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Responses of absolute and specific soil enzyme activities to long term additions of organic and mineral fertilizer

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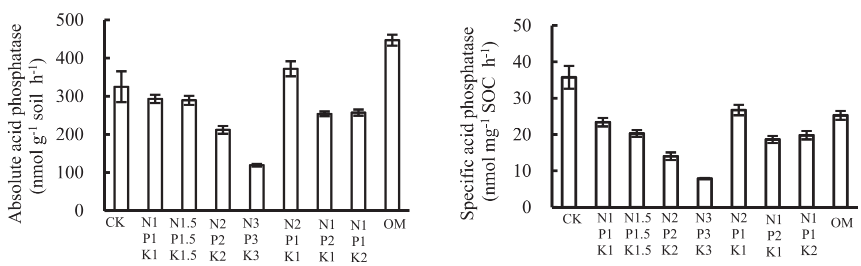
HIGHLIGHTS

- Acid phosphatase activity was negatively correlated with soil pH and P content.
- βG, NAG, and LAP activities were positively correlated with SOC and Total N contents.
- The four enzyme activities were significantly higher under manure than NPK fertilizer.
- Enzyme activities were positively correlated with actinomycete and G^+ bacterium.
- We recommend reducing P fertilizer application rates to subtropical paddy soils.

GRAPHICAL ABSTRACT



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ABSTRACT

Long-term phosphorus (P) and nitrogen (N) applications may seriously affect soil microbial activity. A long-term field fertilizer application trial was established on reddish paddy soils in the subtropical region of southern China in 1998. We assessed the effects of swine manure and seven different rates or ratios of NPK fertilizer treatments on (1) the absolute and specific enzyme activities per unit of soil organic carbon (SOC) or microbial biomass carbon (MBC) involved in C, N, and P transformations and (2) their relationships with soil environmental factors and soil microbial community structures. The results showed that manure applications led to increases in the absolute and specific activities of soil β-1,4-glucosidase(βG), β-1,4-N-acetylglucosaminidase (NAG), and leucine aminopeptidase (LAP). The absolute and specific acid phosphatase (AP) activities decreased as mineral P fertilizer application rates and ratios increased. Redundancy analysis (RDA) showed that there were negative correlations between absolute and specific AP activities, pH, and total P contents, while there were positive correlations between soil absolute and specific βG, NAG, and LAP enzyme activities, and SOC and total N contents. RDA showed that the contents of actinomycete and Gram-positive bacterium PLFA biomarkers are more closely related to the absolute and specific enzyme activities than the other PLFA biomarkers ($P < 0.01$). Our results suggest that both the absolute and specific enzyme activities could be used as sensitive soil quality indicators that provide useful linkages with the microbial community structures and environmental factors. To maintain microbial activity and to minimize environmental impacts, P should be applied as a combination of inorganic and organic forms, and total P fertilizer application rates to subtropical paddy soils should not exceed 44 kg P ha^{-1} $year^{-1}$.

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1. Introduction

Phosphorus (P), nitrogen (N), and potassium (K) are essential elements for plants and microorganisms that often have to be added to soils to sustain agricultural productivity and soil quality. This is especially true for the red soil region of southeast China, where the soils are highly weathered, strongly acidic, and P deficient (Smithson and Giller, 2002; Zhong and Cai, 2007). Farmers' decisions about their fertilizer strategies reflect economic, rather than agronomic, pressure (Chu et al., 2007). Currently farmers apply between 270 and 466 kg N ha^{-1} $year^{-1}$ (Zhang et al., 2013) and between 135 and 160 kg P ha^{-1} $year^{-1}$ (Xie et al., 2014) as chemical compound fertilizers to the reddish paddy soils; organic fertilizer is rarely applied nowadays. Local farmers treat mineral fertilizer as the most convenient and effective method to increase food production and their income, without considering the effects on soil or environmental quality. Continuous excessive applications of mineral fertilizer can lead to nutrient accumulation in soil, and eventual P and N loss from soil to aquatic ecosystems (Qiao et al., 2012; Zhang et al., 2003; Yan et al., 2013). Excessive N and P applications will also deteriorate the soil quality and reduce the soil's production levels. Therefore, a thorough understanding of the soil's response to N and P additions is extremely important so that both soil and environmental quality can be protected and even improved, particularly in these reddish soils. There are gaps in the information currently available about responses of soil microbial communities to sustained fertilizer applications. In particular, the long-term effects of different rates or ratios of fertilizer applications on microbial enzyme activity remain poorly understood.

Soil enzymes have been used as sensitive indicators of soil fertility, soil quality, and productivity (Bandick and Dick, 1999; Ge et al., 2010; Lagomarsino et al., 2009). They are direct measures of the biological metabolism of soil biota (Burns et al., 2013; Das and Varma, 2011; Dick and Kandeler, 2005). Their activities can indicate microbial activity, decay rates, and availability of substrates for microbial or plant uptake (Garcia-Gil et al., 2000; Lagomarsino et al., 2009). Activities of β-1,4-glucosidase (βG), β-1,4-N-acetylglucosaminidase (NAG), leucine aminopeptidase (LAP), and acid (alkaline) phosphatase (AP) control the release of biologically available nutrients from organic compounds of carbon (C), N, and P (Sinsabaugh et al., 2008, 2010; Zhou et al., 2012).

Enzyme activities related to C, N, and P nutrient cycling in soils often increase when organic materials are added (Böhme et al., 2005; Debosza et al., 1999; Garcia-Gil et al., 2000). Organic amendments can stimulate plant growth and microbial activity (Ge et al., 2010; Nayak et al., 2007); protect and maintain soil enzymes in their active forms; serve as readily available sources of energy and nutrients that enhance microbial and crop biomass (Bhattacharyya et al., 2005; Bi et al., 2009), and provide substrates for living microorganisms (Dalal, 1982; Saha et al., 2008). Soil phosphatase plays an important role in soil P transformations and may contribute to the overall P loss potential from paddy fields (Wang et al., 2012). Organic P may be important for short-term biological consumption (Stone, 2011). However, Liang et al. (2013) demonstrated that P concentrations in lateral seepage water increased as manure application rates increased, and suggested a low manure amendment (26 kg P ha^{-1}) to minimize P losses from paddy soils. In comparison, inorganic fertilizers have a weaker effect on soil enzyme activities (Ge et al., 2010; Piotrowska and Wilczewski, 2012; Yu et al., 2012). This may be attributed to an increase in the production of crop residue, which in turn may stimulate the activities of relevant soil enzymes (Bhattacharyya et al., 2005). However, mineral fertilizer may decrease the C, N, and P-related hydrolytic enzyme activities (DeForest et al., 2012; Fan et al., 2012; Wang et al., 2012; Zhou et al., 2012), perhaps because enzymes are not required by microbes to acquire N and P nutrients.

Compared to the absolute enzyme activities per unit of soil, specific enzyme activities per unit of soil total organic C (SOC) or microbial biomass C (MBC) can be used to normalize differences in SOC or MBC contents, thereby permitting a reliable comparison of soil microbial functions under different soil management practices, and providing ecological information about the immobilized extracellular enzyme activities and the metabolic status of variations in the microbial community or enzyme efficiency (Lagomarsino et al., 2011; Raiesi and Beheshti, 2014). They can also indicate the physiological capacity of enzymes since the activities are the result of the current or most recently viable microbial community (Waldrop et al., 2000).

Studies have shown that different land use treatments have an influence on soil specific enzyme activities per unit of SOC or MBC (Medeiros et al., 2015; Raiesi and Beheshti, 2014; Trasar-Cepeda et al., 2008; Waldrop et al., 2000), but to date, little information is available about the effects of fertilization on specific enzyme activities. The responses of soil specific enzyme activities may be clearer than those of the absolute enzyme activities and so could act as indicators to support farmers' decision-making to avoid soil degradation (Medeiros et al., 2015; Raiesi and Beheshti, 2014). Soil specific enzyme activities may act as functional indicators that have immediate ecological implications, and hence could be more easily related to changes in soil microbial community structure (Waldrop et al., 2000). An understanding of the relationships between soil specific enzyme activities and soil microbial community structures is particularly important in agroecoystems. Currently, we have little information about how the forms and rates of fertilizer applied to soil influence the soil specific enzyme activities and their relationships with soil microbial community structures.

The results of our previous study, in which substantial increases were observed in SOC, Gram-positive bacteria, and actinomycetes in red soils, highlighted the influence of swine manure or N, P, and K fertilizers on SOC contents and soil phospholipid fatty acid (PLFA) biomarkers in paddy soils (Dong et al., 2012, 2014). However, there is a lack of information about the influence of fertilization practices on soil enzyme activities and their linkages with the structure of the soil microbial community in this region. The red soils cover a total area of 1.13 million km^2 in tropical and subtropical regions of China and account for about 11.8% of the national land area. As such, they represent a significant portion of China's arable land (Zhong and Cai, 2007). We collected soil samples from a 15-year fertilizer trial on a reddish paddy soil in subtropical southeast China where soils were treated with different levels and ratios of either chemical NPK compound fertilizer or swine manure. The absolute and specific enzyme activities (AP, βG, NAG, and LAP), microbial indicators (MBC, PLFA biomarkers), and soil environmental factors (pH values, SOC, total N (TN), total P (TP) contents) of soils were analyzed. Our objectives were (1) to assess the effects of the different types, amounts, and ratios of fertilization treatments on the absolute and specific enzyme activities, and (2) to explore the correlations between the enzyme activities, soil microbial community structures, and soil environmental factors, under different (organic and inorganic) fertilizer management practices.

2. Materials and methods

2.1. Study sites

The field site (115°03′29.2″E, 26°44′29.1″N) was located in a typical red earth region in Taihe County, Jiangxi Province, China. It has a subtropical monsoon climate, with a mean annual temperature of 18 °C and annual average precipitation of 1470 mm. A long-term fertilization experiment on a double rice cropping system was set up in 1998 and has continued ever since. Rice fields are located in a flat floodplain where the paddy soil (Anthrosol) formed from waterlogged silt loam deposits. Prior to the start of the experiment, the soil had been used for rice (*Oryza sativa* L.) cropping. Before treatment, the basic soil properties of the plow layer (0–20 cm) were as follows: bulk density = 1.5 g cm^{-3}; pH = 6.0; SOC = 9.7 g kg^{-1}, TN = 1.0 g kg^{-1}, and available P = 1.6 mg kg^{-1}.

2.2. Fertilizer treatments

All treatments were arranged in a randomized block design with three replicates. Each plot was 15 m^2 (3 m × 5 m) and was isolated by concrete walls (50 cm beneath soil). Border plots were established to reduce edge effects. We selected nine treatments in total: control (CK), swine manure (OM), four different mineral fertilizer rates ($N_1P_1K_1$, $N_{1.5}P_{1.5}K_{1.5}$, $N_2P_2K_2$, $N_3P_3K_3$), and three different mineral fertilizer ratios ($N_2P_1K_1$, $N_1P_2K_1$, $N_1P_1K_2$) (Table 1). In this study, we focused on the effects of organic or mineral forms of N and P fertilizers on soil C, N, or P related enzyme activities. K was increased at the same rate as N and P except in $N_1P_1K_2$, which ensured that K was not a limiting nutrient for rice in the mineral fertilizer treatments.

No fertilizer was applied to the CK. The OM plot was treated with swine manure with an NPK ratio of 0.55%–0.09%–0.17% on a dry-weight basis, applied at a rate of approximately 41 000 kg·ha^{-1} year^{-1} dry weight. This was equivalent to an NPK amendment of 225–37–70 kg ha^{-1} year^{-1}. It was applied as base fertilizer before rice was transplanted in April each year.

Inorganic fertilizer was applied to the mineral fertilizer plots as urea, calcium–magnesium phosphate, and potassium chloride. The mineral fertilizer treatments were represented as $N_1P_1K_1$, $N_{1.5}P_{1.5}K_{1.5}$, $N_2P_2K_2$, $N_3P_3K_3$, $N_2P_1K_1$, $N_1P_2K_1$, and $N_1P_1K_2$ (Table 1). The N:P:K ratios were 1:0.13:0.41 except for the $N_2P_1K_1$, $N_1P_2K_1$, and $N_1P_1K_2$ treatments, in which the N, P, or K input ratios were doubled. Fertilizer was applied four times a year: 44% was applied over two applications in April for early rice and 56% was applied across two applications in July for late rice. For both the early and late rice, 60% of the NPK fertilizer was applied as base fertilizer when rice was being transplanted, while the remaining fertilizer was applied as top-dressing 7 to 10 days after the rice was transplanted.

2.3. Field management

The early rice was sown at the end of April and harvested in mid July. The late rice was sown at the end of July and harvested in early November. We followed the field practices (field preparation, tillage, puddling, and irrigation) of the local farmers. In this study, the waterlogging conditions and water regimes for each treatment were similar. Irrigation water was maintained at 5 to 10 cm above the soil surface layer during field preparation and rice growing, and then was drained after the rice grain matured. No pesticides were applied during the growing season and weeds were controlled manually. About 5 cm of the rice stalk and the roots were left in situ after harvest.

2.4. Soil sampling and analysis

Soil samples were collected in November 2012, immediately after the late rice harvest. The soil samples reflected the cumulative effects of 15 years of flooding and draining. In each plot, soils were sampled to a depth of 20 cm with a 5-cm diameter auger at five randomly selected locations and then mixed as one composite sample. All fresh soil samples were sieved through a 2-mm mesh and stored at 4 °C for enzyme activity, microbial community, and pH value analysis. A subsample was air dried, and then sieved through a 0.25 mm mesh for soil SOC, TN, and TP analysis.

Soil pH was measured in a soil–water suspension (1:2.5 v: v) by glass electrode (Bao, 2008) Soil was digested using H_2SO_4–$HClO_4$ and TP was measured by the spectrophotometric method with a continuous flow automated analyzer (AA3, Bran + Luebbe, Germany) at 700 nm (Bao, 2008). Soil TN and SOC were determined using a CN Analyzer (Vario Max, Elementar, Germany). MBC was measured using the chloroform fumigation and direct extraction technique (Vance et al., 1987). A conversion factor of 2.64 was used to convert extracted C to biomass C.

We measured the βG, NAG, LAP, and AP activities using fluorogenically labeled substrates, i.e. 4-MUB-β-D-glucoside for βG, 4-MUB-N-acetyl-β-D-glucosaminide for NAG, L-Leucine-7-amino-4-methylcoumarin for LAP, and 4-MUB-phosphate for AP (Saiya-Cork et al., 2002; DeForest, 2009). Soil suspensions were prepared by adding 1 g soil to 125 ml of 50 mM acetate buffer and homogenizing for 1 min with a Vortex-Genie 2. Because enzyme activity is pH sensitive, the pH of the buffer was adjusted to within 0.5 units of the mean soil pH of each treatment (DeForest, 2009). The microplates were incubated in the dark at 20 °C for up to 4 h. Fluorescence was measured using a microplate fluorometer (Synergy^{H4}, BioTek) with 365 nm excitation and 450 nm emission filters. After correcting for homogenate control, substrate control and quenching, absolute activities were expressed in units of nmol g^{-1} soil h^{-1}. We calculated the specific activities of the enzymes by dividing enzyme activities by either the SOC (Trasar-Cepeda et al., 2008) or the MBC (Waldrop et al., 2000; Raiesi and Beheshti, 2014) values to normalize the activity to the size of the SOC or MBC pools.

Soil PLFAs were extracted using the procedure of Bossio and Scow (1998) and was determined using an Agilent 6890N Gas Chromatograph with MIDI peak identification software (Version 4.5; MIDI Inc., Newark, DE) and an Agilent 19091B-102 (25.0 m × 200 μm × 0.33 μm) capillary column. The sum of the following PLFA biomarkers were considered to represent the total PLFAs of the soil microbial community (Gram-positive bacteria (G^+) by i14:0, i15:0, a15:0, i16:0, a16:0, i17:0, a17:0, Gram-negative bacteria (G^-) by 16:1ω9c, cy17:0, 18:1ω5c, 18:1ω7c, cy19:0, fungi by 18:3ω6c, 18:1ω9c and 16:1ω5c, and actinomycetes by 10Me16:0, 10Me17:0 and 10Me18:0). Detailed results have been reported in Dong et al. (2014).

2.5. Statistics

All results were reported as means ± standard errors (SE) for the three replicates. One-way analysis of variance (ANOVA) and Duncan's multiple comparisons were used to determine the differences between the soil absolute and specific enzyme activities, PLFAs, MBC contents, and environmental factors attributable to the different fertilizer treatments using SPSS 17.0 (Statistical Graphics Crop, Princeton, USA). Soil environmental factors, microbial biomass, and the soil absolute enzyme activities were compared and analyzed by principal component analysis (PCA) using SPSS 17.0 (Statistical Graphics Crop, Princeton, USA). Redundancy analysis (RDA) was applied to investigate the responses of soil absolute and specific enzyme activities to environmental variables and soil microbial community structures using CANOCO software 4.5. The significance of the variables was tested by automatic selection of means by Monte Carlo permutations. The vectors of greater magnitude that formed smaller angles with an axis were more strongly correlated with that axis. A significance is deemed if $P < 0.05$.

Table 1
N, P, and K inputs from different fertilizer treatments in the long term experimental plots (kg ha^{-1} year^{-1}).

	CK	$N_1P_1K_1$	$N_{1.5}P_{1.5}K_{1.5}$	$N_2P_2K_2$	$N_3P_3K_3$	$N_2P_1K_1$	$N_1P_2K_1$	$N_1P_1K_2$	OM
N	0	225	338	450	675	450	225	225	225
P	0	29	44	59	88	29	59	29	37
K	0	93	140	187	280	93	93	187	70

3. Results

3.1. Soil physical, chemical, and biological properties

Mineral NPK fertilizer additions significantly neutralized soil pH (Table 2). Soil pH values in the CK (mean value 5.4) were lower than the background pH values (mean value 6.0) measured in 1998. Soil pH values for the OM treatment (mean value 5.9) were higher than those for the CK, but were similar to the background pH values (Table 2). In the mineral fertilizer treatments, the pH values ranged from 5.9 to 6.7 (Table 2) and generally increased as application amounts increased. There was a significant positive correlation between soil pH values and soil TP contents ($P < 0.01$) (Fig. 1).

Manure and mineral fertilizer applications led to significant increases in the SOC and TN contents, but no significant differences were observed between the different mineral fertilizer treatments (Table 2). However, the soil SOC/TN increased in the $N_2P_2K_2$, $N_3P_3K_3$, and $N_2P_1K_1$ treatments. Manure and mineral fertilizer treatments led to significant increases in the soil TP contents, resulting in decreases in the soil SOC/TP and TN/TP ratios. The soil TP contents increased, and the soil SOC/TP and TN/TP ratios decreased, as the amounts and ratios of mineral fertilizer additions increased (Table 2).

With the exception of the highest mineral fertilizer treatment ($N_3P_3K_3$), which caused a decrease in the MBC contents, applications of manure and mineral fertilizer led to a significant increase in the MBC and total PLFA biomarker contents in the soil ($P < 0.05$) (Table 2). From the different rates and ratios of mineral fertilizer treatments, we found that the MBC and total PLFA contents improved most under the $N_{1.5}P_{1.5}K_{1.5}$ and $N_2P_1K_1$ treatments.

3.2. Soil absolute enzyme activities

Soil absolute activities of βG, NAG, and LAP increased by 191%, 516%, and 279%, respectively, under the OM treatment (Table 3). Increases in soil βG ranged from 44% to 164% as a result of the mineral fertilizer treatments, with the higher βG for the $N_2P_1K_1$ treatment. NAG increased under the mineral fertilizer treatments, but there was no significant difference between the different rates and ratios of mineral fertilizer treatments. The increased effects of mineral fertilizer treatments on soil LAP activities were significant for the $N_{1.5}P_{1.5}K_{1.5}$ and $N_2P_2K_2$ treatments. OM additions caused an increase of 38% in soil AP activity, while, with the exception of $N_2P_1K_1$, the mineral fertilizer treatments inhibited AP activity by between 11% and 63% (Table 3). As fertilizer application rates increased, AP activity decreased, and a significant decrease in AP activity was observed for the 59 and 88 kg P ha^{-1} $year^{-1}$ mineral fertilizer treatments ($N_2P_2K_2$, $N_3P_3K_3$, $N_1P_2K_1$).

Results from principal component analysis (PCA) further indicated that the absolute enzyme activities and environmental factors changed under different fertilization treatments (Fig. 2A). The first principal component (PC1) explained 47.7%, and the second (PC2) explained 28.3%, of the total variance (Fig. 2A). The loading values of the enzyme activities and the other soil properties indicated that PC1 was closely related to SOC, TN, NAG, PLFA, βG, and LAP, and that PC2 was closely related to AP, pH, TP, and MBC (Fig. 2B). The separation of the samples observed along PC1 was mainly influenced by swine manure, while the separation of the samples observed along PC2 was due to mineral NPK fertilizations. The effects of fertilization on the absolute enzyme activities and the other soil properties showed an obvious sequential shift along the PC1 axis. The OM treatments with higher PC1 scores were observed on the right of the PC1 axis, whereas CK treatments with lower scores were observed on the left of the PC1 axis (Fig. 2A). The enzyme activities and the other soil properties under the different rates and ratios of NPK fertilizer treatments were similar along the PC1 axis but were different along the PC2 axis (Fig. 2A). Therefore, PC1 and PC2 separated the samples according to their responses to swine manure and mineral NPK fertilizations, respectively.

3.3. Soil specific enzyme activities on the basis of SOM or MBC

Changes in the specific enzyme activities per unit of SOC with fertilization were similar to the variations in the absolute enzyme activities. The specific activities of βG, NAG, and LAP per unit of SOC were significantly greater under the OM treatment than the CK treatment, while the specific activities of AP per unit of SOC were significantly lower under mineral fertilizer treatments than the CK treatment (Fig. 3). This emphasizes the fact that fertilization can influence soil enzyme activities without changing the SOC content.

Swine manure applications resulted in increases in the specific enzyme activities of βG, NAG, and LAP per unit of MBC (Fig. 3). The specific enzyme activities of AP per unit of MBC did not change significantly as a result of the swine manure applications but decreased significantly because of the mineral NPK applications. The specific enzyme activities of βG and NAG per unit of MBC increased significantly after $N_3P_3K_3$ applications because of the low MBC contents under the treatment. However, the specific enzyme activities of the four enzymes per unit of MBC were not significantly different for the other mineral NPK additions, which indicates that changes in the soil enzyme activities are dependent on alterations in the MBC content.

Compared with the $N_1P_1K_1$ treatment, increasing the ratio of K ($N_1P_1K_2$) had no significant effects on soil physical, chemical, and biological properties, soil absolute, and specific enzyme activities.

3.4. Relating soil absolute and specific enzyme activities to soil environmental factors and microbial community structures

The relationships between the soil absolute and specific enzyme activities and environmental variables and microbial community structures are shown in the redundancy analysis (RDA) ordination biplot (Fig. 4). The first ordination RDA axis (horizontal) was mainly correlated with SOC, TN, PLFA, MBC, G^+, G^-, fungi, and actinomycete PLFAs, and explained 42.5% of the total variability. The second ordination axis (vertical), which was strongly associated with pH and TP, explained 29.1% of the total variability (Fig. 4).

pH and TP predominantly formed negative associations with the absolute and specific AP activities. SOC and TN formed positive associations with the absolute and specific βG, NAG, and LAP activities. The MBC and the PLFAs (including actinomycete, G^+, G^-, fungi, and bacteria PLFAs) were associated with the four enzyme activities (Fig. 4). The results of a Monte Carlo permutation test showed that the contents of actinomycete and G^+ PLFA biomarkers were more closely related to the absolute and specific enzyme activities than the other PLFA biomarkers ($P < 0.01$), which suggests that actinomycete and G^+ ($P < 0.01$) microbial communities explained most of the variations in the enzyme activities under the experimental conditions.

4. Discussion

4.1. Effects of fertilizer practices on soil properties

In this study, the background soil pH tended to be acidic, and soil acidification became worse in the CK, which may have been due to acid deposition in the study area (Liu et al., 2010). Results from our previous long-term monitoring suggest that swine manure and mineral fertilizer may increase soil acidity (Dong et al., 2012), which is in agreement with Steiner et al. (2007). The positive relationships between soil pH and TP contents in this study confirmed that calcium–magnesium phosphate fertilizers could neutralize acidity in acid soils (Fig. 1). Calcium–magnesium phosphate fertilizer can return alkaline substances to soils, resulting in increases in soil pH (Wu et al., 2008). Increases in soil pH because of swine manure applications may reflect the proton consuming ability of humic materials (Materechera and Mkhabela, 2002), and may have been due to the steady formation of organic

Table 2
Effects of fertilization treatments on soil physical, chemical, and biological properties, mean (SE) with ANOVA results (n = 3). Different letters in each column represent significant differences between different mineral fertilizer and manure additions ($P < 0.05$; Duncan's test).

Treatment	pH	MBC (ug g^{-1})	PLFA (ug g^{-1})	SOC (g kg^{-1})	TN (g kg^{-1})	TP (mg kg^{-1})	SOC/TN	TN/TP	SOC/TP
CK	5.4 (0.0)c	307 (3)e	5.8 (0.2)e	9.1 (0.7)e	1.0 (0.04)d	123 (7)e	8.9 (0.4)b	8.3 (0.5)a	74 (4)a
$N_1P_1K_1$	6.2 (0.3)b	435 (21)bc	8.2 (0.7)cd	12.5 (0.3)d	1.3 (0.03)c	267 (10)d	9.4 (0.4)ab	5.0 (0.3)cd	47 (3)bc
$N_{1.5}P_{1.5}K_{1.5}$	6.2 (0.0)b	561 (5)a	8.5 (0.2)bc	14.2 (0.2)bc	1.5 (0.05)bc	362 (13)c	9.6 (0.2)ab	4.1 (0.1)de	39 (1)cd
$N_2P_2K_2$	6.6 (0.1)a	445 (5)b	8.6 (0.5)bc	15.1 (0.4)b	1.5 (0.08)bc	521 (22)b	10.4 (0.5)a	2.8 (0.1)f	29 (2)de
$N_3P_3K_3$	6.7 (0.1)a	171 (9)f	8.5 (0.2)bc	15.1 (0.1)b	1.5 (0.04)bc	655 (44)a	10.1(0.3)a	2.3 (0.2)f	23 (2)e
$N_2P_1K_1$	5.9 (0.1)b	555 (13)a	10.2 (0.1)ab	13.9 (0.2)bcd	1.4 (0.05)c	213 (16)d	10.4 (0.5)a	6.4 (0.3)b	66 (6)a
$N_1P_2K_1$	6.6 (0.1)a	409 (6)c	8.0 (0.8)cd	13.7 (0.6)bcd	1.6 (0.07)b	512 (8)b	8.8 (0.6)b	3.1 (0.1)f	27 (1)e
$N_1P_1K_2$	6.1 (0.0)b	441 (8)b	6.6 (0.8)de	13.1 (0.6)cd	1.5 (0.04)bc	260 (22)d	9.0 (0.4)ab	5.7 (0.4)bc	51 (6)b
OM	5.9 (0.1)b	366 (7)d	10.7 (0.7)a	17.7 (0.4)a	1.8 (0.07)a	458 (36)b	9.7 (0.2)ab	4.0 (0.4)e	39 (4)cd

material with functional groups during decomposition, such as carboxyl and phenolic groups, and by the low solubility of $CaCO_3$ (Steiner et al., 2007). However, the influence of mineral fertilizer on soil pH might be partially controlled by the soil characteristics and fertilizer properties. For example, when soil pH values are approaching neutral, manure has less effect on soil pH, but mineral fertilizer containing a large proportion of urea can lead to decreases in soil pH (Fan et al., 2012; Ge et al., 2010).

Two recent meta-analyses on Chinese paddy soils showed that organic manure and NPK treatments sequestrated significant amounts of C compared with the control (Tian et al., 2015; Zhu et al., 2015). The meta-analysis, based on 274 samples collected from 44 paddy field experimental sites in South China, showed that SOC improved more under organic fertilization than NPK fertilizer, with increments of 0.26 ± 0.24 g kg^{-1} $year^{-1}$ and 0.15 ± 0.09 g kg^{-1} $year^{-1}$, respectively, in double cropping systems (Zhu et al., 2015). Similarly in this study, we observed that applications of swine manure and mineral NPK fertilizers resulted in increased SOC, TN, and TP contents and that the increases were greater under swine manure than under the NPK treatment. These results are in agreement with those from other long term experiments (Giacometti et al., 2014). However, increased rates or ratios of NPK fertilizers did not have a significant influence on SOC and TN contents (Table 2). Brown et al. (2014) also found that increased N fertilizer applications had little effect on SOC and TN, while other research has shown that mineral fertilizer could either increase SOC by increasing crop residue inputs to the soil, or decrease SOC by increasing C mineralization (Russell et al., 2009). The results from this study suggest that mineral fertilizers increased crop residue input and SOC mineralization concurrently.

Without P fertilizer applications, the TP contents of the red earth soils are extremely low. Proper management of soil P is important for maintaining soil fertility and crop yields (Chu et al., 2007; Shen et al., 2004). However, surplus P from manure and mineral fertilizers has accumulated in the soil at the study site. Based on similar amounts of P fertilizer, manure fertilizer contributed more to soil TP contents than the mineral fertilizer (Table 2). This is in agreement with long-term results from paddy soil trials (Dong et al., 2012; Wang et al., 2012). Organic manure contains a large amount of organic P, which will be released slowly, and will be beneficial to soil quality and crops over the long-term (Bi et al., 2009). The SOC/TP and TN/TP ratios in mineral fertilizer treatments were lower than those in both the CK and cropland on a global scale (63.9 and 4.4, respectively) (Xu et al., 2013). The build-up of TP due to the continuous use of fertilizer P highlights the need to reduce P fertilizer application rates so as to avoid unnecessary expenditure and environmental problems such as P surface water runoff (Wang et al., 2012).

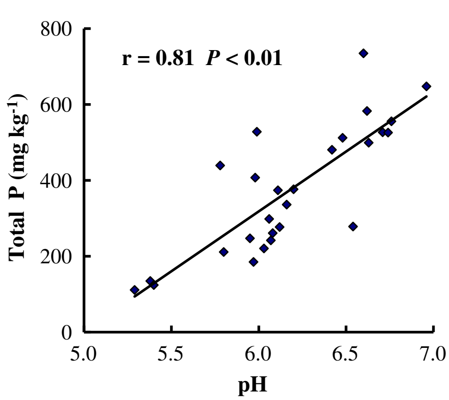

Fig. 1. Correlations between soil total phosphorus (TP) contents and soil acidity (pH values) in the 0–20 cm soil layer.

4.2. Effects of fertilizer practices on soil absolute enzyme activities

Similar to this study, other studies have reported increases in enzyme activities after organic fertilizer applications (Böhme et al., 2005; Giacometti et al., 2014; Islam et al., 2011; Kandeler et al., 1999; Liang et al., 2014). A meta-analysis based on 1160 cases around the world showed that there were positive linear relationships between βG, NAG, and LAP activities and SOC contents (Sinsabaugh et al., 2014). Redundancy analysis showed that changes in the composition of the soil microbial communities and soil environmental factors were important for maintaining soil enzyme activities. Our previous studies clearly demonstrated that swine manure could result in significantly increased contents of SOC and TN (Dong et al., 2012), and PLFAs, with increases in G^+ and actinomycetes (Dong et al., 2014), than the other treatments. This may help explain the enhanced effects of the swine manure on the hydrolytic enzyme activities and suggests that swine manure may promote secretion of hydrolytic enzyme activities by G^+ and actinomycetes.

The meta-analysis of Sinsabaugh et al. (2014) also showed that there was a positive linear relationship between AP activities and SOC contents. Analysis of AP activity showed that manure may contain phosphatase, or may stimulate secretion of phosphatase, thereby enhancing transformations from organic to inorganic P (Stone, 2011). Results showed that mineral P fertilizer applications of 59 and 88 kg P ha^{-1} $year^{-1}$ significantly inhibited soil AP activities. However, none of the mineral P fertilizer treatments, even at rates of 29 and 44 kg P ha^{-1} $year^{-1}$ that were considered low by local farmers, increased soil AP activities. In P-limited soils, soil phosphatase may mineralize P from organic matter (Burns et al., 2013), which could explain why soil AP activities were higher under the control treatment than under the mineral fertilizer treatments. The AP activity values suggest that there is sufficient inorganic P in the red soils under all the NPK fertilization treatments.

RDA showed that soil AP activity was inhibited by TP content and soil pH, and is in agreement with other studies that have also demonstrated inhibition of soil phosphatase activity as a result of mineral P

Table 3
Soil absolute enzyme activities (nmol g^{-1} soil h^{-1}). βG, β-1,4-glucosidase; NAG, β-1,4-N-acetylglucosaminidase; LAP, L-leucine aminopeptidase; AP, acid phosphatase. Different letters in each row represent significant differences between different mineral fertilizer and manure additions ($P < 0.05$; Duncan's test).

	CK	$N_1P_1K_1$	$N_{1.5}P_{1.5}K_{1.5}$	$N_2P_2K_2$	$N_3P_3K_3$	$N_2P_1K_1$	$N_1P_2K_1$	$N_1P_1K_2$	OM
βG	55 (4)d	93 (5)c	118 (4)b	79 (5)c	93 (3)c	145 (5)a	86 (2)c	87 (4)c	160 (11)a
NAG	6 (1)e	24 (3)d	32 (3)bc	32 (3)bc	25 (2)cd	36 (2)ab	40 (1)a	30 (1)bcd	37 (3)ab
LAP	3 (0.2)cd	2 (0.7)cd	5 (0.3)b	5 (0.7)b	1 (0.3)d	5 (0.9)bc	5 (0.8)bc	2 (1.0)cd	11 (0.6)a
AP	325 (40)bc	293 (11)cd	289 (12)cd	212 (10)e	119 (3)f	372 (20)b	254 (6)de	257 (8)de	447 (14)a

fertilizer applications (Shaw and DeForest, 2013; Wang et al., 2012). Giacometti et al. (2014) found that AP activity and soil pH were negatively related and suggested that either the rate of synthesis and release by soil microorganisms or its stability was related to soil pH. Antibus and Linkins (1992) showed that liming reduced soil phosphatase activity when the pH was between 5.0 and 7.0. The mineral fertilizer effect on soil AP activities in our study may be partly due to the lime-like effect of calcium–magnesium phosphate.

A meta-analysis based on 107 datasets from 64 long-term trials worldwide showed that mineral fertilizer applications led to a 15% increase in MBC contents in fertilized plots relative to CK plots. This increase in MBC contents is most likely attributable to increases in SOC because of higher crop productivity (Geisseler and Scow, 2014). Similarly in this study, we observed that, relative to the CK, mineral fertilizers generally enhanced the MBC, PLFA, and SOC contents. However, increased rates of NPK fertilizer did not significantly increase the soil SOC, TN, and PLFA contents (Table 2), which might explain why there were no significant differences in soil C and N hydrolytic enzyme activities between the different rates of NPK fertilizer treatments (Table 3). An increased ratio of N ($N_2P_1K_1$) resulted in increased MBC and PLFA contents, which might have caused associated increases in the βG and NAG activities. Zhong and Cai (2007) showed that the effects of mineral fertilizer on soil enzyme activities were mainly due to enhanced growth of rice crops and accumulation of SOC through increased root turnover and rhizodeposition. However, Ge et al. (2010) found that mineral fertilizer applications of 135 kg N ha^{-1} $year^{-1}$ and 69 kg P ha^{-1} $year^{-1}$ decreased soil sucrase activity, suggesting that some C hydrolytic enzyme activities were highly sensitive to the inhibitory effects of large applications of mineral fertilizers. We did not find that mineral fertilizers inhibited soil βG and NAG activities, but we found that soil LAP activities were lower under the 675–88–280 NPK kg ha^{-1} $year^{-1}$ fertilizer treatment than for the control, suggesting that LAP activity may be sensitive to the inhibitory effects associated with these amounts of NPK fertilizer applications.

4.3. Effects of fertilizer practices on soil specific enzyme activities

Soil specific enzyme activities of βG, NAG, and LAP per unit of SOC or MBC were improved by swine manure. Our results showed that the substrate availability (i.e. SOC) and soil microbial community structure (G^+ and actinomycetes) were the main factors that influenced the soil specific enzyme activities. While changes in βG, NAG, and LAP activities were greater than changes in the SOC or MBC contents, our results suggest that there was either an increased tendency for stabilized extracellular enzyme activity, or that the metabolic status of the microbial biomass was modified to a higher degree by organic manure (Raiesi and Beheshti, 2014). Organic manure may increase enzyme synthesis and production by soil microorganisms, and promote the release of enzymes that are either immobilized by clay and humic particles or are entrapped within soil aggregates (Trasar-Cepeda et al., 2008). While the soil specific AP activities per unit of SOC or MBC did not increase under organic manure applications, the results suggest that the responses of stabilized extracellular AP activity, or the metabolic status of microbial biomass to hydrolyze organic P by organic manure, were different.

The decreases in AP per unit of SOC or MBC in NPK fertilized soils were mainly caused by decreases in the AP activity, and are attributable to the same mechanism that led to decreases in the absolute AP activities in NPK fertilized soils. The decline in the specific activity of P acquiring enzymes may indicate alleviation of P limitation because of organic matter decomposition in the soils. The results indicate that soil specific AP activities are sufficiently sensitive to changes in NPK and can be used as responsive indexes for measuring changes in both AP activity and MBC and SOC contents in the study area.

However, βG and NAG activities per unit of MBC were higher under the $N_3P_3K_3$ treatment than the CK treatment. This may be possible because the lower soil microbial biomass under $N_3P_3K_3$ might promote either similar enzyme activity production by the surviving microorganisms or enzyme release from dead microbial cells (Raiesi and Beheshti,

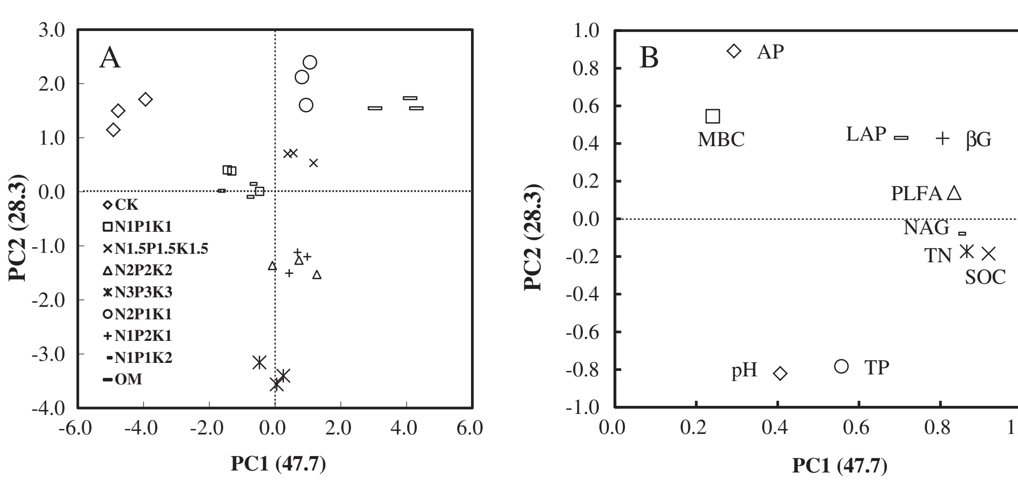

Fig. 2. PCA of the soil absolute enzyme activities and environmental factors under the different fertilization treatments. Absolute enzyme activities were β-1,4-glucosidase (βG), β-1,4-N-acetylglucosaminidase (NAG), leucine aminopeptidase (LAP), and acid phosphatase (AP). Environmental variables were soil acidity (pH), total organic carbon (SOC), total nitrogen (TN), total phosphorus (TP), microbial biomass carbon (MBC), and total PLFAs (PLFA).

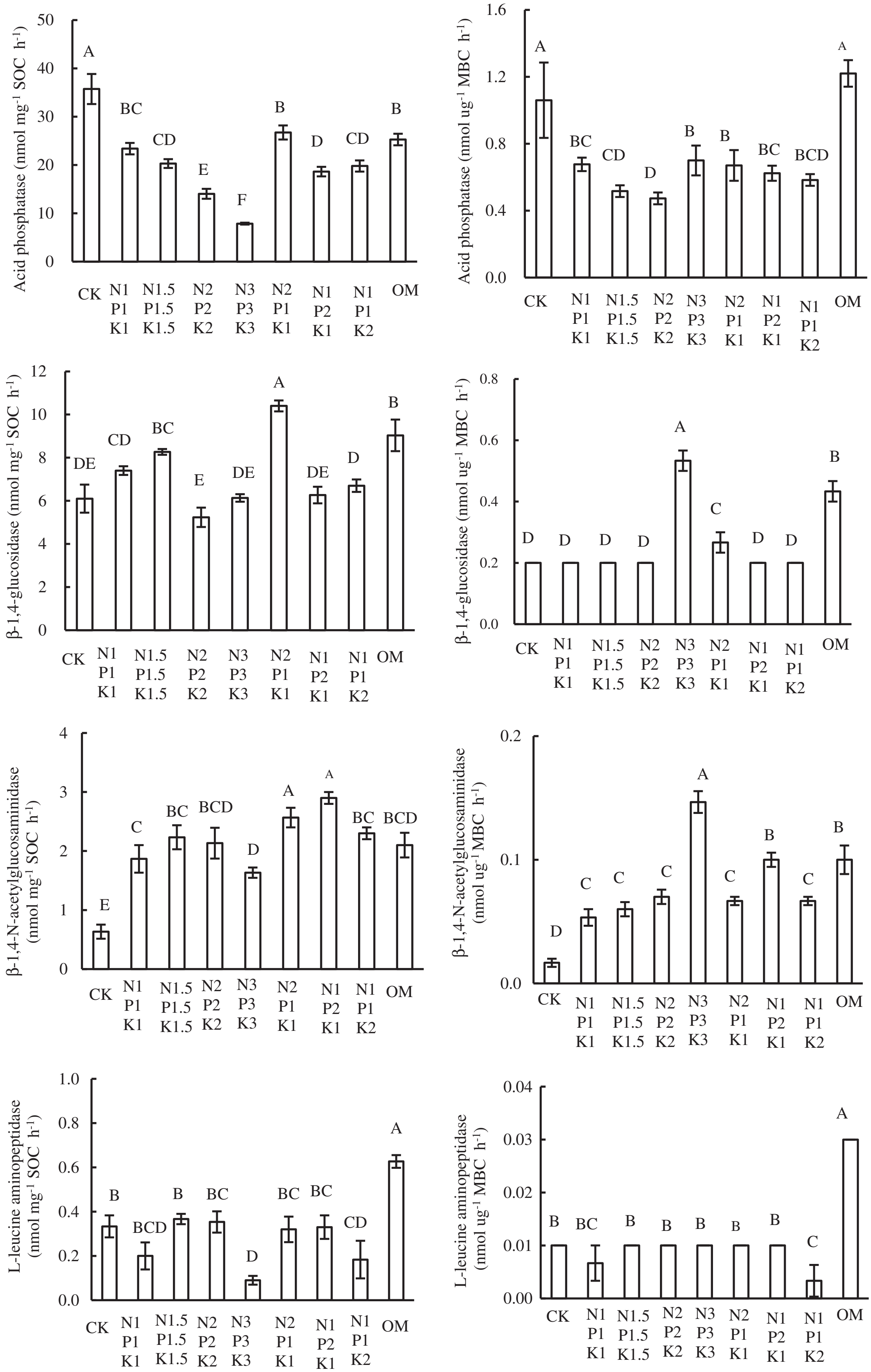

Fig. 3. Soil specific enzyme activities per unit of SOC and MBC, mean (SE) with ANOVA results (n = 3). Different letters represent significant differences between different mineral fertilizer and manure additions ($P < 0.05$; Duncan's test).

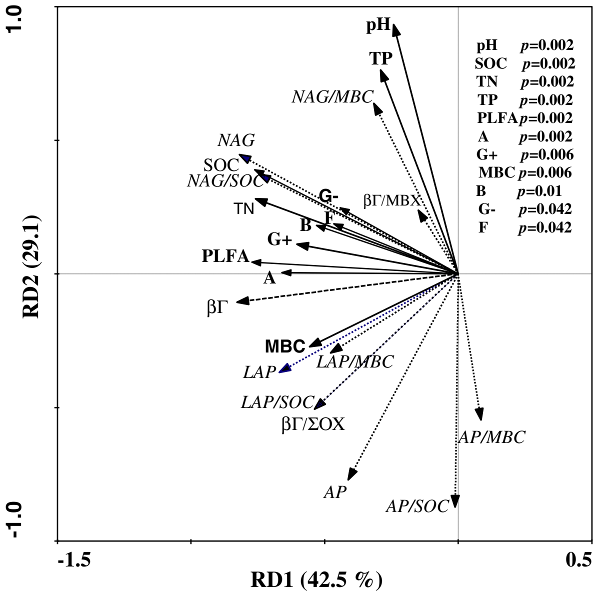

Fig. 4. Redundancy analyses (RDA) of the soil absolute and specific enzyme activities, environmental factors and microbial communities. The explanatory variables are indicated by different arrows, and enzyme activities are indicated by dotted lines. Enzyme activities specific to SOC were βG/SOC; NAG/SOC, LAP/SOC, and AP/SOC. Enzyme activities specific to MBC were βG/MBC; NAG/MBC, LAP/MBC, and AP/MBC. Environmental variables and soil microbial communities are represented by solid lines (bacterial PLFAs (B), Gram-positive bacteria PLFAs (G^+), Gram-negative bacteria PLFAs (G^-), fungi PLFAs (F), and actinomycete PLFAs (A)).

2014). A greater soil βG and NAG per unit of MBC under $N_3P_3K_3$ may also suggest that the soil microorganisms are metabolically more active, with increasing efficiency of enzyme production and release by soil microorganisms. The responses of specific βG, NAG, and LAP enzyme activities per unit of MBC to the other NPK additions were similar, hinting at some commonality in the physiological response of microbes to NPK additions.

Our results are in agreement with those reported by Waldrop et al. (2000), who observed that the absolute and specific enzyme activities per unit of MBC could provide a useful linkage between the microbial community structure and organic matter processing. Through understanding the correlations between the absolute and specific enzyme activities and the microbial community structures, we were able to relate the actinomycetes and G^+ with potential rates of degradation of macromolecular carbon compounds in the study sites.

4.4. Suggestions for fertilization of the reddish paddy soils

The positive responses of SOC, TN, and TP contents, soil microbial biomass, and absolute and specific enzyme activities to swine manure applications demonstrate that farmers should be instructed to reuse organic fertilizer on the reddish paddy soils (Dong et al., 2012, 2014). Because of the inhibitory effects of $N_2P_2K_2$ and $N_3P_3K_3$ on AP absolute and specific activities and the potential risk of soil TP pollution, mineral fertilizer applications should be maintained below 338 (N)-44 (P)-140 (K) kg ha^{-1} year^{-1}. Mineral N and P fertilizers are currently applied at excessive rates to the reddish paddy soils, and priority should be directed at their reduction (Xie et al., 2014; Zhang et al., 2013). P applications, including organic and inorganic forms, should not exceed 44 kg P ha^{-1} year^{-1}. The results from the $N_1P_1K_1$, $N_2P_1K_1$,$N_1P_2K_1$ and $N_1P_1K_2$ treatments indicate that the application ratios of mineral P and K should not be increased and that the N–P–K ratio should be maintained between 1:0.13:0.41 and 2:0.13:0.41.

5. Conclusions

Both long-term swine manure and mineral NPK fertilizers may improve the soil absolute and specific βG and NAG activities. Further, there were significant positive correlations between the soil absolute and specific βG, NAG, and LAP activities and the SOC and TN contents. Long-term swine manure applications resulted in significant increases in the soil AP activity and TP contents. Soil AP activities decreased significantly when mineral P fertilizer applications were 59 kg P ha^{-1} year^{-1} ($N_2P_2K_2$) or more ($N_3P_3K_3$, $N_1P_2K_1$). Soil absolute and specific AP activities were negatively significantly correlated with soil pH values and TP contents ($P < 0.01$). The mineral fertilizers neutralized soil acidity and increased soil TP contents due to direct inputs of mineral P, and sustained P accumulation in the study soils, which may inhibit the microbial physiological capacity of AP. RDA showed that the contents of actinomycete and G^+ PLFA biomarkers are more closely related to the absolute and specific enzyme activities than the other PLFA biomarkers ($P < 0.01$). The results suggest that the absolute and specific enzyme activities could be used as sensitive soil quality indicators and could provide useful linkages with microbial community compositions and environmental factors. To maintain microbial activity and to minimize environmental impacts, P applications, including inorganic and organic forms, should not exceed 44 kg P ha^{-1} year^{-1}.

Conflict of interest

The authors declare no conflict of interest.

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