of manure substitution and reduction of mineral fertilizers on soil physio-chemical properties, soil microbial community structure, and soil biological functional stability; 2) to isolate the effects of organic amendment from those of mineral fertilization on soil biologic functional stability; and 3) to elucidate the controlling mechanisms on the soil functional stability. Soils were sampled from the field treatments, no fertilization (CK), mineral N, P and K (NPK), two doses of NPK (2NPK), manure amendment (OM) and OM in combination with NPK (NPK+OM). The nutrient inputs were similar in treatments OM and 2NPK. The functional stability was quantified by measuring the decomposition rate of crop litter added to the soils following Cu addition and heating. Soil nutrients, organic carbon and pH increased due to mineral fertilization and organic amendment. The principal component analysis of phospholipid fatty acid (PLFA) profiles demonstrated that the structure of soil microbial communities shifted between the mineral-fertilized soils and manure-amended soils and the shifts were not due to nutrient limitation because the soil microbial communities were not separated between the treatments of NPK and 2NPK. The manure amendment enhanced the resistance and resilience to Cu and heating more than the mineral fertilization, to a larger extent in treatment NPK+OM than in treatment OM. The resistance and resilience to Cu addition was positively correlated with soil organic matter, soil aggregate stability, while only the resistance to heating was positively correlated to soil aggregate stability. Moreover, the resistance and resilience were correlated with the shifts of functional and physiological structure of soil microbial communities due to long-term manure amendment and mineral

fertilization. In conclusion, the partial substitution of mineral fertilizers with manure (NPK+OM) increased soil functional stability to heavy metal pollution and global warming through altered structure and physiology of soil microbial communities due to improved soil aggregation with higher soil organic matter.

Keywords: Soil microbial resilience; Microbial community structure; Soil aggregation; Soil organic matter; Organic amendment; Fertilization.

1. Introduction

Healthy soils are intrinsically able to adapt to either natural or anthropogenic perturbations in order to deliver soil functions for sustaining ecosystem goods and services [1]. The ability is defined as functional stability and comprises both resistance and resilience [2, 3]. Soil microbes play a central role in determining soil functions such as soil organic matter decomposition, nutrient transformation and soil structure formation and stabilization. Therefore, soil biological stability is quantified by measuring changes in the short-term microbial functional groups or specific functions in response to experimental perturbation to understand the controlling mechanisms and the effects of land use and soil management [4]. Soil biological stability is related to many soil properties, among which soil organic matter and soil aggregation are most important soil properties and to a less extent clay and soil pH [5-9]. As a consequence, soil management can influence soil biological stability as being observed using paired long-term experiments [8, 10, 11]. Mineral fertilization has greatly contributed to crop production and food security, but excessive mineral fertilization leads to decreasing on soil quality and environmental pollution. Organic amendment and substitution of mineral fertilizers are increasingly recommended for the development of sustainable agriculture to sustain crop yield and soil quality while minimizing environmental issues [12, 13]. However, to our knowledge there is only one study which reported that the microbial functional stability of a tropical sandy clay loam soil to heat was enhanced most effectively in the treatment of 36-y application of farmyard manure with mineral N, P and K compared with the treatments of mineral fertilization only and no fertilization [11]. Meanwhile, the effects of organic amendment have not been isolated and the controlling factors are largely unknown.

Soil biological stability is hypothesized to be primarily generated by inherent diversity of soil microbial communities with functional redundancy (the insurance hypothesis) [14] or by soil resource heterogeneity (the resource heterogeneity hypothesis) [15]. Both hypotheses

have been intensively validated, but no consensus has been reached [3, 10, 16-18]. Griffiths et al., [5] proposed soil biological stability is likely governed by the physio-chemical structure of the soil through its effect on microbial community composition and microbial physiology. Many long-term studies have demonstrated that soil microbial communities changed under mineral fertilization and organic amendment [7, 19-22]. Since the growth of soil microorganisms is mainly limited by C or N [23] and also sensitive to application of P and K [24], the factors controlling the shifts of soil microbial communities may be then different between organic amended soils and mineral-fertilized soils. Therefore, understanding the controlling factors is critical to isolate the effects of organic amendment and mineral fertilization on soil biological stability.

Soil microbial communities may change in different mechanisms between long-term mineral fertilization and organic amendment. Long-term mineral fertilization changes soil pH and nutrient environments and then influences soil microbial communities [20, 25]. For example, long-term application of mineral N in excess can reduce soil pH, to which soil bacteria are more sensitive to change than fungi and actinomycetes [19, 26]. In addition, long-term mineral fertilization can increase soil organic matter due to increased crop production and crop litter inputs [27]. Thus soil microbial communities may shift with the increase in soil organic matter and its effect on soil water and temperature and soil aggregation [21, 28-30]. Therefore, if soil pH, soil nutrient and soil organic matter do not change with increasing amounts of mineral fertilizers in combination, soil microbial communities may not change. In contrast, long-term application of organic materials, e.g. crop straw and manure, may favor the growth of cellulolytic microorganisms, such as fungi, in relation to those that were not able to degrade cellulose [20, 31, 32]. Therefore, it is expected that the quality and quantity of soil organic matter will increase with increasing amount of organic amendments, leading to continuous shifts in soil microbial communities. In such conditions, the effects of organic

amendment and mineral fertilization on soil biological stability can be isolated and the redundancy and resource heterogeneity hypothesis of functional stability can be tested.

In this study, we aimed to isolate the effects of manure amendment on soil biological functional stability from those of mineral fertilization by using a 22-yr field experiment. The mineral N, P and K fertilizers were applied in combination and the nutrient inputs were not less than under manure amendment than under mineral fertilization to avoid the effects due to nutrient limits. The specific objectives were 1) to determine the effects of manure substitution and reduction of mineral fertilizers on soil physio-chemical properties, soil microbial community structure, and soil biological functional stability; 2) to isolate the effects of organic amendment from those of mineral fertilization on soil biologic functional stability; and 3) to elucidate the controlling mechanisms on the soil functional stability. The aboveground biomass was removed to eliminate the additional effects. We hypothesized that manure amendment would increase functional stability compared with balanced mineral fertilization and soil organic matter would play a crucial role in controlling soil microbial communities and functional stability.

2. Materials and methods

2.1. Study site description

The research site was located at the Institute of Red Soil, Jiangxi Province, China (116°26' E, 28°37' N, and about 26 m above sea level). The area has a typical sub-tropical humid climate, with mean annual temperature of 17.5°C, ranging from 5.5°C in January to 29.9°C in July. Annual rainfall averaged 1727 mm with majority of the precipitation falls between March to July. The soil was derived from Quaternary red clay and classified as Ultisol according to USDA soil taxonomy [33].

The long-term fertilization experiment was established in 1986 to determine the

influence of the increasing use of mineral fertilizers on crop yield and soil properties compared with the traditional agriculture with only manure amendment. The 2NPK treatment represented the conventional agriculture with high inputs of mineral fertilizers in balance, while the treatments of NPK, OM and NPK+OM represented those alternative models of manure substitution and reduction of mineral fertilizers.

The field experiment was arranged following a randomized complete block design with three replicates. Each experimental plot was 22.22 m². Five treatments from the experiment were selected for this study considering different nutrient inputs (Table 1). The treatments were no fertilization control (CK), application of mineral N, P and K fertilizers (NPK) and that with double doses of NPK rates (2NPK), organic manure alone (OM), and NPK plus organic manure (NPK+OM). The amounts of N, P and K fertilizers in NPK were adopted by local farmers in 1980s and were projected to be doubled for high yield since then. The amount of N input fixed to be equivalent between OM and 2NPK though the measured value was slightly different. The mineral fertilizers used were urea, calcium magnesium phosphate and potassium chloride and the application rate was 60, 13 and 50 kg ha⁻¹ for N, P and K at each growing season, respectively. The organic manure was pig manure applied at 15.0 t ha⁻¹ y⁻¹ (dry weight), which had 23.9% of total C, 2.0% of total N, 0.7% of total P and 0.7% of total K. Two thirds of inorganic N fertilizer, all P and K fertilizers as well as pig manure were applied as basal fertilization, and the rest of N fertilizer was applied as top dressing. The cropping system was double maize (*Zea mays* L.) cropping with winter fallow each year. Approximately 37.8% and 14.4% of annual rainfall occurred for the first (April to July) and second maize growing season (July to October), respectively, and irrigation was applied if necessary.

2.2. Soil sampling and analysis

Surface soils (0-20 cm) were collected at five random points in each plot to make a

composite sample in March, 2008. The soil samples were immediately shipped to the laboratory and temporally stored at 4°C before the preparation for the incubation experiment and soil analysis. The soil samples were gently destructed to pass through a 4-mm mesh after removing visible roots and organic debris for the incubation experiment. Part of the soils was air-dried and further sieved through a 2-mm mesh for chemical analysis. Part of the non-destructed aggregates (2-4 mm) were dried at 40°C for 24 hr and used to measure aggregate size distribution by the wet sieving method and calculating the mean weight diameter. Soil pH was measured by a glass/calomel electrode (Model 206-C, Shanghai San-Xin Instrument Co. China) at a soil: water ratio of 1:2.5 after 1 h end-over-end shaking. Soil organic carbon was determined using Walkley-Black method [34] and total N with micro Kjeldahl digestion. Alkali hydrolysable N was determined following standard procedure, total P and available P following the molybdenum-blue method, and total K and available K with flame photometry [35]. Soil microbial biomass C (SMB-C) and N (SMB-N) were measured by fumigation-extraction [36] and it was calculated using a K_C factor of 0.38 and a K_N factor of 0.45, respectively [37].

2.3. Microbial community structure

Soil microbial community structure was assessed by determining the profiles of phospholipid fatty acids (PLFA) and the community-level physiological profiles (CLPP). PLFAs were measured following the modification of method described by Bligh and Dyer [38] and Bossio et al. [39]. Briefly, 2 g of fresh soils was extracted in a 2:1:0.8 solution of methanol, chloroform and phosphate buffer, and phospholipid was subsequently separated by silicic acid bonded solid-phase-extraction columns. Phospholipid was saponified and methylated to fatty-acid methyl esters (FAME), which were analyzed by MIDI Sherlock Microbial Identification System (MIDI Neward, DE, USA). Concentration of individual PLFAs was obtained by comparing peak area with that of the internal standard (C19:0

FAME). Signature PLFAs were used as indicators for specific microbial groups. Phospholipid fatty acids were grouped according to the nomenclature proposed in the literature [40].

The community-level physiological profiles were assessed by using Biolog Eco-microplates with 31 C substrates (Biolog, Inc., Hayward, CA, USA) after minor modification of the method of Garland [41]. Three replicates of 10-g soil were suspended in 100 mL sterile phosphate buffer solution (0.05 M K_2HPO_4/KH_2PO_4 , pH 7.0) and shaken for 30 min. Then, one mL soil suspension solution was subjected to two series of 10-fold dilution with phosphate buffer solution, 150- μ L diluted suspension was added in each well in the microplates using 8-channel repeating pipette. The microplates were incubated at 25°C in dark and the absorbance at 590 nm was recorded using Microlog Rel 4.2 software (Biolog, Inc., Hayward, CA, USA) between 24 to 192 h at 12 h interval. The reading at 96th hour was suggested to represent approximately 50% saturation which would show the greatest difference among treatments [41]. Therefore, it was used to calculate mean well color development (AWCD), Shannon index (H'), Simpson index (D') and Evenness index (E') according to Insam and Goberna [42].

2.4. Soil functional stability

Soil functional stability was determined by measuring CO_2 emission in a 56-day incubation experiment following the procedures described by Griffiths et al. [17] and Zhang et al. [29]. Pre-incubation was performed for 7 d at 40% of field water-holding capacity (WHC) and 28°C to reactivate the microbial activity. This pre-incubation was carried out to reduce the priming effect of soil organic matter decomposition. Briefly, 20 g soil (dry weight) was mixed with peanut (*Arachis hypogaea* L.) leaf powder (C/N ratio 20:1) at 10 g kg^{-1} soil after pre-incubation, and short-term decomposition rate of added peanut leaf at sampling days was measured over 56 days after subjecting soil to either a transient heat perturbation (50 °C for 24 h) or a persistent copper perturbation (500 mg Cu g^{-1} dry soil)

using CuSO₄ solution or no perturbation as control. The perturbations represent soil pollution by heavy metal or warming due to global change or in greenhouse. The foreign organic matter rather than indigenous pine litter was amended to avoid the home-field advantage [43] or the effect of resource use history [44]. Copper addition and heating may cause persistent and transient stresses, respectively, which are increasingly compounded by anthropogenic soil pollution and global warming. Immediately after pre-incubation, soils in the control treatment were put into the 240-mL glass jars and incubated at 28°C in dark. For the perturbation treatments, the soils were transferred into glass jars after stresses and incubated in dark as in the control. In total, 270 jars were prepared to ensure destructive sampling at days 1, 3, 7, 14, 28 and 56. For each measurement, three jars for each treatment were randomly selected, and 20 mL gas was sampled from the headspace by using syringe to determine CO₂ concentration by gas chromatography (Shimadzu GC-14B, Japan) equipped with a thermal conductivity detector. The standards of CO₂ were supplied by the BOC Inc (Shanghai, China).

The relative decomposition rate ($f(t)$, %) of perturbation treatment relative to control was calculated using equation (1) described by Zhang et al. [7]. Integrative resistance (IR) and integrative resilience (IS) were calculated using equation (2) and (3), respectively.

$$f(t) = \frac{G_{as_{stressed}}(t)}{G_{as_{control}}(t)} * 100 \quad (1)$$

$$IR = \int_0^i f(t)dt/i \quad (2)$$

$$IS = \int_0^{56} f(t)dt/(56 - i) \quad (3)$$

where, the relative CO₂ emission rate was lowest at the i^{th} day.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) was performed to determine the effects of field treatments on soil properties using SPSS (SPSS for Window©, version 13.0, Chicago, IL, USA). Three-way ANOVA was performed to determine the effects of field treatments, perturbation type and incubation time on decomposition rate and relative decomposition rate.

One-way ANOVA was performed to determine the effects of field treatments on IR and IS. Multiple comparisons were used to determine the significant difference between treatments at $P < 0.05$, unless otherwise noted. Correlation between quantitative descriptors was performed by applying the Pearson correlation matrix.

A total of 50 PLFAs were detected, and 42 were of microbial origin. A correlation-based principal component analysis (PCA) of the profiles of 42 PLFAs expressed in mole percentage and mean AWCD at 96th h were applied to determine the effect of field treatments on soil microbial community structure, with ANOVA applied to principal component scores to assess the significance of treatment effects. PCA was interpreted graphically by constructing biplots, with factor scores to specify the loadings of individual PLFAs or substrates on the principal components. The soil properties were included as a supplementary variable to reflect its relations with individual PLFAs or carbon substrate groups.

3. Results

3.1. Soil physical and chemical properties

The maize grain yield was highest in 2NPK and NPK+OM, followed by NPK and OM and lowest in the no fertilization control (CK) (Table 1). There were no differences between NPK+OM and 2NPK and between NPK and OM ($P < 0.05$). The organic amendment and fertilization significantly ($P < 0.05$) increased most of the selected soil properties compared to the control CK, with a few exceptions (Table 1). There were no differences in soil pH, and the concentrations of SOC and total N and P between the treatments of NPK and CK. However, the aggregate stability was lower in treatment NPK than in treatment CK. Most of the soil properties increased in treatment 2NPK than in treatment NPK. There were no differences in aggregate stability, and the concentrations of total P and total K between the treatments of 2NPK and NPK. Only hydrolysable N and extractable P concentration were higher in

NPK+OM than in OM. The manure-amended soils (OM and NPK+OM) were higher than the mineral-fertilized soils (NPK and 2NPK) in most of the soil properties. The total K content was higher in the soils with mineral fertilization (NPK, 2NPK and NPK+OM) than in treatment OM. The extractable K content was higher in treatments NPK+OM and 2NPK than in treatments OM and NPK. The hydrolysable N was highest in treatment NPK+OM.

3.2. Soil microbial properties

The total soil microbial biomass was affected by the field treatment in total PFLAs than that measured using fumigation extraction method (SMB-C) (Table 2). Compared to the control CK, the total microbial biomass and the biomass of total bacteria, G(-) bacteria and fungi increased to a larger extent due to organic amendment than due to the mineral fertilization. The SMB-C concentration was not significant ($P < 0.05$) between treatments NPK and 2NPK and between treatments OM and NPK+OM. The concentration of total PLFAs was greater in treatment OM than in treatment NPK+OM, and in treatment 2NPK than in treatment NPK. The G(-) bacterial and fungal biomass were not different between treatments NPK and 2NPK, while the fungal biomass was not different between treatments OM and NPK+OM. The total bacterial biomass was greater, while the G(-) bacterial biomass was lower in treatment NPK+OM in treatment OM. The G(+) bacterial biomass was the greatest in treatment OM, followed by treatment 2NPK, then by treatments NPK and NPK+OM, and the lowest in CK. The actinomycetes biomass was larger in treatments 2NPK and OM than treatments NPK+OM and NPK. The ratio of G(+) bacteria to G(-) bacteria was greater in the mineral-fertilized soils than in the manure-amended soils. The ratio of fungi to bacteria was greater in treatment NPK than in treatments OM and NPK+OM than in treatment 2NPK.

The effects of field treatments were larger on mean AWCD than on functional diversity (Table 3). Compared to the control CK, the mean AWCD increased, to a larger

extent due to organic amendment than due to the mineral fertilization. There was no significant difference between treatments 2NPK and OM. Compared to the control CK, the functional diversity indices increased, however, to a less extent due to organic amendment than due to the mineral fertilization. The differences were significant between treatments 2NPK and NPK+OM in Shannon-Weiner index (H'), between treatments NPK+OM and NPK or 2NPK in Simpson index (D'), and between treatments 2NPK and OM or NPK+OM in Evenness index (E').

3.3. Soil microbial community structure

Forty-two microbial originated PLFAs were subjected to principle component analysis (PCA). The first two components, PC1 and PC2 explained 59.8% and 22.6% of the total variance (Fig. 1). All the treatments lay in the diagonal across the second and the fourth quadrant, with the PC1 score decreasing and the PC2 score increasing from treatment CK, through the mineral-fertilized soils to the manure-amended soils (Fig. 1a). The treatments OM and NPK+OM were largely separated from other three treatments PC1. There were no separations between OM and NPK+OM and among CK, NPK and 2NPK except between NPK and CK via PC1. Whereas all the treatments were separated from each other except between NPK and 2NPK via PC2. The separations were driven by G(-) bacteria (cy 19:0, 16:1 2OH), G(+) bacteria (i16:0) and prokaryotes (15:0; 16:0) associated with CK and by G(-) bacteria (20:1 ω 9c, 18:1 ω 7c), G(+) bacteria (a15:0), fungi (18:1 ω 9c, 18:2 ω 6c,9c) and actinomycetes (10Me18:0) associated with manure-amended soils (Fig. 1b).

The community-level physiological profiles (CLPP) using Biolog Eco-plates were subjected to principal component analysis. The first two components, PC1 and PC2 explained 50.6% and 29.3% of the total variance (Fig. 2). All the treatments were separated from each other via PC1 and PC2, with exceptions between treatments 2NPK and OM via PC1 and between CK and NPK+OM via PC2. The organic amendment treatments (OM, NPK+OM)

were associated with all substrates used except for phenolics, the selected soil properties, G(-) bacteria and fungi were with treatments OM and NPK+OM on the side of the most positive PC1 score. In contrast, the CK and NPK treatment was associated with G(+) bacteria, non-special bacteria and actinomycetes on the side of the most negative PC1 score. The phenolics, polymers and G(+) bacteria were associated with NPK and 2NPK on the side of most positive PC2 core, while the amines, amino acids, carbohydrates and G(-) bacteria were associated with treatments CK and NPK+OM on the side of the most negative PC2 score.

3.4. Decomposition rate and soil functional stability

The dynamics of decomposition rate of amended crop residue was affected by field treatment and perturbation type (Fig. 3). The decomposition rate decreased slowly for 3 or 7 days after the beginning of the incubation and then increased in all control soils, while it decreased largely with one day after application of the stress and then recovered the stressed soils. The magnitudes of the decreases were larger following Cu addition than heating and the magnitudes of the recovery were reversely larger following heating than Cu addition. The initial and final decomposition rates were larger in treatments NPK+OM and OM than in treatments 2NPK and NPK and the lowest in the control CK in all perturbation types.

The decomposition rate of the stressed soil relative to that of the unstressed control demonstrated different responses of the soils following Cu addition and heating (Fig. 4). The relative decomposition rate decreased to 40-75% of the control soils one day after Cu addition and recovered to 50-80% after the 56-d incubation, with the magnitude of the changes smaller in the manure-amended soils than in other soils (Fig. 4). The relative decomposition rate decreased to 35-45% of the control soils one day after heating in the treatments of CK, NPK and 2NPK and to 10-20% of the control soils in the treatments of OM and NPK+OM with one week after heating. Thereafter, the relative decomposition rate recovered slightly.

The integrative resistance (IR) and resilience (IS) were significantly affected by field

treatment and perturbation type (Table 4 and Table 5). The integrative resistance (*IR*) to Cu and heating perturbations were greater in the treatments of OM and NPK+OM than in other treatments, with no difference between treatments CK and NPK (Table 5). The integrative resilience (*IS*) to Cu perturbation was greatest in OM and NPK+OM, followed by 2NPK, and the lowest in treatments NPK and CK. Whereas, the integrative resilience to heating was greatest in treatment OM, followed by treatment NPK+OM and then treatments of NPK and CK, and the lowest in 2NPK.

3.5. Correlations

All soil properties and microbial community indices except for extractable K, SBM-N, fungal biomass and PC2 of PCA based CLPP data were correlated with the resistance or resilience following Cu and heating perturbations (Table 6). The functional diversity indices were correlated only with the resilience following Cu addition. The correlations were positive for all soil properties and microbial community indices except for bacterial biomass, G(+) bacterial biomass and PC2 of PCA of PLFA profiles. The correlations were consistent for both the resistance and the resilience following Cu addition. Following heating, only the resistance was correlated with MWD, soil pH and Olsen P. Both the resistance and resilience were not correlated with SOC, total N, hydrolysable N, PC2 of PCA of PLFA profiles and PC1 and PC2 of PCA of CLPP profiles.

4. Discussion

The manure amendment and mineral fertilization were designed to ensure balanced supply of N, P and K nutrients (Table 1). Although the total nutrient inputs were about 1.7-2.4 fold more in N and 2.7-4.2 fold more in P in the manure-amended soils, the crop yield was not higher in treatment NPK+OM compared to treatment 2NPK, and in treatment OM compared to treatment NPK. These results indicate that N and P were not limiting nutrients

for the crop yield or the excess nutrients from the organic manure did not match nutrient demands by maize crop [45]. The lower K application and lower extractable K concentration in the manure amendment treatments (Table 1) suggested that K would be a limiting factor for a higher yield in the manure-amended soils than in the mineral-fertilized soils. The resistance and resilience to Cu addition and heating increased in treatments NPK+OM and OM and was not different in treatment NPK compared with treatment 2NPK (Table 5). The microbial functional stability in CK was significantly lower than that in treatment 2NPK, but was not different from those in treatment NPK except for higher resistance to heating than in treatment NPK. These results highlighted the importance of either soil organic matter or manure amendment in promoting microbial functional stability against environmental perturbations as demonstrated in previous studies using long-term field experiments [8,11]. Moreover, our results clarified that the functional stability was enhanced larger by organic amendment in combination with mineral fertilizers compared to mineral fertilization or manure application only

The PCA analysis of PLFAs demonstrated that the soil microbial community structure shifted from the control (Fig. 1). Since the microbial community structure was not different between the treatments of NPK and 2NPK, the shifts in the microbial community structure between the manure-amended soils and the mineral-fertilized could not be attributed to nutrient limits despite of the lowest nutrient concentration in treatment NPK among the fertilized soils (Table 1). Therefore, only soil aggregate stability, soil organic matter and soil pH can explain the shifts in the structure of soil microbial communities, with fungi, actinomycetes and G(-) bacteria associated with the manure-amended soils and different G(-) and (+) bacteria associated with the mineral-fertilized soils and the control (Fig. 1). This was consistent with previous study [46]. Because the microbial community structure was not different between treatments NPK and 2NPK as expected, comparing the treatments of NPK

and 2NPK supported the soil resource heterogeneity hypothesis. However, all the soil properties measured were not different between in treatment NPK than in treatment 2NPK, and so no soil properties affecting soil resource heterogeneity could be identified, indicating the failure of this hypothesis. The PCA of CLPP profiles demonstrated that treatment 2NPK was separated from the treatments of NPK, OM and NPK+OM, with treatment 2NPK associated with G(+) bacteria and phenolics (Fig. 2). This was also in contrast with the negative correlation between the resilience to heating with G(+) bacterial biomass (Table 6). Therefore, the lower resilience to heating in treatment 2NPK than in treatment NPK could be attributed to the physiological differences of specific communities although the soil microbial communities were similar. The larger biomass of G(+) bacteria in treatment 2NPK than in treatments NPK and NPK+OM was likely related to the lower resilience to heating, indicating that G(+) bacteria preferentially using phenolics than other substrates were likely less resistant to heating. Because there are large variations not only in the structure of soil microbial communities, but also in soil properties between the soils, the microbial redundancy hypothesis could not be separated from the resource heterogeneity hypothesis by using this long-term experiment.

The relative decomposition was more largely reduced and recovered following Cu addition than following heating in all soils (Fig. 4). This is inconsistent with the previous studies under the similar perturbation condition [7, 17, 18, 29, 47], which has been attributed to the nature of the stresses and the adaptation of soil microbial communities to the stresses. Different soil microbial communities have different strategy to survive from different perturbations [49]. Therefore, soil microbial communities change differently after Cu addition [7, 50, 51] and heating [7, 18]. Zhang et al. [29] suggested that the transience of heating would cause non-selective death of microorganisms, while the persistency of the added Cu would cause selective death of microorganisms [29]. Griffiths et al. [18] postulated that the lack of

resilience to copper perturbation was due to altered physiological tolerance rather than changed microbial community. In addition, the soil microbial communities in the treatments likely have experienced high temperature stress and adapted to the hot and dry summer (with an average air temperature of 29.9°C in July). Therefore the microbial communities were not sensitive to heating at 50°C and then showed litter recovery in the experiment. However, they received litter Cu pollution even in the field treatments with manure application as the manure was from a local farmer. Therefore, the microbial communities recovered relative quickly from the Cu stress.

The microbial functional stability of the treatments of 2NPK, OM and OM+NPK were larger following Cu addition than following heating, while the resilience of the treatments of NPK and CK was larger following heating than following Cu addition (Table 5). These results of the treatments of 2NPK, OM and OM+NPK are inconsistent with the previous studies except for the manure-amended studies [7, 17, 29, 47, 48]. The resistance and resilience to Cu addition were correlated with soil organic matter and aggregate stability, while only the resistance to heating was correlated with aggregate stability (Table 6). These results indicate that soil organic matter and aggregation affected differently the microbial functional stability following different perturbations [52]. Soil organic matter can protect heat- and Cu-resistant microbes through soil aggregation and retaining more water [53, 54] and then affect the resistance to these stresses [7, 8, 11]. Moreover, soil organic matter can protect Cu-resistant microbes due to reduced bioavailability [6]. Furthermore, soil organic matter also affects the resilience to Cu addition through the effects on the physiological adaption or changes in microbial communities with time [5]. However, soil organic matter had little impact on the resilience to heating due to its transient characteristics and stress history. The soils from the subtropical region in this study and our previous studies [7, 29] might have experienced temperature stress in the summer. The soils with low soil organic

matter may have been more stressed in the field and then was not sensitive to the temperature applied (50°C), resulting in higher resistance following heating than following Cu addition in these soils. The larger microbial functional stability following heating than following Cu addition [7, 17, 29, 47, 48] were possibly because those soils had much higher SOM and not experienced heating before, while the soils in the study had relative lower SOM and had experienced heating in the summer. The contrasting results of the treatments of NPK and OK compared to other soils on the resilience further suggest the effects of soil organic matter on soil microbial functional stability to different perturbation type and stresses in history. In addition, our studies suggested that repeated application of manure, particularly in combination with mineral fertilizers can enhance the microbial resistance and resilience to Cu stress as indicated in other studies for other soil perturbation such as soil disinfection using chloropicrin and sodium methyl dithiocarbamate [51, 55]

5. Conclusion

The 22-yr field experiment demonstrated that the partial or total substitution of mineral fertilizers with manure had little effect on crop yield increase possibly due to limited N and K supply. The mineral fertilization only had little influence on microbial activity and soil functional stability compared with no fertilization, while the manure substitution increased the resistance and resilience to Cu addition and heating more than the mineral fertilization, to a larger extent in treatment NPM+OM than in treatment OM. The biological resistance and resilience to Cu addition was determined by the functional structure of soil microbial communities mediated by soil organic matter and soil aggregation, while the resistance to heating was determined by physiological structure of soil microbial communities mediated by soil aggregation during the long-term soil management. Therefore, organic amendment is more sustainable than mineral fertilization to environmental changes such as heavy metal

pollution and global warming.

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Figure captions

Figure 1. Biplots of the first two principal components (PC1 and PC2) of PLFA profiles in the soils from different field treatments (a), and the loading of individual PLFAs to PC1 and PC2 (b). Bars represent the least significant differences at $P < 0.05$ ($N = 3$).

Figure 2. Biplots of the first two principal components (PC1 and PC2) of AWCD data at 96th hour in the soils from different field treatments (a), and the loading of specific microbial groups, soil properties and carbon substrate groups to PC1 and PC2 (b). Bars represent the least significant differences at $P < 0.05$ ($N = 3$).

Figure 3. Decomposition rate during the incubation after addition of crop straw in the soils from different field treatments subjected to no perturbation (a), Cu (b) and heating (c). Bars represent standard errors ($N = 3$).

Figure 4. Relative decomposition rate (%) during the incubation after addition of crop straw for the soils from different field treatments after being perturbed with Cu (a) and heating (b) compared to the un-perturbed control soils. Bars represent standard errors ($N = 3$).

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655 Table 1. Fertilizer application rate, yearly average crop yield and selected physical, chemical properties of soils taken in 2008 under different
656 fertilization treatments.

Treatment ^a	Fertilization rate and				Average crop yield ^c	Soil physical and chemical properties ^c								
	carbon input ^b					MWD ^d	pH	SOC	Total N	Total P	Total K	Hydrolysable	Olsen	Extractable
	N	P	K	C								N	P	K
	----- kg ha ⁻¹ yr ⁻¹ -----					mm	----- g kg ⁻¹ -----			----- mg kg ⁻¹ -----				
Initial soil	-	-	-	-	-		6.0	9.4	1.0	1.4	15.7	60.3	12.9	102
CK	0	0	0	147	1750 c	1.80 b	4.9 c	7.8 c	0.9 c	0.5 c	12.2 b	52.6 d	18.2 d	103.5 c
NPK	120	26	100	603	7178 b	1.71 c	4.9 c	8.0 c	1.0 c	0.6 bc	12.7 a	78.6 c	37.0 c	198.7 b
2NPK	240	52	199	816	9713 a	1.78 bc	5.4 b	9.5 b	1.3 ab	0.7 b	12.9 a	119.4 b	63.3 b	283.2 a
OM	298	108	103	4159	6895 b	1.91 a	6.5 a	10.8 a	1.3 ab	1.4 a	12.1 b	120.8 b	152.0 a	217.6 b
NPK+OM	418	134	202	4374	9452 a	1.98 a	6.3 a	11.3 a	1.4 a	1.5 a	12.5 ab	134.7 a	187.5 a	269.4 a

657 ^a Initial soil represents soil sample taken in 1986.

658 ^b The C input calculated is based on the C concentration of maize root (442 g C kg⁻¹ residue) and the ratio of maize root to crop yield of 0.19,
659 assuming that all the above-ground residue was removed.

660 ^c Different letters in the same column indicate significant differences at $P < 0.05$ (N = 5).

661 ^d MWD refers to mean weight diameter.

Table 2. Concentrations of soil microbial biomass C (SMB-C) and N (SMB-N), total PLFAs and microbial subgroups of PLFAs and the ratios between G(+) to G(-) bacterial PLFAs and between fungal to bacterial PLFAs in the soils under different fertilization treatments.

Treatment	SMB-C mg kg ⁻¹	SMB-N mg kg ⁻¹	PLFAs, nmol g ⁻¹					G(+) /G(-)	Fungi / Bacteria
			Total	Total bacteria	G(+) bacteria	G(-) bacteria	Fungi Actinomycetes		
CK	462.4	45.7	187.				10.3	1.1	
	c	cd	9 e	85.8 e	37.0 d	33.4 d	c	b	0.12 d
NPK	507.0	43.9	282.				39.1	1.3	
	b	d	8 d	124.2 d	61.8 c	47.5 c	b	a	0.32 a
2NPK	542.6	51.9	314.				35.8	1.3	
	b	b	7 c	163.4 c	69.5 b	51.8 c	b	a	0.22 c
OM	670.3	47.2	407.				59.4	0.8	
	a	c	6 a	192.3 b	73.2 a	87.7 a	a	c	0.31 ab
NPK+O M	701.0	56.7	371.				62.5	0.8	
	a	a	5 b	210.5 a	60.2 c	77.0 b	a	c	0.30 b

Different letters indicate significant differences among treatments at *P* < 0.05.

667 Table 3. Mean of average well-color development (AWCD) and functional diversity
 668 (Shannon-Weiner index (H'), Simpson index (D') and Evenness index (E') calculated based
 669 on carbon substrate utilization in Biolog Eco-microplates at 96th hour of incubation in the
 670 soils under different fertilization treatments.

Treatment	Mean AWCD	Functional diversity		
		H'	D'	E'
CK	0.27 d	2.75 c	0.928 c	0.84 b
NPK	0.42 c	2.88 ab	0.937 b	0.86 ab
2NPK	0.52 bc	2.83 b	0.935 b	0.85 b
OM	0.56 b	2.90 ab	0.939 ab	0.88 a
NPK+OM	0.66 a	2.96 a	0.943 a	0.88 a

671 Different letters indicate significant differences among the treatments at $P < 0.05$.

672
 673
 674

675 Table 4. Analysis of variation (ANOVA) of the effects of field treatment, perturbation type
676 and incubation time on soil functional stability (decomposition rate, relative decomposition
677 rate ($f(t)$), integrative resistance (IR) and integrative resilience (IS) among the fertilization
678 treatments.

Variation	Decomposition rate	$f(t)$	IR	IS
Field treatments (F)	***	***	*	*
Perturbations (P)	***	***	*	*
Time (T)	***	***	NA	NA
F×P	**	***	*	*
F×T	*	*	NA	NA
P×T	***	**	NA	NA
F×P×T	***	***	NA	NA

679 NA, not applicable.

680 *, **, *** indicate significant difference at $P < 0.05$, 0.01 and 0.001, respectively.

681

Table 5. Average decomposition and integrative resistance (*IR*) and resilience (*IS*) to Cu addition and heating of the soils under different fertilization treatments.

Treatment	Average decomposition (mg-C kg ⁻¹ d ⁻¹)	Cu addition		Heating	
		<i>IR</i> _{Cu}	<i>IS</i> _{Cu}	<i>IR</i> _{heat}	<i>IS</i> _{heat}
CK	257.0 c	64.5 c	56.1 d	64.5 c	68.8 c
NPK	348.8 b	64.8 c	58.7 cd	58.7 d	66.5 cd
2NPK	358.4 b	70.1 b	61.6 c	53.0 de	49.3 e
OM	609.8 a	83.9 a	76.8 ab	99.0 a	94.4 a
NPK+OM	616.0 a	83.2 a	79.7 a	93.1 b	89.3 b

Different letters indicate significant differences among the treatments at $P < 0.05$.

Table 6. Correlation coefficient matrix of soil functional stability indices (integrative resistance (*IR*) and integrative resilience (*IS*)) following Cu and heating perturbations in relation to soil properties, microbial community structure and principle component (PC) scores from the principle component analysis (PCA) of PLFA and CLPP profiles.

Soil property	Functional stability			
	<i>IR</i> - <i>Cu</i>	<i>IS</i> - <i>Cu</i>	<i>IR</i> - <i>heat</i>	<i>IS</i> - <i>heat</i>
MWD	0.91*	0.92*	0.89*	0.79
pH	1.00 ***	0.97 ***	0.89 *	0.75
SOC	0.97 ***	0.96 ***	0.79	0.63
Total N	0.87 *	0.87 *	0.60	0.41
Total P	0.99 ***	1.00 ***	0.93 **	0.84 *
Hydrolysable N	0.83 *	0.83 *	0.54	0.35
Olsen P	0.98 ***	1.00 ***	0.88 **	0.77
Extractable K	0.54	0.55	0.16	-0.02
SMB-C	0.98 ***	1.00 ***	0.87 *	0.76
SMB-N	0.60	0.63	0.34	0.17
Total PLFAs	0.90 **	0.88 **	0.72	0.59
Fungal PLFAs	0.71	0.78	0.56	0.51
Bacterial PLFAs	-0.90 **	-0.95 **	-0.89 **	-0.86 *
G(+) bacterial PLFAs	-0.82 *	-0.84 *	-0.92 **	-0.89 **
G(-) bacterial PLFAs	0.91 **	0.90 **	1.00 ***	0.96 ***
Actinomycetous PLFAs	-0.71	-0.80	-0.68	-0.69
Shannon index	0.76	0.83 *	0.65	0.61
Simpson index	0.78	0.84 *	0.63	0.56
Evenness index	0.90 **	0.93 **	0.86 *	0.81 *
PC1-PLFA	-0.96 **	-0.99 ***	-0.94 **	-0.88 *
PC2-PLFA	0.88 *	0.94 **	0.78	0.70
PC1-CLPP	0.88 *	0.91 **	0.66	0.53
PC2-CLPP	-0.26	-0.31	-0.58	-0.70

*, **, *** indicates significant difference at $P < 0.05$, 0.01 and 0.001, respectively.