Carbon-to-nutrient stoichiometry shapes microbial carbon utilization and soil organic carbon storage in agricultural soils

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Abstract

Most agro-ecosystems receive inputs of anthropogenic-derived nutrients, which has an important influence on soil organic matter (SOM) and plant growth in agricultural soil. However, the process of the microbial-mediated SOM decomposition and storage as well as the microbial nutrients-acquisition strategy in response to fertilization remains unclear. In this thesis, I combined a meta-analysis and short-time incubation experiments to explore microbial organic carbon mineralization and stabilization in soil along with the nutrients-acquisition strategy in response to fertilization.

The results show that fertilization has a positive effect on soil organic carbon (SOC) stocks, with intermediate N and K fertilization but high P fertilization affecting SOC the most. Mineral N, P, and N plus P input decreased cumulative litter-derived CO_2 emissions but increased litter-derived dissolved OC due to increasing litter-C use efficiency and alleviating microbial nutrient limitation. Straw (or litter) plus intermediate N fertilization levels increased SOC in both meta-analysis and short-time incubation experiments, illustrating that additional provision of mineral nutrients (with a wide C:nutrients ratio) can increase plant residues retention and induce N immobilisation, increasing C sequestration. P fertilization removed microbial P limitation and produced amount of labile-C from root exudates in P-limited paddy soil, resulting in protecting SOM due to labile-C was preferentially utilized by microorganism. Two inverse linear relationships in model rhizosphere and bulk soil were observed due to labile-C regulate microbial P-acquisition strategy. Specially, a positive relationship was observed between the P:C acquisition ratio and the dissolved OC:Olsen-P ratio in bulk soil but a negative relationship in a model rhizosphere soil due to relative higher labile-C content in the latter, which was dominated by r-survival strategy microorganisms. Therefore less organic P was mineralized in rhizosphere than in bulk soil. I conclude that microbial C and nutrient limitation impede SOC accumulation and P availability in agricultural soil. Plant residues return with intermediate N fertilization levels has a good potential to increase SOC stocks in agricultural soils. A moderate mineral fertilizer application along with crop residues return to the field thus contribute to sustainable utilization of agricultural soils.

Keywords: Soil organic carbon; Fertilization; Microbial C or P limitation; Rhizosphere and bulk soil

Zusammenfassung

Den meisten Agrarökosystemen werden anthropogene Nährstoffe zugeführt, was einen wichtigen Einfluss auf die organische Bodensubstanz (SOM) und das Pflanzenwachstum in landwirtschaftlichen Böden hat. Der Prozess des mikrobiell gesteuerten Abbaus und der Speicherung von SOM sowie die Strategie der mikrobiellen Nährstoffaufnahme in Mikroorganismen als Reaktion auf die Düngung ist jedoch bislang unzureichend erforscht. In dieser Arbeit habe ich eine Meta-Analyse mit Kurzzeit-Inkubationsexperimenten kombiniert, um die mikrobielle Mineralisierung und Stabilisierung von organischem Kohlenstoff im Boden sowie die Strategie der Nährstoffaufnahme als Reaktion auf Düngung zu untersuchen.

Die Ergebnisse zeigen, dass sich Düngung positiv auf die organischen Kohlenstoffvorräte im Boden (SOC) auswirkt, wobei eine mittlere N- und K-Düngung, aber eine hohe P-Düngung SOC am stärksten anreichern. Die Zufuhr von mineralischem N, P sowie N plus P verringerte die kumulativen CO_2-Emissionen aus der Streu, steigerte jedoch den aus der Streu stammenden gelösten OC, da die Effizienz der Streu-C-Nutzung erhöht und die mikrobielle Nährstofflimitierung verringert wurde. Stroh (oder Streu) plus Niveaus mittlerer N-Düngung erhöhten den SOC sowohl in der Metaanalyse als auch in Kurzzeit-Inkubationsexperimenten, was verdeutlicht, dass die zusätzliche Zufuhr von mineralischen Nährstoffen (mit einem breiten C:Nährstoff-Verhältnis) die Rückhaltung von Pflanzenrückständen erhöhen und eine N-Immobilisierung bewirken kann, wodurch die C-Sequestrierung erhöht wird. Die P-Düngung hob die mikrobielle P-Limitierung auf und führte zu höheren Gehalten an labilem C aus Wurzelexsudaten in ursprünglich P-limitierten Nassreisböden, was zu einem Schutz der SOM führte. Es wurden zwei inverse lineare Beziehungen in der Modell-Rhizosphäre und im Boden beobachtet, die auf die Strategie der mikrobiellen P-Akquisition durch labiles C zurückzuführen sind. So wurde eine positive Beziehung zwischen dem P:C-Akquisitionsverhältnis und dem Verhältnis von gelöstem OC:Olsen-P im Matrixboden beobachtet, aber eine negative Beziehung in der Modell-Rhizosphäre. Dies lässt sich mit dem relativ höheren Gehalt an labilem C in letzterem erklären, die von Mikroorganismen mit r-Überlebensstrategie dominiert wurde. Daher wurde in der Rhizosphäre weniger organisches P mineralisiert als im nicht-durchwurzelten Boden. Schlussfolgernd zeigen meine Daten, dass die mikrobielle C- und Nährstofflimitierung die SOC-Akkumulation und die P-Verfügbarkeit in landwirtschaftlichen Böden behindert. Die Rückführung von Pflanzenrückständen mit mittlerem N-Düngungsniveau hat ein gutes Potenzial, die SOC-Vorräte in landwirtschaftlichen Böden zu erhöhen. Eine moderate Mineraldüngerausbringung mit Rückführung von Ernterückständen auf landwirtschaftliche Flächen trägt daher zu einer nachhaltigen Nutzung landwirtschaftlicher Böden bei.

Schlüsselwörter: Organischer Kohlenstoff im Boden; Düngung; mikrobielle C- oder P-Limitierung; Rhizosphäre und Hauptboden

Table of Contents

List of Tables

Study 3

Study 3

List of Figures

supplemented with N fertilizers; P, soil supplemented with P fertilizers; NP, soil supplemented with N and P fertilizers; litter, soil supplemented with *Dactylis glomerata* L. (litter); litter + N, soil supplemented with litter and N fertilizers; litter + P, soil supplemented with litter and P fertilizers; litter + NP, soil supplemented with litter, N and P fertilizers. Samples were taken at four times days 2, 6, 14 and 37 days, respectively. All results are means ± standard error (n = 4).

Fig. 3 Soil microbial biomass C (MBC) (a), the metabolic quotient (qCO_2) (b), excess microbial biomass ^{13}C ($MB^{13}C$) (c), and C use efficiency (CUE) of added litter, (d) over 37 days of soil incubation period under eight different treatments, i.e., CK (only soil); N, soil supplemented with N fertilizers; P, soil supplemented with P fertilizers; NP, soil supplemented with N and P fertilizers; litter, soil supplemented with *Dactylis glomerata L.* (litter); litter + N, soil supplemented with litter and N fertilizers; litter + P, soil supplemented with litter and P fertilizers; litter + NP, soil supplemented with litter, N and P fertilizers. Samples were taken at four times days 2, 6, 14 and 37 days, respectively. All results are means ± standard error (n = 4).

Fig. 4 The maximal velocity (V_{max}) of β-1.4-glucosidase (BG) (a), β-1, 4-N-acetyl-glucosaminidase (NAG) (b) and β-1,4-phosphate (AP) (c) over 37 days of soil incubation period under eight different treatments, i.e., CK (only soil); N, soil supplemented with N fertilizers; P, soil supplemented with P fertilizers; NP, soil supplemented with N and P fertilizers; litter, soil supplemented with *Dactylis glomerata* L. (litter); litter + N, soil supplemented with litter and N fertilizers; litter + P, soil supplemented with litter and P fertilizers; litter + NP, soil supplemented with

litter, N and P fertilizers. Samples were taken at four times days 2, 6, 14 and 37 days, respectively. All results are means ± standard error (n = 4).

Fig. S1 Soil organic C (SOC) content (a) and excess organic ^{13}C ($SO^{13}C$) content (b) over day 37 day of the incubation period of the step soil under four different treatments, i.e., CK (only soil) ; N, soil supplemented with N fertilizers; soil supplemented with P fertilizers; soil supplemented with N and P fertilizers; litter, soil supplemented with *Dactylis glomerata* L. (litter); litter + N, soil supplemented with litter and N fertilizers; litter + P, soil supplemented with litter and P fertilizers; litter + NP, soil supplemented with litter, N and P fertilizers at four times (at 2, 6, 14 and 37 days, respectively). All results are means ± standard error (n = 4).

Study 3

Fig. 1 Contents of soil phosphorus fractions based on the biological availability $CaCl_2$-P (a), Citric-P (b), Enzyme-P (c) and HCl-P (d) in soil after 38 days of incubation under four different treatments, i.e., no substrate addition (CK), phosphorus addition (P), *Dactylis glomerata* L. addition (D), *Dactylis glomerata* L. and phosphorus addition (DP). All results are means ± standard error (n = 4).

Fig. 2 Soil DOC (a) and Olsen-P (b) contents, the ratio of DOC to Olsen-P (c), MBC (d) and MBP (e) contents, and the ratio of MBC to MBN (f) in soil after 38 days of incubation under four different treatments, i.e., no substrate addition (CK), phosphorus addition (P), *Dactylis glomerata* L. addition (D), *Dactylis glomerata* L. and phosphorus addition (DP). All results are means ± standard error (n = 4).

Fig. 3 The maximal velocity (V_{max}) of C acquisition enzymes (the sum of the maximal

velocity of β-1, 4-glucosidase (BG), β-1,4-cellobioside (CBH), and β-1,4-xylosidase (XYL)) (a), the maximal velocity of P acquisition enzymes (β-1,4-phosphate (AP)) (b), and the ratio of C- to P acquisition enzyme ($EEA_{C:P}$, c) in soil after 38 days of incubation under four different treatments, i.e., no substrate addition (CK), phosphorus addition (P), *Dactylis glomerata* L. addition (C), *Dactylis glomerata* L. and phosphorus addition (DP). All results are means ± standard error (n = 4).

Fig. 4 Extracellular enzyme stoichiometry of the relative proportions of C to N acquisition versus C to P acquisition (a), the variation of vector length (b) and angle (c) and their relationships (d) in soil after 38 days of incubation under four different treatments, i.e., no substrate addition (CK), phosphorus addition (P), *Dactylis glomerata* L. addition (D), *Dactylis glomerata* L. and phosphorus addition (DP). All results are means ± standard error (n = 4).

Fig. 5 Spearman correlation heat map of between microbial nutrient (N or P) limitation and soil physicochemical properties.

Fig. S1 Soil organic carbon (SOC) contents in soil after 38 days of incubation under four different treatments, i.e., no substrate addition (CK), phosphorus addition (P), *Dactylis glomerata* L. addition (D), *Dactylis glomerata* L. and phosphorus addition (DP).

Fig. S2 The maximal velocity (V_{max}) of N acquisition enzymes (the sum of the potential maximum activity of β-1,4-N- acetyl-glucosaminidase (NAG) and L-leucine aminopeptidase (LAP)) in soil after 38 days of incubation under four different treatments, i.e., no substrate addition (CK), phosphorus addition (P), *Dactylis*

glomerata L. addition (D), *Dactylis glomerata* L. and phosphorus addition (DP).

Study 4

Fig. 1 Pearson correlation between physicochemical properties and enzyme kinetic parameters (V_{max} and K_m) of rhizosphere soil (RS) and bulk soil (BS) 45 days after rice transplantation. The symbols *, **, and *** represent significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. DOC, dissolved organic C; Olsen-P, available P; NH_4^+, ammonium nitrogen; NO_3^-, nitrate; MBC, microbial biomass C; MBP, microbial biomass P; V_{max}, maximal velocity; K_m, half-saturation constant; BG, β-1,4-glucosidase; CBH, β-cellobiohydrolase (β-1,4-cellobioside); ACP, acid phosphomonoesterase; ALP, alkaline phosphomonoesterase.

Fig. 2 Linear relationship of C (V_{max} of [BG + CBH]) and P (V_{max} of [ACP + ALP]) acquisition and their ratio (P:C) in relation to soil available nutrients (DOC:Olsen-P) (a–c) and microbial biomass (MBC:MBP) (d–f) in rhizosphere soil (RS) and bulk soil (BS) 45 days after rice transplantation. DOC, dissolved organic C; Olsen-P, available P; MBC, microbial biomass C; MBP, microbial biomass P; V_{max}, maximal velocity; BG, β-1,4-glucosidase; CBH, β-cellobiohydrolase (β-1,4-cellobioside); ACP, acid phosphomonoesterase; ALP, alkaline phosphomonoesterase.

Fig. 3 Schematic of stoichiometric theory that shapes enzyme kinetics in the bulk soil (but not the rhizosphere soil) of rice after cellulose and phosphorus addition.

Abbreviations

ACP	Acid phosphomonoesterase
AP	Phosphomonoesterase
ALP	Alkaline phosphomonoesterase
Al	Aluminum
BG	β-glucosidase
C	Carbon
Ca	Calcium
CBH	β-cellobiohydrolase
CO_2	Carbon dioxide
CUE	Carbon use efficiency
D	Dactylis glomerata *L.*
DOC	Dissolved organic carbon
F	Farmyard manure
Fe	Iron
K	Potassium
Km	Half-saturation constant
MBC	Microbial biomass carbon
M	Mineral fertilization
Mg	Magnesium
MO	Mineral + organic fertilization
N	Nitrogen
NAG	β-N-acetyl-glucosaminidase
O	Organic fertilization
P	Phosphorus
P_i	Inorganic P
P_o	Organic P
qCO_2	Metabolic quotient
SOC	Soil organic carbon
SOM	Soil organic matter
S	Straw
V_{max}	The maximal velocity
XYL	β-Xylanase
LAP	L-leucine aminopeptidase

1 General Introduction

1.1 SOC stocks

Soil contains approximately 2344 Gt (1 gigaton = 1 billion tonnes) of organic carbon (OC) globally and is the largest terrestrial pool of OC (Stockmann et al., 2013). Agro-ecosystems occupy more than about one third of the global land surface (Smith et al., 2008). OC stocks in croplands (111–170 Pg C) accounts for approximately 10% of total soil C up to1 m soil depth (1500 Pg C) globally (Eswaran et al., 1993; Feng et al., 2014). Average C stocks up to depths of 35 cm in upland soils were 31 Mg C ha^{-1} (Wei et al., 2021).

Besides acting as a global C sink or source, soil organic carbon (SOC) is an important parameter for soil fertility in agro-ecosystems (Lal, 2006). The SOC stock is related to land use and management practices such as fertilizer application, crop rotation, soil cultivation (Zhang et al., 2010; Tian et al., 2013; Khan et al., 2019). Small changes in the SOC stock could result in significant impacts on the atmospheric C concentration (Fig.1a), soil quality, and crop yield (Fischlin et al., 2007; Zang et al., 2017). So, Han et al. (2018) reported that increasing SOC by 0.35 Mg C ha^{-1} $year^{-1}$ increased the wheat grain yield by 13.4%. Intensive fertilization, i, e., nitrogen (N), phosphate (P) and potassium (K) directly and indirectly affects the C input and SOC stocks (Zang et al., 2016; Liu et al., 2018). Fertilization can increase (Obour et al., 2017), decrease (Hao et al., 2017), or unchange (Liang et al., 2014) the SOC content

or stocks in agriculture land. This is related to the level of fertilization (Follett et al., 2005; Li et al., 2020; Liu et al., 2020). Chen et al. (2016) reported an increase in SOC by approximately 8% with increasing N (300 kg ha^{-1}) fertilization. It match well with Kätterer et al. (2012), who reported in Swedish long-term cropland fertilization experiments an annual increase in SOC of 1–2 kg ha^{-1} for each kg of mineral N fertilizer applied was identified. A meta-analysis also reported that the addition of mineral fertilization significantly increased SOC content compared to the unfertilized control by an average of 12.8% with long-term mineral fertilization in global agriculture upland (Geisseler & Scow, 2014). However few research quantify the influence of the types and levels of fertilization can resolve uncertainties regarding the spatial and temporal variations in SOC related to fertilization. This is a pressing hot topic currently.

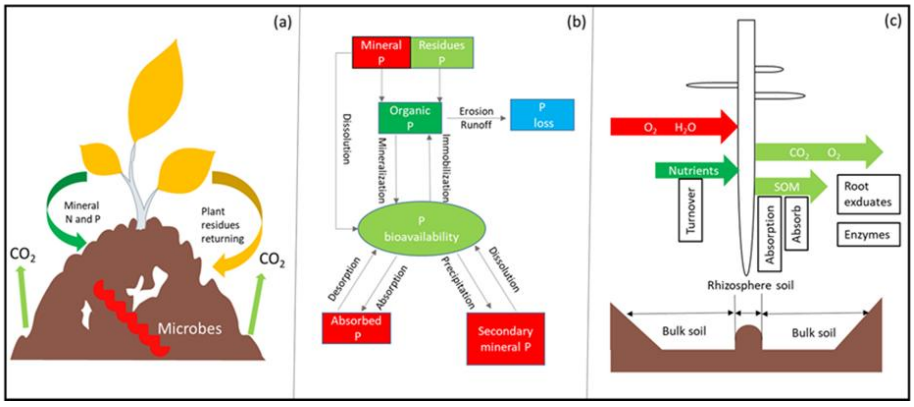

Fig1. Concertation figure of substrates with mineral N and P decomposition (a), P turnover (b) and nutrients turnover of rhizosphere and bulk (c)

1.2 SOC mineralization

The release of CO_2 from soil, as a result of microbial decomposition of SOM, is an important component in the exchange of C between agro-ecosystems and the

atmosphere (Liu et al., 2018). Soil CO_2 emissions are highly sensitive to environmental change, and even small changes in soil C pool will have strong effects on atmospheric CO_2 concentration (Heimann & Reichstein, 2008).

Intensive mineral fertilization has divergent effects on soil CO_2 emission (SOM decomposition), it can decrease (Burton et al., 2004; Zang et al., 2016), increase (Cleveland & Townsend, 2006) or unchange it (González Polo et al., 2015). In a previous study, we found that high levels of intensive fertilization (with N, P, K, Ca, and S) decreased (3–21%) CO_2 emission, while low levels of fertilization increased (12–17%) CO_2 emission in paddy soil (Liu et al., 2018). Likely, high levels of mineral fertilization could satisfy the needs of microbial growth thought decreasing microbial biomass and net N mineralization, thereby decreasing the dependency of the organisms on the original nutrients from SOM decomposition to induce a negative priming effect (PE) (Chen et al., 2009; Kirkby et al., 2014). In contrast, low levels of mineral fertilization increases microbial biomass and nutrient turnover rates to produce positive PE under nutrient-limited conditions, owing to the stimulation of microbial biomass production after low amount of exogenous nutrient input (Kuzyakov & Xu, 2013; Liu et al., 2018). These results are consistent with a previous meta-analysis, which found that small amount of N fertilization (< 10% total N) caused positive PE and high N addition induced negative PE based on 158 observations (Zang et al., 2016).

Mineral N and P fertilizer change the available organic C: available inorganic nutrient (N or P) ratios in soil, regulating the turnover and decomposition of soil

organic matter (SOM) by substrates input (Soong et al., 2018). In a previous study, we found that at a high available organic C: available inorganic N ratio CO_2 emission was intensified due to high microbial N demand (Liu et al., 2020). In line with that, Wang et al. (2019) found split NP addition (1/3 of the total N and P fertilizer at the start of the experiment, 1/3 after 15 days, and the final 1/3 after 30 days) decreases rice straw mineralization (19%) and the priming in paddy soil due to increasing the microbial straw-derived C use efficiency. As microorganisms prefer fresh C substrates over native SOC (Lu et al., 2003; Zhu et al., 2016), quite much work focuses on the impact of plant residues on SOM decomposition and CO_2 mineralization under alleviating microbial available nutrients limitation (Wang et al., 2019), and usually such studies are based on labelled plant residues (^{13}C). Unanimous conclusions are a positive PE for native soil. In the initial phase of rapid decomposition of plant residues the and high nutrient availability, the activity of primarily of r-strategists of the microorganisms is promoted (Chen et al., 2014; Shahbaz et al., 2017). However in later phase of slow decomposition and under microbial C limitation microorganisms produce extracellular enzymes to degrade relatively recalcitrant C substrates, leading to a positive PE (Wang et al., 2019). However how exogenous nutrients (i.e. N and P), influence the rates of soil organic matter (SOM) formation from plant residues and the microbial decomposition of soil indigenous SOM in nutrients limitation soils has few known.

1.3 Microbial C: P acquisition stoichiometry

The growth and metabolism of microorganisms are expressed by almost

enzymatic reactions (Moorhead & Sinsabaugh, 2006). Though the extracellular enzyme activities reflect the nutrient demands of microbial communities (Sinsabaugh et al., 2009b; Shahbaz et al., 2017), the potential enzyme activities do not necessarily mirror the microbial activity (Nannipieri et al., 2018). Because an index of nutrient mineralization rates, enzyme assays reflect the potential rather than the actual *in situ* enzyme activity rates. However, the enzyme stoichiometric balance (i.e., ratio of C:N or C:P related hydrolase) corresponds to the microbial biomass stoichiometry and soil nutrient conditions due to being expressing by microbes C, N, and P acquisition (Wei et al., 2019; Liu et al., 2020). Main extracellular hydrolase involved in acquisition of the different elements are for C: β-1, 4-glucosidase (BG, hydrolyze cellobiose into glucose), β-cellobiohydrolase (CBH, hydrolysis of cellobiose from cellulose), and β-1, 4-xylanase (XYL, degradation of hemicellulose and lignin to cellobiose); for N: β-1,4-N-acetyl-glucosaminidase (NAG, degrade chitin) and L-leucine aminopeptidase (LAP, proteolysis), and for P: phosphatase (AP, hydrolyzed phosphoric acid) (Sinsabaugh et al., 2009b; German et al., 2012; Spohn et al., 2015).

The elemental stoichiometry of microbial biomass in relation to the nutrient availability in the soil environment determines the microbial nutrient demand (Moorhead & Sinsabaugh, 2006). Considering the plant input material to soil, plant litter have a high C: nutrients ration. The mean C:N:P ratio of plant litter is about 3000:46:1 (Reich & Oleksyn, 2004). Microbial utilization and transformation of the plant litter leads to the formation of soil organic matter with a mean redfield C:N:P ratio of 186:13:1 and soil microbial biomass with a mean C:N:P ratio of from 42:6:1

to 60:7:1 (Cleveland & Liptzin, 2007; Xu et al., 2013). These two "Redfield ratio" values with the acquisition ratio of C:N:P enzymes are used to investigate into the balance of microbe's nutrients acquisition. Concerning P acquisition, Spohn & Kuzyakov (2013) suggested microbial-derived phosphatase mineralizes organic P to improve P availability thought microbes C allocation (Wei et al., 2019).

Extracellular enzymatic stoichiometry models (microbial nutrient limitation) was quantified by calculating the vector lengths and angles of the V_{max} of C, N and P acquisition enzymes. The models were used to explain microbial C, N and P limitation in response to changes environment nutrient availability (Moorhead et al, 2013; Cui et al., 2019, 2020). According to Cui et al., (2020), appropriate P limitation under N fertilization could decrease the loss of N, because microbial P limitation negatively affected the abundance of AOA *amoA*, AOB *amoA* (involved in nitrification) and *nirK*, *nirS*, *nosZ* (involved in denitrification) in semi-arid agriculture. Inorganic P addition led to an unbalance between C and P, decreasing microbial SOC mineralization due to improving P bioavailability (Liu et al., 2018; Cui et al., 2019, 2020). SOC mineralization induces simultaneous organic P mineralization and thus improve P bioavailability (Wang et al., 2019; Wei et al., 2019). Wei et al., (2019) showed that P fertilization in a P-poor (4.96 mg kg^{-1} Olsen P) paddy soil decreased the C:P ratio of root-detritus from 171 to 59. Microbial mineralization of this P-rich plant litter leads to a smaller microbial P immobilization than of P-poor litter and thus improves soil P availability. The C:P ratio (59) of the root-detritus in the P-rich soil (80 mg kg^{-1} Olsen P) soil met well the microbial community stoichiometry (C:P = 60)

(Cleveland & Liptzin, 2007). Accordingly, there was no significant change for the microbial biomass C:P ratio in P-fertilized after 150 days of incubation. Thus, the balance between microbial demand and resource supply of C and P indicated that microorganisms did not need to invest excess energy for P mining (Sinsabaugh et al., 2008, 2009; Zhang et al., 2013). Thus the decomposition of root-detritus with a low C: P ratio has potential to improve soil P availability; however, C and P imbalance may increase during the decomposition of root-detritus with a high C:P ratio (Wei et al., 2019). However, it is always unclear that the mechanism of the stoichiometry relationship of soil C and P effect soil bioavailable P in semi-arid available nutrients limitation soils in response to mineral P fertilizer and plant residues returning.

1.4 Rhizosphere effect

After fixation of atmospheric CO_2 plants translocated organic C to soil either by shoot or root residues input after plant death or as rhizodeposits (Lu et al., 2003; Hinsinger et al., 2009; Phillips et al., 2011; Liu et al., 2019). The latter represents the release of organic compounds by living roots into the soil (Kuzyakov & Xu, 2013; Pausch & Kuzyakov, 2018). Thus, plant roots not only take up nutrients from soil but also return them to the soil in the form of exudates, which are considered a labile source of energy for microorganisms (Liu et al., 2020; Fig. 3). The organic C, N, P, and S released can be immobilized by microorganisms (Elser & Urabe, 1999; Hinsinger et al., 2009). The soil volume that is influenced by rhizodeposition is defined as the rhizosphere and accounts for commonly 5–10% in topsoil and less than 5% in subsoil (Nannipieri et al., 2003; Kuzyakov & Blagodatskaya, 2015). Root

exudates usually consist of a mixture of organic compounds, including sugars, organic acids, amino acids, phenols, and other secondary metabolites, which are released during plant growth at different quantities (Bertin et al., 2003; Yuan et al., 2017).

Liu et al.(2020) simulated root exudate addition with three different C:N ratios (10, 20, and 40) to explore its effect on SOM decomposition adjusted by different proportions of the low-molecular-weight organic compounds glucose, oxalic acid, and glutamate to represent sugars, organic acids, and amino acids, respectively (Jones et al., 2004; Yuan et al., 2017). Ammonium sulfate was selected as a source of mineral N. The results revealed an increasing CO_2 emission with increasing C:N ratios of the root exudates due to higher microbial N demand. Thus labile C from root exudates have an important effect on SOM decomposition (Yuan et al., 2017; Liu et al., 2020). However the rhizosphere environment can become nutrients (i.e., P) limited due to high C input through root exudates (Kuzyakov, 2002; Wei et al., 2019). Already nowadays P is limiting for crop yield on > 30% of the world's arable land and world resources of inexpensive P may be depleted by 2050 (Vance et al., 2003). Plants have evolved a diverse array of strategies to obtain adequate P under limiting conditions, including modifications to root architecture, carbon metabolism and membrane structure, exudation of low molecular weight organic acids, protons and enzymes, and enhanced expression of the numerous genes involved in low-P adaptation (Vance et al., 2003). Higher liable C content in rhizosphere and lower liable C content bulk soils lead to the activity of two difference microbes' survival strategies (r- and K-strategists) for acquiring P in rhizosphere and bulk soils. The available C:P ratio (dissolved

organic C: Olsen-P) in soil is regulated by extracellular hydrolases for the C and P acquisition of microbes and plants.

The rhizosphere is the most active area concerning microbe–soil–plant interactions (Marschner et al., 2011; Kuzyakov & Xu, 2013). There a large amount of roots exudates are released into soils that are a readily available C source for microorganisms, turning the rhizosphere into a hotspot of microbial abundance and activity (Watt et al., 2006; Jones et al., 2009), which holds particularly true for P mineralization by rhizosphere microorganisms (Spohn et al., 2015). Plant–microbe interactions can be mutualistic as well as competitive (Spohn et al., 2015). Microorganisms in the rhizosphere can strongly mineralize organic P and solubilize bound inorganic P (Richardson et al., 2009). But they can also decrease the availability of P to plants by immobilizing P in their biomass, by decomposing P-mobilizing organic compounds released by roots, and by counteracting root-induced pH decreases by proton consumption during plant growth (Richardson et al., 2009; Marschner et al., 2011). However, the enzyme profiles of rhizosphere and bulk soils have rarely been distinguished, particularly in paddy soils. Additionally, the effect of P fertilization and root exudates (rhizosphere soil) on microbial acquisition of P from SOM in paddy soils is yet to be established.

1.5 Objective and Hypotheses

SOM and the release of nutrients by its mineralization is an important aspect in sustainable agriculture. The available carbon (C) to phosphorus (P) ratio in soil is regulated by extracellular hydrolases for C and P acquisition by microbes and plants.

The relation of total C:P ratios as well as available C:P ratios can be influenced by agricultural management strategies, e.g. the crop type and fertilization (Zhang et al., 2010; Wei et al., 2019; Yuan et al., 2019). The relationship of C:P interaction can be explore by ecological stoichiometry theory, using elemental ratios to predict nutrient retention and biomass production (Sinsabaugh, et al., 2009b; Sinsabaugh & Follstad Shah, 2012). However, the impact of different fertilizer types and levels of N, P, and K fertilization on SOC, the mechanism of exogenous N and P effect on microbial-mediated SOM decomposition and storage as well as the microbial nutrients-acquisition strategy remains unclear. On the hand, the overarching goal of this thesis was to elucidate the consequences of fertilization on SOC stock and the SOC mineralization as well as P availability. On the other hand, the objective of this thesis is improve the current understanding of the characteristics of microbial C and P acquisition to optimize P fertilizer application in P-limited paddy soil. Specifically, we examined the underlying mechanisms of C and P acquisition stoichiometry in model rhizosphere and bulk soils in response to P fertilization and C substrate addition.

To achieve these objectives, the following hypotheses were tested:

H1 Intensive fertilization increases SOC stocks in agricultural soils, being more pronounced at addition of organic substrates. The optimal amount of N, P, and K for SOC sequestration exist in agricultural upland soils.

H2 N and P fertilization decrease litter mineralization and priming effect in a semiarid agricultural soil duo to alleviating microbial nutrient limitation and

increasing litter-C use efficiency

H3 The decomposition of organic residues eliminated microbial P limitation and increased P availability by allocating C and P acquisition enzymes to balance the stoichiometric ratio of microbial C and P demand

H4 Microorganisms use different strategies to acquire P though microbial SOM mineralization in the model rhizosphere with high labile-C content and bulk soils with low labile-C content, leading to a reduced P:C acquisition ratio in model rhizosphere soil and an increase in bulk soil due to large amounts of P was clustered in the model rhizosphere soil.

To test these hypotheses, I carried out a combination of a meta-analysis and experimental studies to test them in the following way:

Firstly, in a meta-analysis I investigated the effects of types and levels of N, P, and K fertilization on SOC in global agricultural upland soils in order to quantify the different effect levels from 217 published studies. Then combining environmental variables (i.e., temperature, precipitation, water conditions, crop rotation and tillage type) explain the effect of SOC in agricultural upland soil under fertilization, thus testing **H1**.

Secondly, I differentiated the specific interactions of N and P fertilization on litter and SOC mineralization in a semiarid agricultural soil. I determined the fate of litter-C, dissolved organic C, microbial biomass C, and the maximal velocity (V_{max}) of BG, NAG, and AP, along with the associated CO_2 emission after exogenous nutrients, i.e. fertilized nitrogen (N) and phosphorus (P), and litter, thus contributing to **H2** and

H3.

Thirdly, I explored how the available C:P ratio affects the P bioavailability under mineral P and plant litter addition in semi-arid agricultural areas. Four bioavailable P fractions content ($CaCl_2$-P, Citrate-P, Enzyme-P, and HCl-P), dissolved organic C, Olsen-P, microbial biomass C and P, the V_{max} of C acquisition enzymes (BG, CBH, and XYL), the N acquisition enzymes (NAG and LAP), and the P acquisition enzyme (AP) were measured. Microbial nutrient limitation was quantified by calculating the vector lengths and angles of the V_{max} of C, N and P acquisition enzymes, exploring P availability under increasing or decreasing microbial C limitation in semi-arid Kazakhstan steppe soil, thus contributing to **H3**.

Fourthly, I evaluated the C:P acquisition stoichiometry in model rhizosphere and bulk paddy soils in response to P fertilizer and an cellulose amendment (to stimulate straw return). Dissolved organic C, Olsen-P, microbial biomass C and P, the V_{max} and saturation affinity constant (K_m) of C acquisition enzymes (BG and CBH) and P acquisition enzymes (acid and alkaline phosphomonoesterases) in model rhizosphere and bulk soil were measured. Linear relationships of C:P ratio between in available, microbial biomass and acquisition were used to clarify the mechanism of acquiring C and P in paddy model rhizosphere and bulk soils, thus contributing to **H4**.

With these experiments I envisaged to improve our understanding in the role of the stoichiometry of the C derived from plant litter to the main plant nutrients N and P for SOC storage and to provide a basis for SOC management by optimizing plant residue return and fertilization.

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2 Study 1

Meta-analysis on the effects of types and levels of N, P, and K fertilization on organic carbon in agricultural upland soils

Contribution: I participated in data collection, performed data analysis, prepared tables and figures, and wrote the manuscript.

Meta-analysis on the effects of types and levels of N, P, and K fertilization on organic carbon in agricultural upland soils

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Highlights

- A meta-analysis was used to investigate the effect of fertilization on SOC
- Multi-nutrient fertilization has shown the potential to improve SOC storage
- Intermediate N and K with high P fertilization affected SOC the most
- Climates and human activities under fertilization affect SOC

Abstract Most agroecosystems receive inputs of anthropogenically derived nutrients, which have an impact on soil organic carbon (SOC). However, the impact of different fertilizer types, as well as of different levels of nitrogen (N), phosphorus (P), and potassium (K) fertilization on SOC remains unclear. Here, we reviewed 217 published studies to identify the consequences of different types and levels of N, P, and K fertilization on SOC across global agricultural upland soils. The average effect size of fertilization on SOC was 0.2704 ± 0.0085 (95% confidence interval: 0.2538–0.2871, $p < 0.0001$). Categorical variable analysis revealed that the fertilization type significantly positively influenced the effect size, in the order of mineral plus organic fertilization > pure organic fertilization > pure mineral fertilization. The increasing of available nutrients led to farmyard manure or straw crop retention and limited nutrients loss, increasing C sequestration. Intermediate N (100 − 300 kg ha^{-1} $year^{-1}$) and K (50 − 150 kg ha^{-1} $year^{-1}$) application with high P (≥ 60 kg ha^{-1} $year^{-1}$) fertilization produced the largest effect on the SOC stocks. Heterogeneity analysis revealed that the annual average precipitation, annual average temperature, water conditions, and tillage type significantly affected the average effect size. Overall, the meta-analysis revealed that multinutrient fertilization, with intermediate N and K levels, and a high P level, decreased the dependency of the organisms on the original nutrients from SOM decomposition and had strong positive effects on increasing SOC in agroecosystems.

Keywords: Fertilization; Meta-analysis; Soil organic carbon; Effect size; Agricultural upland soils

1. Introduction

Agricultural land occupies 37% of the Earth's land surface (Smith et al., 2008), providing sustenance for over seven billion people globally (Dai et al., 2018). Soil organic carbon (SOC) stocks in croplands (111–170 Pg C) account for approximately 10% of total soil C up to a depth of 1 m (1500 Pg C) globally (Eswaran et al., 1993; Feng et al., 2014). The average SOC stocks of the arable layer (≤ 35 cm) in upland soils (aerated soils or not water affected soils in long term) are 31 Mg C ha^{-1} (Wei et al., 2021). As nutrients are exported during harvest, fertilization is necessary to ensure plant production and increase crop yield (Khan et al., 2019). However, mineral fertilizers alone can have negative consequences on ecosystems, such as soil degradation, groundwater pollution, surface water eutrophication, and greenhouse gas emissions (Conley et al., 2009; Divito et al., 2011; Honeycutt et al., 2020; Toljander et al., 2008; Wang et al., 2020). Hence, the type and amount of fertilizer must be adjusted to prevent detrimental effects (Chen et al., 2016). Fertilization can also reportedly help maintain SOC levels (Ashraf et al., 2020; Templer et al., 2012), a key parameter for sustaining soil fertility and productivity (Demyan et al., 2012; Khan et al., 2019; Wang et al., 2020). Han et al. (2018) reported that in typical agricultural region of subtropical China increasing SOC by 0.35 Mg C ha^{-1} $year^{-1}$ is related to an increase in wheat grain yield by 13.4%. According to Feng et al. (2014) and Mi et al. (2018), fertilization type (i.e. mineral, organic, combined mineral plus organic fertilization) and fertilization level are important factors for maintaining the SOC

balance or increasing the SOC stocks.

The main plant nutrients include nitrogen (N), phosphorus (P), and potassium (K) in organic and mineral forms, and their various combinations (i.e. NP, NK, PK, and NPK). Körschens et al. (2013) reported from 20 European long-term experiments that mineral fertilizer (NPK) increased SOC by approximately 10%, as compared with no fertilization, due to the increased plant productivity and crop residue input to soil. These results match well with the meta-analysis of Geisseler and Scow (2014), in which SOC increased by 8.5% on average upon the addition of mineral fertilizer. Kätterer et al. (2012) also reported an annual increase in SOC by 1–2 kg ha^{-1} for each kg of mineral N fertilizer applied in Swedish long-term cropland fertilization experiments.

Organic fertilizers, either as farmyard manure or crop residues, are an important source of soil organic matter (SOM) and an effective substitute for mineral fertilizer inputs (Chen et al., 2016; Ding et al., 2017). They can supply nutrients to crops and are beneficial for soil quality, providing prolonged nutrient effects after application (Feng et al., 2014). The combined application of mineral and organic fertilizers increases crop yield and SOC content when compared with only mineral fertilizer application (Hua et al., 2020; Morra et al., 2010). Chivenge et al. (2011) reported in a meta-analysis that organic resources with N fertilizers increased SOC by 12% in sub-Saharan Africa.

Despite these finding over a wide range of climates, soil types and farming practices, the effect of fertilization on SOC has been controversially discussed

because of the application of different levels of fertilizer. Different fertilization levels to agricultural soils can increase (Obour et al., 2017) or decrease (Hao et al. 2017) the SOC content or cause no change at all (Liang et al., 2014). Chen et al. (2016) reported an increase in SOC by approximately 8% after mineral N (300 kg ha^{-1}) fertilization. In contrast, Zhong et al. (2015) found that mineral N (360 kg ha^{-1}) fertilization decreased SOC by approximately 35%.

Besides the types and levels of fertilization, SOC levels in agroecosystems are also closely related to the climate (precipitation and temperature), water conditions (alternate wetting and drying), tillage types, crop rotation, etc. (Khan et al., 2019; Trumbore et al.,1996;Wei et al., 2021; Six et al., 1999). Wei et al. (2021) reported that SOC in paddy soils was generally highest under humid conditions in subtropical climates, while according to Gupta Choudhury et al. (2018), Tian et al. (2013), Witt et al. (2000) SOC in upland soils was highest under dry conditions in temperate climates. In addition, soils under continuous paddy had higher SOC stocks than soils experiencing intervals of aerobic and anoxic conditions (Keiluweit et al., 2018; Wei et al., 2021). Thus, water conditions are an important factor that affect SOC in agricultural upland soils (Qiu et al., 2018; Ashraf et al., 2020). Furthermore, when compared with conventional tillage (CT), no tillage (NT) causes the least amount of soil disturbance, stimulates biological activity, and enhances aggregate formation, and is thus a significant component of conservation agriculture (Nicoloso et al., 2016; Šimanský et al., 2017). The tillage also affects SOC stocks. So Luo et al. (2010) in a meta-analysis based on global data from 69 paired-experiments found an increase in

SOC stocks of the surface layer (0–10 cm) by 3.15 ± 2.42 t ha^{-1}, but declined by 3.30 ± 1.61 t ha^{-1} in the 20–40 cm soil layer.

To elucidate the relationship between the SOC of agricultural uplands and fertilization along with management and environmental factors, we conducted a meta-analysis, which is as a powerful tool to compute site specific, temporally variable results and to draw general conclusions at a global scale (Geisseler & Scow, 2014; Jian et al., 2016; Luo et al., 2010; Ren et al., 2017). Thus, quantifying the influence of the types and levels of fertilization can resolve uncertainties regarding the spatial and temporal variations in SOC related to fertilization. Previous meta-analyses have mainly analysed the impact of mineral and organic fertilization on SOC from a microbial and C emission perspective (Geisseler & Scow, 2014; Ren et al., 2017; Wei et al., 2021), few researches focuses on the effect of fertilizer types and levels on SOC. Therefore, the objective of the current study was to analyse the impact of fertilization, particularly with respect to fertilizer type and amount of nutrient application, on SOC in agricultural upland soils. We hypothesised that (1) fertilization significantly increases the SOC in agricultural upland soils; (2) the combination of organic and mineral nutrient application has the largest effect due to OC input by organic fertilizer and increasing crop residue input with fertilization; and (3) precipitation and temperature as well as tillage types under fertilization affect SOC sequestration in agricultural upland soils.

2. Materials and methods

2.1 Selection criteria and data collection

To quantify the effect of fertilization on SOC, we analyzed the results from peer reviewed articles indexed by the Web of Science (http://apps.webofknowledge.com/) database from 1945 to 2020 in a meta-analysis using the following search terms: “fertilization” AND (“SOM” OR "soil organic matter" OR “SOC” OR "soil organic carbon") NOT “forest”, NOT “grassland”, NOT “paddy”.

Only primary studies that satisfied the following criteria were included: (i) all studies reported must include an unfertilized control and a treatment with fertilization; (ii) fertilization must not include biochar, Cu, Zn, and Mo; (iii) the experimental duration were clearly recorded, and measurements of the variables in the experimental and control groups were performed at the same spatial and temporal scales; (iv) at least two replicates for each treatment were conducted; and (v) the means, sample sizes, and standard deviations (SDs) or standard errors (SEs) of the chosen variables (SOC) were directly provided. When the studies reported data from several soil layers, data only on topsoil were included in the present study. Finally, 1137 data points from 217 articles across the world met our criteria and were included in the synthesis analysis (Fig. 1). Missing latitude, longitude, and elevation data were estimated using Google Maps (https://maps.google.com/). Climatic data included mean annual precipitation (MAP) and mean annual temperature (MAT), and if climatic data were not provided in the manuscripts, they were obtained from https://en.climatedata.org/. In addition to climatic data, water conditions (alternate wetting (W) and drying (D)), tillage type (CT: conventional tillage (20–30 cm); DT: deep tillage (30–45 cm); RT:

reduced tillage (10–20 cm); MT: minimum tillage (≤ 10 cm); and NT: no tillage), and crop rotation were considered as moderators, i.e. supporting variables that help to explain the effect of fertilization on the SOC content or stock. Some data were extracted from published figures using the Getdata software (Version 2.20).

2.2 Data analysis

When data on the SOM content or stock were reported, they were divided by 1.724 to calculate the SOC content or stocks (Allison, 1965). When different amounts of N, P, and K fertilizers were applied for different years, their average value was calculated as the rate (kg ha^{-1} $year^{-1}$). Two categorical variables, fertilization type and level, were introduced. The fertilization types were differentiated into mineral (N, P, and K), organic (manure, slurry, compost, and straw) (Liang et al., 2014), and mineral plus organic. With respect to the effects of the amount of fertilization, low (≤100, 20, and 50 kg ha^{-1} $year^{-1}$), intermediate (100–300, 20–60, and 50–150 kg ha^{-1} $year^{-1}$), and high (≥ 300, 60, and 150 kg ha^{-1} $year^{-1}$) levels for N, P, and K, respectively, were differentiated for mineral fertilization (M), organic fertilization (O), and mineral plus organic fertilization (MO) to explain the intensity of the effect size. The means, standard deviations (SD), and sample sizes (n) of the selected variables were extracted from the articles for each case study. If only the standard errors (SE) were given in a paper, the SD was calculated according to the following formula:

$$SD = SE\sqrt{\mathrm{n}}$$

where n represents the number of replicates (sample size).

The natural log of the response ratio (effect size) was used as the effect size according to the following equation (Hedges et al., 1999):

$$Effect ln\left(\frac{X_t}{X_c}\right) = ln\left(X_t\right) - ln\left(X_c\right)$$

where X_t and X_c represent the means of SOC in the fertilised and control treatments, respectively.

The variance (υ) was estimated according to Chen et al. (2016), as follows:

$$\upsilon = \frac{S_t^2}{n_t x_t^2} + \frac{S_c^2}{n_c x_c^2}$$

where n_t and n_c represent the sample sizes for the fertilization treatments and control, respectively. S_t and S_c represent the SD for fertilization treatments and control, respectively.

Average effect sizes and 95% confidence intervals (CI) were calculated using random effect models (Geisseler & Scow, 2014), including the following analyses:

Weight of individual (w_i) study conclusions: $w_i = 1/(v_i + \tau^2)$

Average effect size: $\acute{y} = \frac{\sum_{i=1}^{k} w_{i=1}^{k} y_i}{\sum_{i=1}^{k} w_i}$

Overall standard error: $SE = \sqrt{\frac{1}{\sum_{i=1}^{k} w_i}}$

95% confidence interval of average effect value: $CI = \acute{y} \pm 1.96 \times SE$

where v_i is the intra study variance, τ^2 the inter study variance, and y_i is the single study effect value.

Heterogeneity test of effect size (Q_t):

$$Q_t = \sum_{i=1}^{k} w_i \left(yi - \acute{y}\right)^2$$

Test of the influence of explanatory variables on effect size (Q_m):

$$Q_m = \sum_{j=1}^{p} \sum_{i=1}^{ni} w_i (y_{ij} - \acute{y})^2$$

The ratio of total (residual) heterogeneity to total (unaccounted) variability (I^2):

$$I^2 = \frac{\tau^2}{\tau^2 + S^2}$$

where S^2 is the residual variance:

$$S^2 = \frac{(k-1)\sum w_i}{(\sum w_i)^2 - \sum W_i^2}$$

Effect size variation (R^2):

$$R^2 = \frac{\tau^2_{\Re} - \tau^2_{ME}}{\tau^2_{\Re}}$$

where $\tau_{\Re}$ is the inter study variance in the random effects model without the explanatory variables, and τ_{ME} is the inter study variance in the mixed effects model with all explanatory variables added.

All analysis and figures were made using R software with the "metafor" package (BenítezLópez et al., 2017; Viechtbauer, 2010). The variance–covariance matrix was computed due to non-independence of the effect sizes (Midolo et al., 2019). The estimated effect size and standard error were analysed using the random effect model, in which SOC was used a random factor (independent factor). Fertilization types and levels were used categorical variables. The residual heterogeneity with different moderators was explained using a mixed effects model, in which MAP, MAT, water conditions, tillage types, and crop rotation were used as moderators. The explained moderator heterogeneity statistic (Qm) was calculated to test for significance in single covariate meta regressions (Du et al., 2021). Two by two comparison in fertilization

types and levels, MAP, MAT, water condition, tillage types and crop rotation on SOC used *holm* method.

3. Results

3.1 Effect size of fertilization on SOC

In the individual studies chosen for this meta-analysis, fertilization effects on SOC in agricultural upland soils were mostly positive. Over all studies, the calculated value of the effect size ranged from -0.30 ± 0.003 to 2.19 ± 0.039, with an average effect size of 0.2704 ± 0.0085 (95% confidence interval (CI): 0.2538–0.2871) (Fig. 2), thus illustrating significant (P < 0.0001) overall positive response of SOC to fertilization. In addition, there was a significant residual heterogeneity in the random effects meta-analysis for the SOC dataset (I^2=99.55%, Qt =219212.0023, P < 0.0001; Table 1), which we attempted to explain using different moderators via categorical variable analysis.

3.2 Effect size of different fertilizer types and amounts on SOC

The test of moderators (I^2=99.39%, Qm=1632.3818, p < 0.0001; Table 1) revealed a significant difference in effect size among fertilization types based on a mixed effects model (Fig. 3). The average effect size was significantly affected by the three fertilization types in the order of MO (0.41 ± 0.01) > O (0.38 ± 0.02) > M (0.13 ± 0.01). The order of the effect of different components in M on the effect size was NP > NPK > NK > K > PK > P > N, which has no significant different; the order of

the effect of different components in M plus farmyard manure (F) fertilization on the effect size was PKF > NPKF > F > NPF > NF > PF, in which PKF was significantly higher than NPKF, F, NPF, NF and PF; the order of the effect of different components in M plus straw (S) fertilization on the effect size was NPKS > NPS > NS > S, in which S was significantly lower than NPKS, NPS and NS (Fig. 4). Overall, S fertilization needs always mineral fertilizer as well in order to have a positive response on SOC stocks. In contrast, F is having a direct positive impact on SOC stocks. Interestingly, only if K added as mineral fertilizer and plus farmyard manure, the effect level is larger as for farmyard manure alone.

Different levels of N, P, and K fertilizer from the three different fertilization types had significantly different effect sizes on the OC in agricultural soils ($p < 0.05$), however effect size was no significantly affected between three different levels of N, P, and K fertilizer from the three different fertilization types. In terms of effect size alone, for N, the order of effect size was intermediate N level > high N level > low N level for M, O and MO (Fig. 5a); for P, the order of effect size was high P level > intermediate P level > low P level for M and O, and low P level > high P level > intermediate P level for MO (Fig. 5b); and for K, the order of effect size was intermediate K level > high K level > low K level for M, high K level > intermediate K level > low K level for O, and high K level > low K level > intermediate K level for MO (Fig. 5c). The test for residual heterogeneity (Qe = 155419.6541, $p < 0.0001$; Table 1) showed that the residuals were still heterogeneous, and that other moderators should be included.

3.3 Response of average effect sizes on environmental and management factors

As explanatory variables for the heterogeneity in effects of fertilization on the SOC content or stocks in arable soils. MAP, MAT, water conditions, tillage type, and crop rotation were introduced. The heterogeneity analysis revealed that the explanatory variables MAP, MAT, water condition, and tillage type had a significant impact ($p < 0.0001$) on the average effect size, and that they could explain approximately 1.00%, 1.05%, 0.46%, and 4.01% of effect size variations, respectively (Table 2).

3.4 Symmetry test of effect size of fertilization on OC in agricultural upland soils

A meta-analysis involves the quantitative evaluation of the average effect size of variables. The data are obtained from published papers, which may be affected by selection bias. Therefore, funnel plots with Egger's test (Du et al., 2021) and failsafe numbers (Viechtbauer, 2010) were used herein to test for potential publication bias. The funnel plots were not asymmetric ($z = 4.7484$, $p < 0.0001$) (Fig. S2), however the failsafe analysis indicated that 35705435 additional studies with null results would be needed in the dataset to reduce the significance level to $p = 0.05$. As such, publication bias was not we did not considered as an issue for the interpretation of the results.

4. Discussion

4.1 General effects of fertilization on SOC

The results of this meta-analysis demonstrated that fertilization significantly increased the SOC content or stocks of agricultural upland soils, with an average effect size of 0.2704 ± 0.0085. When examining the three main types of fertilization, the effect size of the mineral fertilization was 0.13 ± 0.01. These results are consistent with a previous meta-analysis that reported an effect size of 0.12 for long-term mineral fertilization on SOC in global agricultural upland soils (Geisseler & Scow, 2014). Although the authors reported a decrease in SOC over time, this decrease was less pronounced in plots that received mineral N compared with the unfertilized control (Ladha et al., 2011). There are multiple processes by which mineral fertilization influences SOC stocks in agricultural soils. Mineral fertilization (i.e., N, P and K) increases photosynthetic C uptake by plants and thus increases crop residue input to soil (Saffigna et al., 1989; Liang et al., 2014). Concurrently, mineral fertilization directly improves nutrient availability in the soil, leading to higher crop root exudation due to crop growth (Willig et al., 2020; Zhu et al., 2016). This in return promotes microbial metabolism, thus increasing the microbial biomass C and microbially derived SOM in the soil (Liu et al., 2020). Finally, mineral fertilization reduces SOM decomposition through increased microbial turnover, decreasing the dependency of the organisms on the original nutrients from SOM decomposition. (Ding et al., 2017; Liu et al., 2018). It can have positively influence on C sequestration. Together, these processes lead to a smaller C loss by microbial mineralization as the input of crop residues, leading to an overall increase in SOC.

4.2 Role of individual nutrients for SOC

When investigating the effects of the main nutrients N, P, and K, our results indicate the increasing of available N, P and K is beneficial to SOC sequestration. Li et al. (2020) reported that single P and K fertilization and their co-application did not significantly change crop yield and SOC, but solo N significantly increased the yield without changing SOC. This illustrates that N is the main limiting factor for the growth of crops in agricultural upland soils. However, to translate the increased plant productivity into raising SOC values, following SOC stabilization must be assured by combining multiple nutrients. The impact of multi-nutrient combined fertilization (NP, NK, PK, NPK) on SOC is higher than that of single nutrient fertilization (N, P, K) (Fig. 4) because multi-nutrient fertilization provides more balanced nutrition for both microbial populations and plants, resulting in higher SOC accumulation in soils (Dai et al., 2018). Hence, our study is in line with the study of Li et al. (2020), who reported that NP and NPK fertilization resulted in 19 – 47% higher SOC stocks than single N, P, and K fertilization. Combined multi-nutrient fertilization thus has the potential to improve not only soil fertility but also SOC stocks.

4.3 Impact of farmyard manure and crop residues on SOC

The effect sizes of organic fertilization and organic plus mineral fertilization on the SOC were 0.38 ± 0.02 and 0.41 ± 0.01, respectively, which were clearly higher than that of mineral fertilization 0.13 ± 0.01. This illustrates that organic manure and residue management are important for improving SOC (Ladd et al., 1994; Mi et al.,

2018). When addressing organic matter application to soil, one needs to differentiate between organic manure and crop residues. Organic manure is an important source of SOM and an effective substitute for mineral fertilizer inputs (Ding et al., 2017). For example, horse and pig manure (18600 and 22500 kg ha^{-1} $year^{-1}$, respectively) increased SOC by 13% over 35 years and 32% over 37 years, respectively (Ashraf et al., 2020; Ding et al., 2017). Under a high input of organic manure, crops have strong and extensive root systems (Zhang et al., 2020). As such, the application of manure helps maintain the soil nutrient balance, improves soil structure and water holding capacity, and is beneficial for environmental protection compared with the application of mineral fertilizers alone (Mwangi & Box, 2010). However, this also leads to spatial heterogeneity in resource distribution, resulting in the microbial decomposition of organic materials, which can release organic and inorganic nutrients for plant uptake (Zhang et al., 2020). Finally, the input of crop residues in the form of roots and stubbles increases as a result of fertilization of organic manure, which in turn increases the SOC content more than that with mineral fertilization alone (Sherrod et al., 2005).

Also straw input has been shown to be important for increasing SOC (Fig. 4). Straw, derived from wheat, maize, soybean and corn, is the main form of crop residue in agricultural practice (Guo et al., 2014; Li & Han, 2016; Yang et al., 2015). These crop residues include easily decomposable as well as more stable substrates (Liu et al., 2020; Mi et al., 2018). The return of crop residues to soil stimulate microbial activity to accelerate the accumulation of microbial residues in SOM and enhance the

contribution of microbial residues to SOM sequestration (Liu et al., 2019). However, the effect level was least, when no nutrients were added with additional fertilization (Fig.4). Residues of wheat and many other cereals are characterized by high C:nutrient (i.e., N, P, K, S, micronutrients) ratios. The high C: nutrients ratio mineralize more organic C to acquire more nutrients for microbes along with a higher investment of extracellular hydrolytic enzymes (Wei et al., 2019), leading to a reduce microbial carbon use efficiency (CUE) (Wang et al., 2019) and increase nutrients use efficiency (Mooshammer et al., 2014).

Application of mineral fertilizer in addition to crop residue return leads to a lower C:nutrient ratios, which modifies the decomposition pathways of crop residues (Soong et al., 2018). Added mineral nutrients (as available nutrients) can be preferentially utilized by microorganisms, leaving crop residue intact (Duan et al., 2021). Furthermore, crop residue retention can decrease mineral N, P, and K fertilizer losses by inducing N, P, and K immobilization in the short term, increasing C sequestration in the soil, and enhancing soil quality (Plante et al., 2006; You et al., 2014; Zhao et al., 2014). Therefore, straw plus mineral fertilization leads to more SOC than straw return alone (Fig. 4). Interestingly, only if K added as mineral fertilizer and plus farmyard manure, the effect level is larger as for farmyard manure alone. This means that low-quality residues (high C:N:P) ratio is simply burned by microorganisms, and additional nutrients are needed to increase the CUE of the residue. On the contrary for high-quality organic substrates, obviously the C:N:P ratio is obviously optimum. Microbial CUE of the residues decrease after mineral

fertilization. Thus, it infers that K is likely an inhibition of oxidative enzymes involved in the degradation of aromatic compounds by K in combination with a reduced energy requirement for microbial K acquisition in the fertilized soils (Spohn et al., 2016). Overall, organic fertilization improves SOC stocks. Particularly, for organic amendments with high C:nutrient ratios additional nutrient dressings are decisive to shift the decomposition more from catabolic to anabolic pathway (Akhtar et al., 2019; Ashraf et al., 2020; You et al., 2014).

4.4 Role of the amount of fertilizer application on SOC

Adapted mineral N, P, and K fertilization contributes not only to the maximum crop yield but also to the amount of plant residues returned to the soil (Geisseler & Scow, 2014). With respect to the amount of fertilization, our results show that N and K have the strongest impact on effect size at intermediate levels (100–300 kg N ha^{-1} $year^{-1}$, Fig 5a, and 50–150 kg K ha^{-1} $year^{-1}$, Fig. 5c) of fertilization, whereas for P, a high fertilizer amount (≥ 60 kg P ha^{-1} $year^{-1}$, Fig. 5b) resulted in the greatest effect size. Hence, for N and K, there appears to be a positive response of the amount of fertilization on SOC stocks, whereas beyond a certain level, higher fertilization rates rather lead to a decline in the SOC level. Compared with intermediate N, excess N fertilization (> 300 kg ha^{-1} $year^{-1}$) combined with a low N use efficiency led to N loss by leaching and deterioration in soil structural quality, causing a negative effect on C sequestration (BlancoCanqui & Schlegel, 2013; Brown et al., 2014; Follett et al., 2005; Zhu et al., 2016). At low N fertilization rates (<100 ha^{-1} $year^{-1}$), roots exudate less

organic substances into the soil to gain nutrients through SOM decomposition for the growth of crops, thus causing a reduction in SOC content (Zhao et al., 2019). Similarly, intermediate K fertilization (50–150 kg ha^{-1} $year^{-1}$) alleviated soil K depletion and increased soil K fertility (Zhao et al., 2014), however excess K fertilization (≥ 150 kg ha^{-1} $year^{-1}$) couldn't stimulate the rate of OC transfer from the crop residues and roots to induce significant changes in SOC pool (Yuan et al., 2021). Differently, the efficient use of P fertilizer with a goal to improve SOC levels ultimately enhancing crop production and simultaneously increasing soil C sequestration, which highly depends on soil initial P fertility (poor or rich P) (BlancoCanqui & Schlegel, 2013;Bansal et al., 2020). In addition, P has a low plant availability due to sorption or occlusion within aluminium and iron in acidic soils, or calcium and magnesium cations in alkaline soils. It leads to an increase in SOC with the amount of P fertilizer increasing.

In addition to the local climate or other management factors, intermediate N and K with high P fertilization may stimulate both the growth and development of plant shoots and roots (Razaq et al., 2017; Sustr et al., 2019), leading to more photosynthetic derived C being allocated to soil through crop residues and rhizodeposition (Zang et al., 2019). Therefore, comparable to the well-known concept of optimum fertilization with respect to crop yield, SOC gains seem to follow a nonlinear correlation with fertilizer amounts. To some extent this might be a result of the input amounts following the optimum concept of crop yield; but our analysis of literature also indicates that soil processes, like triggering of anabolic and catabolic

functions of microbes, are affected by the amount of fertilizers leading to a delicate balance between positive and negative fertilizer effects.

4.5 Modulation of fertilizer effects on SOC by environmental variables

Environmental variables also affected OC stocks in upland agricultural soils. Our results show that MAP, MAT, water conditions, and tillage types caused variation in effect value. Li et al. (1994) and Wei et al. (2021) reported that MAP is an important variable for the impact of fertilization on SOC stocks. Our results are in line with Márton (2008), who stated that precipitation is negatively related to SOC with mineral fertilization. In the 20-year experimental term the site of the lower precipitation (204 mm) observed that SOC stocks from 1.28 $Mg \cdot ha^{-1}$ to 1.79 $Mg \cdot ha^{-1}$ (Márton, 2008). This is because SOC correlates quite well with climate (precipitation) and a number of important soil physical, chemical and microbiological changes as a consequence of fertilization (Adams et al., 1995; Kirschbaum et al., 2001). When it comes to the effects of MAT, our results indicate a decreasing impact of higher temperatures (i.e. >15℃ as compared to 5–15℃) on the effect size of fertilization on SOC. This is because the temperature in the long-term agricultural soil increase can lead to an increased decomposition of SOM (Wiesmeier et al., 2015). Trumbore et al. (1996) reported that decreasing temperature with altitude have been shown to limit SOC turnover, leading to enhanced SOC storage. Soil moisture (water conditions) is an important factor for plants to utilize added nutrients for plant growth (Kramer, 1944), and it is also necessary for soil microbial growth and activity (Cui et al., 2020; Skopp

et al., 1990). Therefore SOM mineralization rates is regulated by oxygen limitations through water conditions (alternating anaerobic wetting and aerobic drying) (Keiluweit et al., 2018; Liu et al., 2016). Even within seemingly well-drained upland soils when oxygen consumption (microbial respiration) in soil microsites outpaces oxygen supply (through diffusion), oxygen limitations may arise in otherwise well-aerated soils (Keiluweit et al., 2018; Wei et al., 2021). Regular intervals of aerobic and anaerobic conditions lead to the accumulation of SOC stocks in fertilized arable systems (Ashraf et al., 2020) because of reduced decomposition of crop residues under anaerobic conditions (Qiu et al., 2018). Another meta-analysis demonstrated that aerobic conditions have a positive effect on soil bacterial diversity, while an anaerobic conditions have a negative effect on soil bacterial diversity with fertilization in agroecosystems worldwide (Dai et al., 2018).

Also different tillage types modulate the effect of fertilization on SOC stocks (Table 2). De Sanctis et al. (2012) reported that over a 50 year simulation period, the SOC content in the top 40 cm of soil was consistently higher under NT than that under CT at a given level of N fertilizer application (90 and 180 kg ha^{-1} $year^{-1}$). The simulated SOC under NT increased at a mean annual rate of 0.43, 0.31 and 0.03 t ha^{-1} in response to 180, 90 and 0 kg N ha^{-1} $year^{-1}$, respectively. This can be explained by the higher crop residue return to the surface soil at NT with increasing fertilization. Also ÁlvaroFuentes et al. (2012) found that at NT, higher N addition resulted in greater C inputs and an increase in SOC, while at CT, N addition did not affect C inputs or SOC stocks at 0–30 cm soil depth. Also Poirier et al. (2009) reported higher

SOC stocks in the surface soil layer at NT at a given mineral fertilization level, reflecting greater residue accumulation in the soil surface. Tillage incorporates crop residues to soil layers of 5–45 cm and induces changes in the soil SOC distribution compared with that in natural soils (Luo et al., 2010). Consequently, in a global meta-analysis, Luo et al. (2010) reported that SOC wasy 3.15 ± 2.42 t ha^{-1} larger under NT in the 0-20 cm soil layer but 3.30 ± 1.61 t ha^{-1} smaller in the 20–40 cm soil layer than under CT. Thus, NT has been considered an effective way to increase the SOC stocks in surface soil (Luo et al., 2010; Šimanský et al., 2017). Thermal conditions and a disturbed soil microbiota community with CT as compared to NT (Coppens et al., 2007; Mazzoncini et al., 2011; Six et al., 1999) might have additional consequences of the different impact of soil management on the effect level of fertilization on SOC stocks.

5. Conclusion

Our study, using a heterogeneity test and categorical variable analysis on a global dataset, demonstrated a significant positive response of fertilization on SOC in agricultural upland soils. Combined organic plus mineral fertilization had the largest impact on SOC stocks, followed by organic fertilization alone, while the effect of mineral fertilization alone was minor. Concerning organic fertilization, the C:nutrient ratio of the substrate is decisive. For low-quality substrates (i.e. high C:nutrient ratios) additional nutrient dressings are necessary to secure a high effect level. Intermediate N (100–300 kg ha^{-1} $year^{-1}$) and K (50–150 kg ha^{-1} $year^{-1}$) application with high P (≥

60 kg ha^{-1} $year^{-1}$) fertilization produced the largest effect on the SOC stocks, indicating moderate fertilization reduces SOM decomposition through increased microbial turnover, which might positively affect C sequestration. The effects of fertilization on SOC stocks is modulated by environmental factors such as MAP and MAT and soil management. The impact of soil properties (i.e., clay and pH) and different microbial taxa on the contribution of fertilization to SOC should be paid more attention in the future.

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Tables

Table. 1 Test of heterogeneity for the effect sizes of fertilization and categorical variable analysis of the different types on fertilization effect size among global agricultural upland soil

	Test for heterogeneity (Qt)	df	*p*	τ^2	I^2
Upland	219212.0023	1135	<0.0001	0.0769	99.55%
Fertilization types	Test for moderators (Qm)	df	*p*	τ^2	I^2
	1632.3818	3	<0.0001	0.0583	99.39%
	Test for residual heterogeneity (Qe)	df	*p*		
	155419.6541	1133	<0.0001		

τ^2, interstudy variance; I^2, ratio of total (residual) heterogeneity to total (unaccounted) variability.

Table. 2 Analysis of the effects of environmental and management factors on the effect size

Moderators	df	Test of moderator (Qm)	intrcpt	*p*	τ^2	I^2	R^2
MAP	1	13.5408	0.3258	0.0002	0.0761	99.54%	1.00%
MAT	1	12.1642	0.2114	0.0005	0.0761	99.54%	1.05%
Water condition	1	5.9378	0.2759	0.0148	0.0765	99.54%	0.46%
Tillage types	4	52.0623	0.2664	<0.0001	0.0738	99.53%	4.01%
Crop rotation	1	0.2606	0.2778	0.6097	0.0769	99.54%	0.00%
Total	8	105.8785	0.2435	<0.0001	0.0707	99.49%	8.10%

MAP, mean annual precipitation; MAT, mean annual temperature; τ^2, interstudy variance; I^2, ratio of total (residual) heterogeneity to total (unaccounted) variability; R^2, effect size variation.

Figure captions

Fig. 1 Geographical location of the 217 studies included in the meta-analysis. Locations are indicated by red dots. Countries that hosted at least one study are green in colour. Red dots may represent multiple effect sizes from multiple individual studies.

Fig. 2 Forest plot of effect of fertilization on SOC in agricultural upland soil. Effect size, response ratios and black dots with 95% confidence intervals (CI). Red dotted line and red dashed line represent effect size = 0 and averaged effect size.

Fig. 3 Forest plot of the effect of three different fertilization types (mineral, organic, mineral plus organic fertilization) on SOC in agriculture upland soil. Effect size, response ratios and black dots are presented with 95% confidence intervals (CI).

Fig. 4 Forest plot of effect of different combinations of N, P, and K fertilization in mineral, farmyard (F) and mineral, and straw (S) and mineral fertilization on SOC in agriculture upland soil. The numbers above and bottom the single points mean sample size.

Fig. 5 Forest plot of effect of N (a), P (b), and K (c) fertilization level (low, intermediate, and high levels) from mineral, organic, and mineral plus organic fertilization on SOC in agriculture upland soil. L, low levels; M, intermediate levels; H, high levels.

Fig. 1

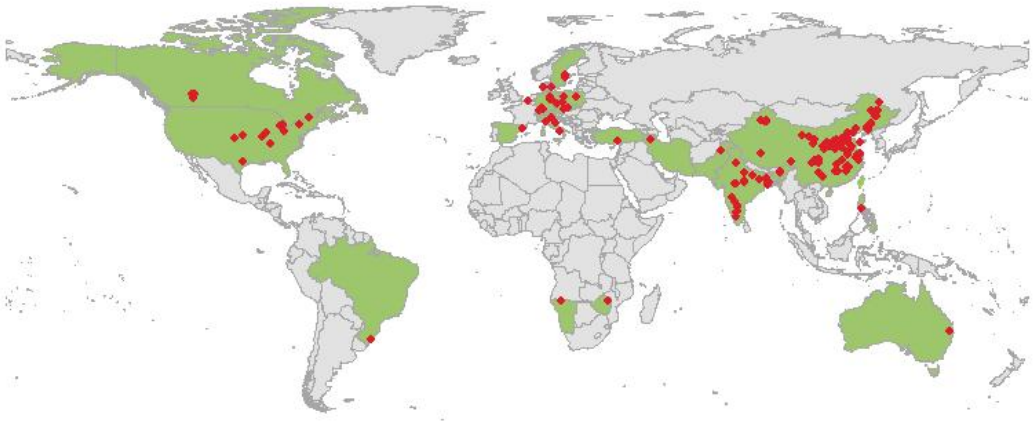


Fig. 2

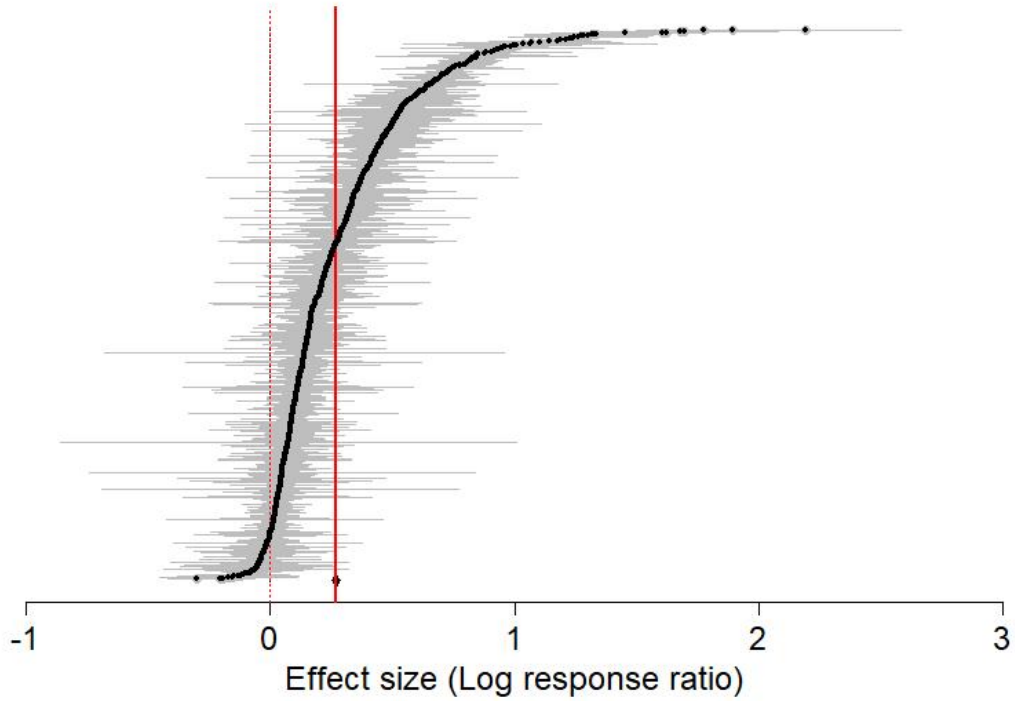


Fig. 3

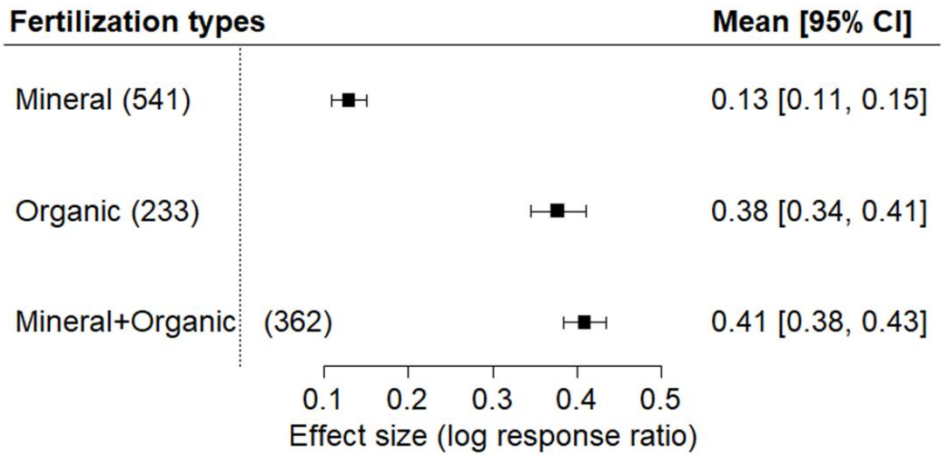

Fertilization types
Mean [95% CI]
Mineral (541)
0.13 [0.11, 0.15]
Organic (233)
0.38 [0.34, 0.41]
Mineral+Organic (362)
0.41 [0.38, 0.43]
0.1
0.2
0.3
0.4
0.5
Effect size (log response ratio)

Fig. 4

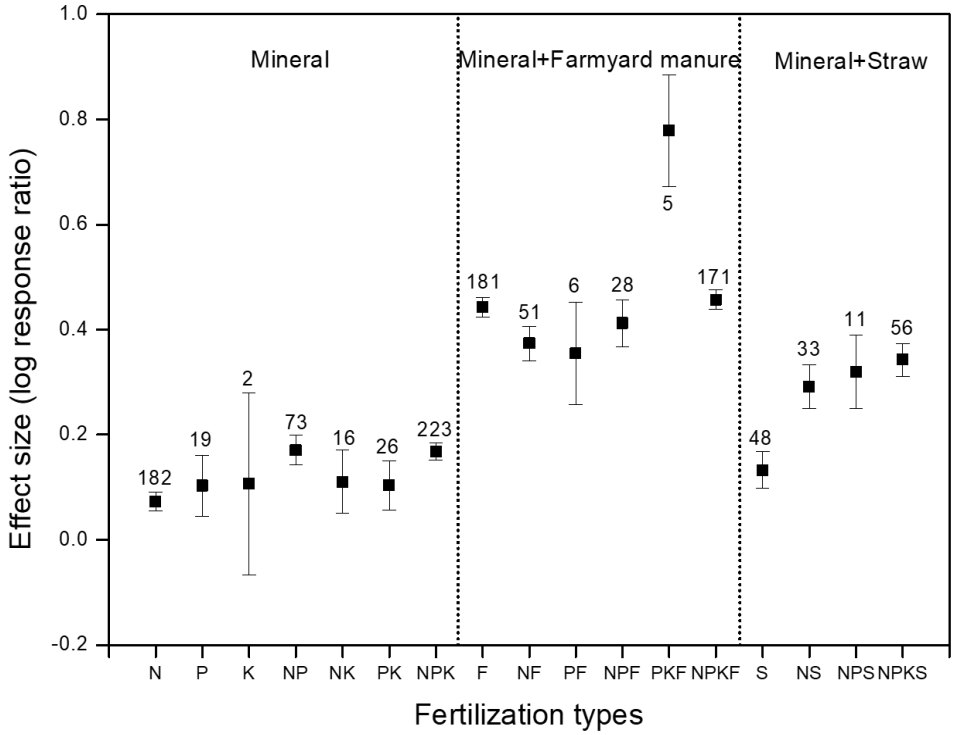
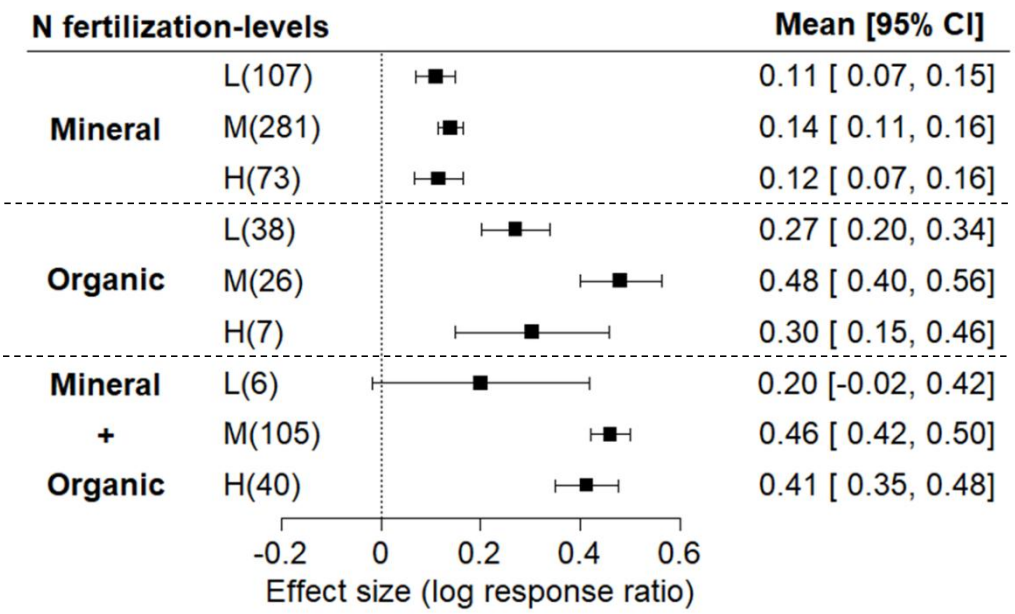
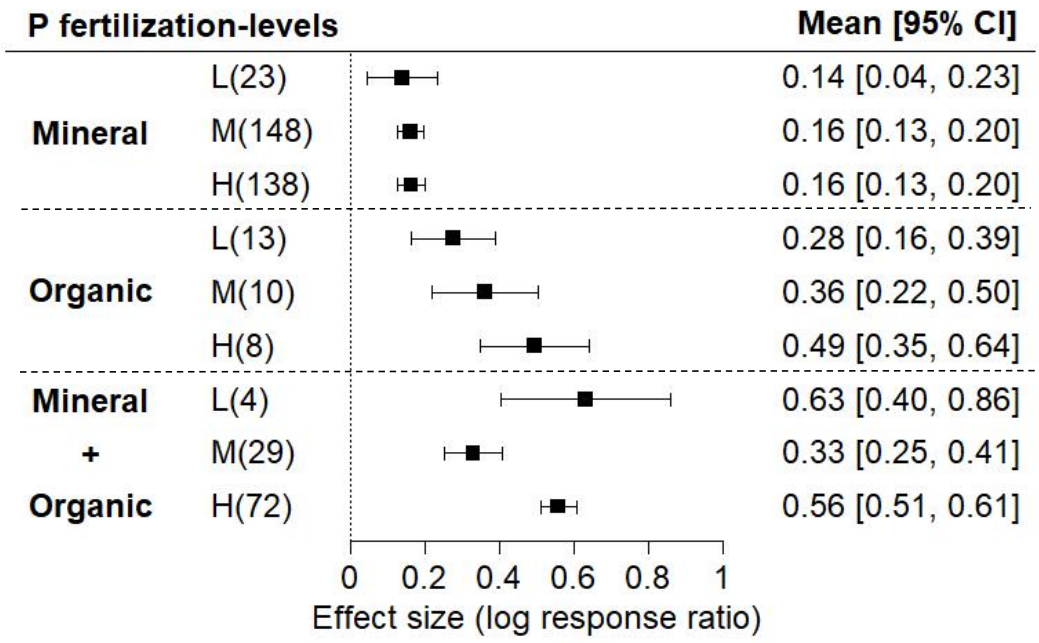

Mineral
Mineral+Farmyard manure
Mineral+Straw
Effect size (log response ratio)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
182
19
2
73
16
26
223
181
51
6
28
5
171
48
33
11
56
N
P
K
NP
NK
PK
NPK
F
NF
PF
NPF
PKF
NPKF
S
NS
NPS
NPKS
Fertilization types

Fig. 5

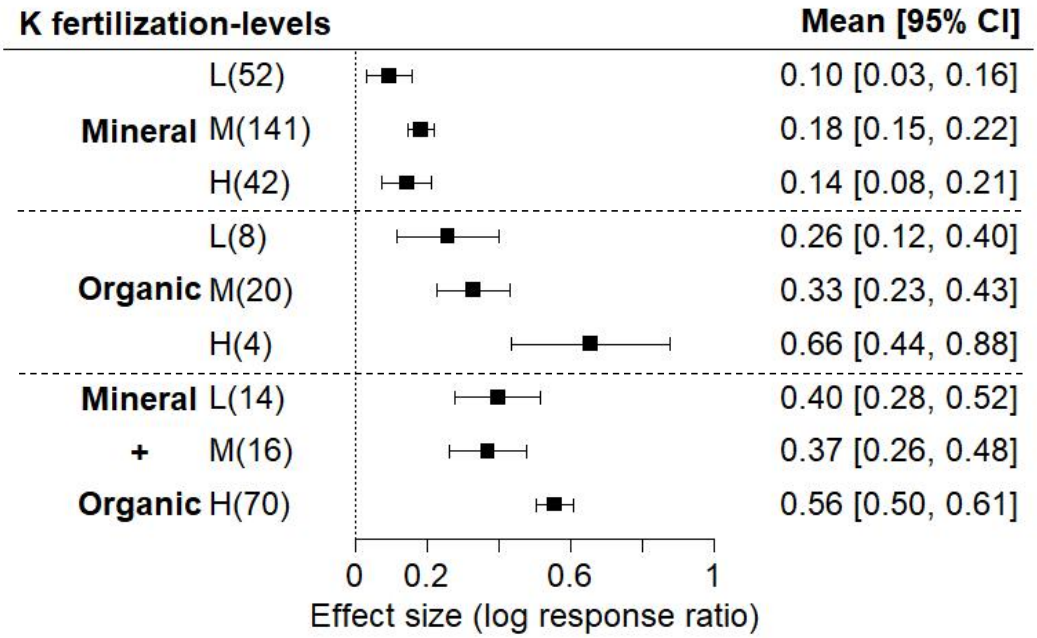
(a)


N fertilization-levels
Mean [95% CI]
L(107)
0.11 [0.07, 0.15]
Mineral
M(281)
0.14 [0.11, 0.16]
H(73)
0.12 [0.07, 0.16]
L(38)
0.27 [0.20, 0.34]
Organic
M(26)
0.48 [0.40, 0.56]
H(7)
0.30 [0.15, 0.46]
Mineral
L(6)
0.20 [-0.02, 0.42]
+
M(105)
0.46 [0.42, 0.50]
Organic
H(40)
0.41 [0.35, 0.48]
-0.2
0
0.2
0.4
0.6
Effect size (log response ratio)

(b)


P fertilization-levels
Mean [95% CI]
L(23)
0.14 [0.04, 0.23]
Mineral
M(148)
0.16 [0.13, 0.20]
H(138)
0.16 [0.13, 0.20]
L(13)
0.28 [0.16, 0.39]
Organic
M(10)
0.36 [0.22, 0.50]
H(8)
0.49 [0.35, 0.64]
Mineral
L(4)
0.63 [0.40, 0.86]
+
M(29)
0.33 [0.25, 0.41]
Organic
H(72)
0.56 [0.51, 0.61]
0
0.2
0.4
0.6
0.8
1
Effect size (log response ratio)

(c)


K fertilization-levels
Mean [95% CI]
L(52)
Mineral M(141)
H(42)
L(8)
Organic M(20)
H(4)
Mineral L(14)
+ M(16)
Organic H(70)
0.10 [0.03, 0.16]
0.18 [0.15, 0.22]
0.14 [0.08, 0.21]
0.26 [0.12, 0.40]
0.33 [0.23, 0.43]
0.66 [0.44, 0.88]
0.40 [0.28, 0.52]
0.37 [0.26, 0.48]
0.56 [0.50, 0.61]
0
0.2
0.6
1
Effect size (log response ratio)

Supplementary information

Fig. S1 PRISMA flow chart showing the procedure of selecting publications

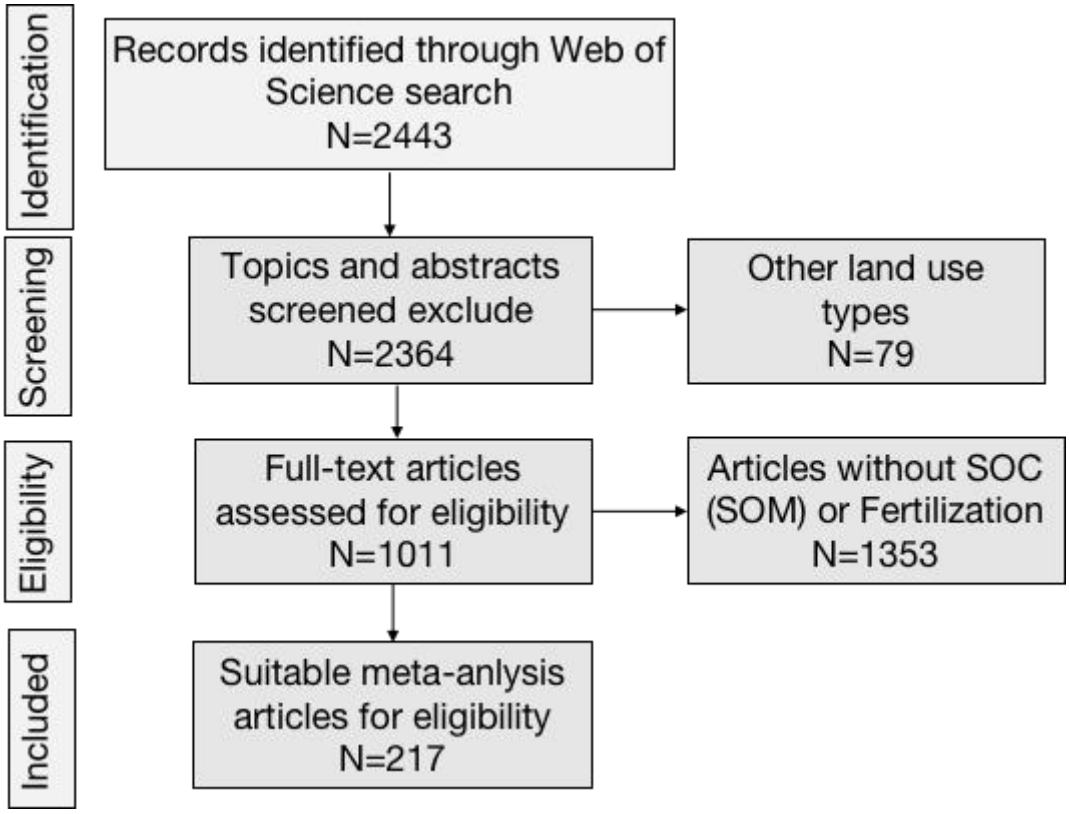


Fig. S2 Funnel plot of effect size on SOC in agriculture upland soil

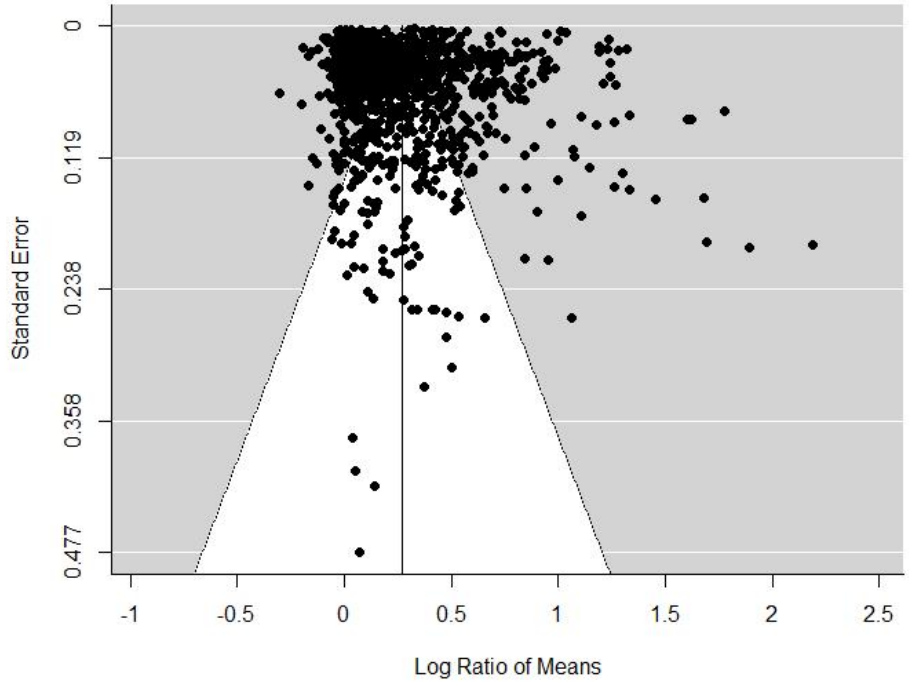
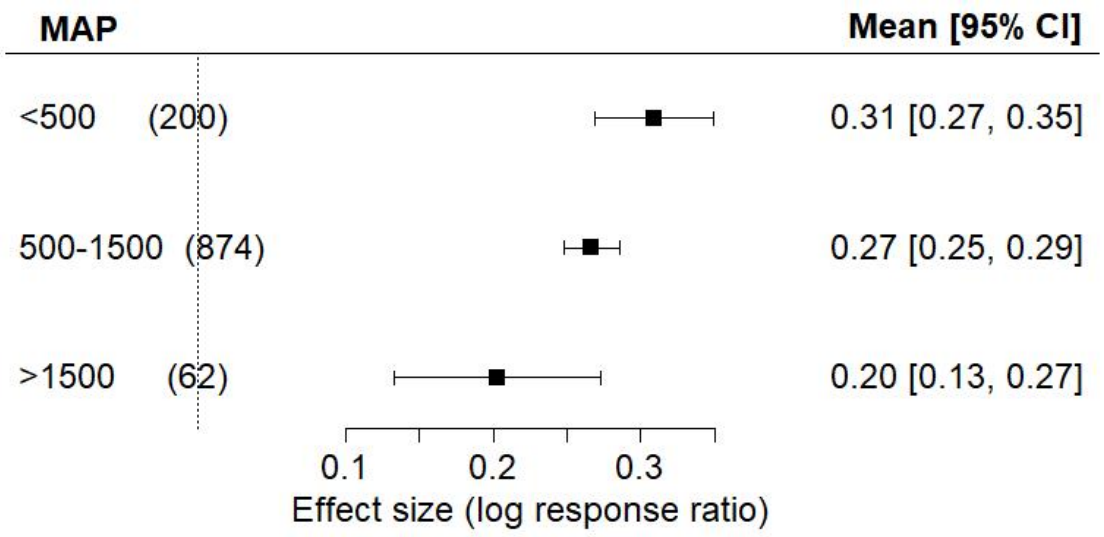
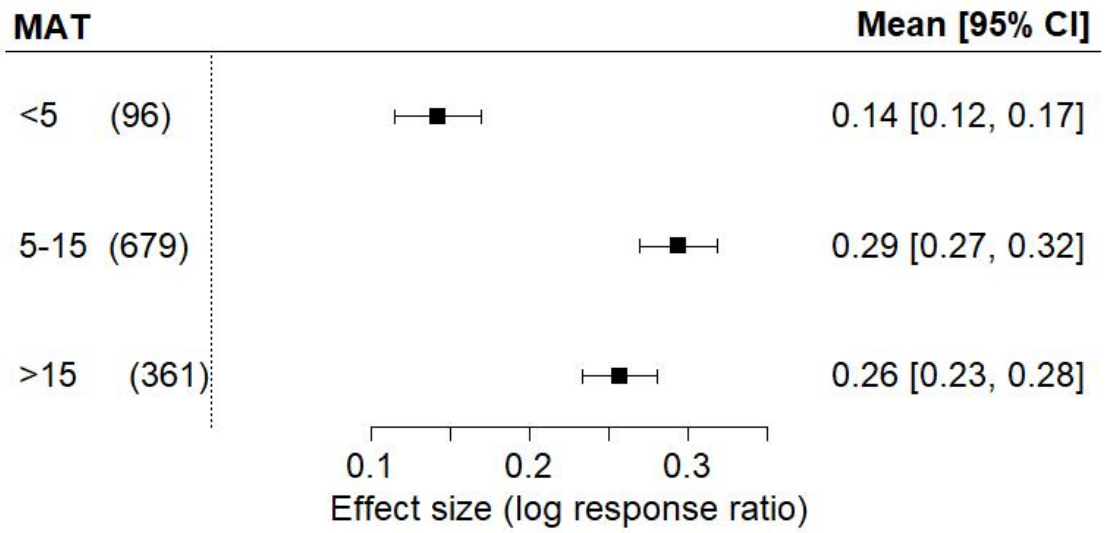


Fig. S3 Forest plot of effect of moderators MAP (a), MAT (b), water condition (c), tillage types (d) and crop rotation (e) on SOC in agriculture upland soil. MAP, mean annual precipitation; MAT, mean annual temperature; CT, conventional tillage; DT, deep tillage; RT, reduced tillage; MT: minimum tillage; NT, no tillage; W, alternate wetting; D, drying.

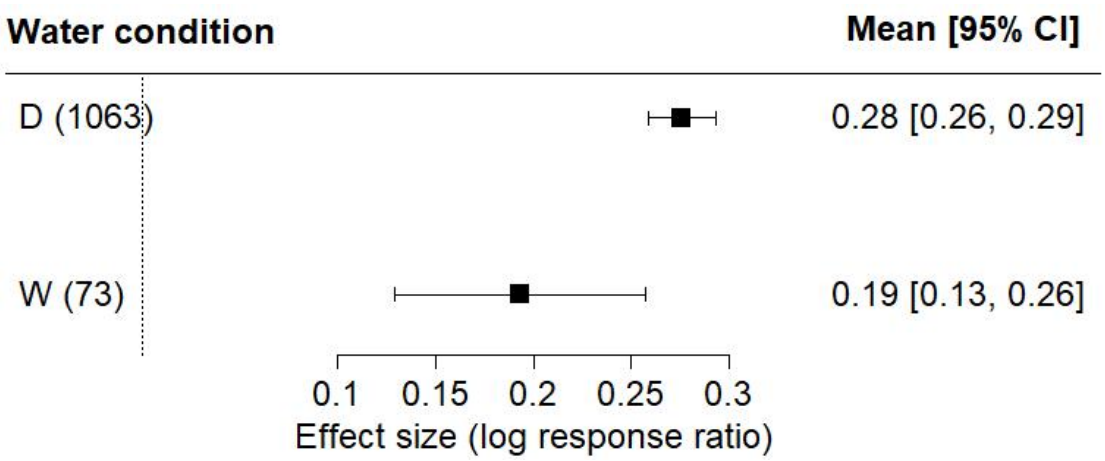
(A)



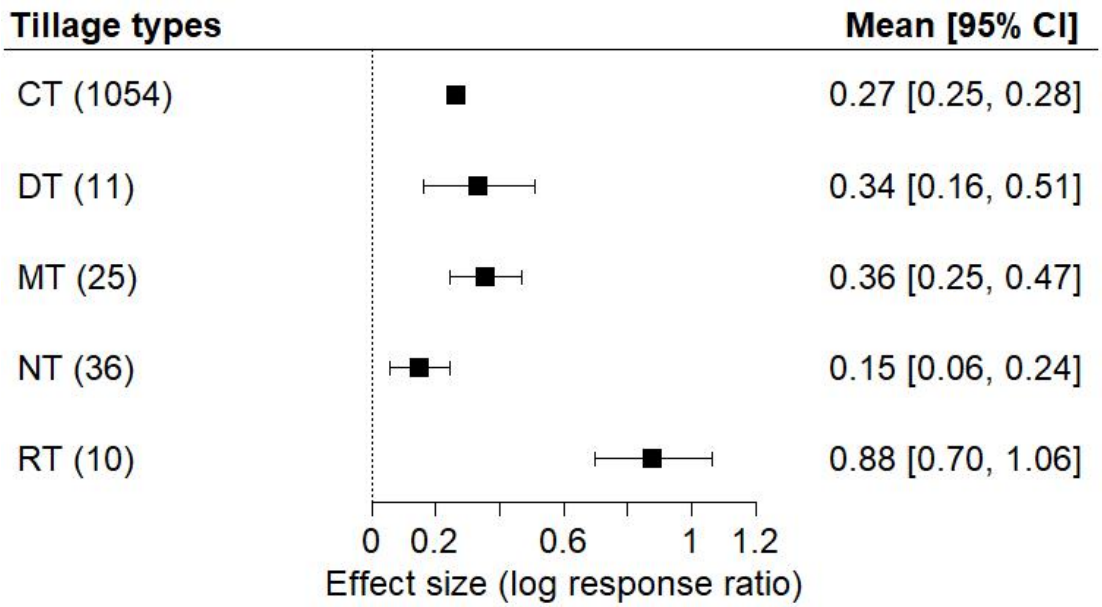
(b)



(c)


Water condition
Mean [95% CI]
D (1063)
0.28 [0.26, 0.29]
W (73)
0.19 [0.13, 0.26]
0.1
0.15
0.2
0.25
0.3
Effect size (log response ratio)

(d)


Tillage types
Mean [95% CI]
CT (1054)
0.27 [0.25, 0.28]
DT (11)
0.34 [0.16, 0.51]
MT (25)
0.36 [0.25, 0.47]
NT (36)
0.15 [0.06, 0.24]
RT (10)
0.88 [0.70, 1.06]
0
0.2
0.6
1
1.2
Effect size (log response ratio)

(e)

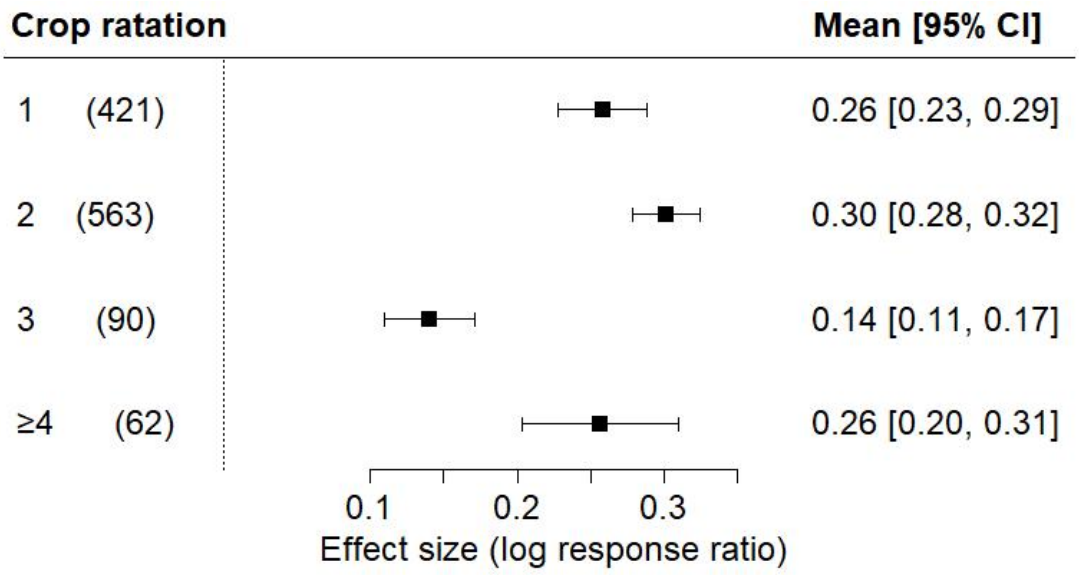

Crop ratation
Mean [95% CI]
1 (421)
0.26 [0.23, 0.29]
2 (563)
0.30 [0.28, 0.32]
3 (90)
0.14 [0.11, 0.17]
≥4 (62)
0.26 [0.20, 0.31]
0.1
0.2
0.3
Effect size (log response ratio)

Table. S1 Two-by-two comparison of fertilization types on SOC in agriculture upland soil

Fertilization types			Estimate	SE	t	*p*
Mineral	vs	Organic	0.24803	0.01981	12.52	***
Mineral	vs	Organic + Mineral	0.27946	0.01699	16.451	***
Organic	vs	Organic + Mineral	0.03144	0.02116	1.486	0.138
K	vs	N	0.0333422	0.1735784	0.192	1
		NK	0.00455	0.1825944	0.025	1
		NF	0.267354	0.1757027	1.522	1
		NP	0.064007	0.1749412	0.366	1
		NPK	0.061376	0.1734052	0.354	1
		NPKF	0.350827	0.1736253	2.021	1
		NPKS	0.236412	0.1755171	1.347	1
		NPF	0.306039	0.1782877	1.717	1
		NPS	0.213316	0.1865161	1.144	1
		NS	0.184873	0.1777389	1.04	1
		F	0.336452	0.1735958	1.938	1
		P	0.0033023	0.1822439	0.018	1
		PK	0.0023385	0.1788772	0.013	1
		PKF	0.672488	0.20282	3.316	0.081142
		PF	0.248382	0.1979453	1.255	1
		S	0.026335	0.1762077	0.149	1
N	vs	NK	0.037892	0.0620223	0.611	1
		NF	0.300696	0.0371143	8.102	***
		NP	0.09735	0.0333233	2.921	0.277291
		NPK	0.094718	0.0239877	3.949	**
		NPKF	0.38417	0.0255302	15.048	***
		NPKS	0.269754	0.0362253	7.447	***
		NPF	0.339381	0.0478804	7.088	***
		NPS	0.246659	0.0727619	3.39	0.062941
		NS	0.218215	0.0457947	4.765	***
		F	0.369794	0.0253287	14.6	***
		P	0.03004	0.0609825	0.493	1
		PK	0.031004	0.050031	0.62	1
		PKF	0.705831	0.1078979	6.542	***
		PF	0.281724	0.0984296	2.862	0.32571
		S	0.059677	0.0394357	1.513	1
NK	vs	NF	0.262804	0.0677402	3.88	*
		NP	0.059458	0.0657397	0.904	1
		NPK	0.056827	0.061536	0.923	1
		NPKF	0.346278	0.0621535	5.571	***
		NPKS	0.231863	0.0672572	3.447	0.05166
		NPF	0.30149	0.0741876	4.064	**

		NPS	0.208767	0.0922256	2.264	1
		NS	0.180323	0.0728589	2.475	0.956531
		F	0.331903	0.062071	5.347	***
		P	0.0078518	0.0832474	0.094	1
		PK	0.006888	0.0755934	0.091	1
		PKF	0.667939	0.1218738	5.481	***
		PF	0.243832	0.1135767	2.147	1
		S	0.021786	0.0690394	0.316	1
		NP	0.2033465	0.0430398	4.725	***
		NPK	0.2059777	0.0362958	5.675	***
		NPKF	0.083474	0.0373333	2.236	1
		NPKS	0.0309417	0.0453238	0.683	1
		NPF	0.038685	0.0550864	0.702	1
		NPS	0.0540374	0.0776935	0.696	1
NF	vs	NS	0.0824811	0.0532835	1.548	1
		F	0.069098	0.0371957	1.858	1
		P	0.2706561	0.0667896	4.052	**
		PK	0.2696923	0.0569656	4.734	***
		PKF	0.405135	0.1112832	3.641	*
		PF	0.0189719	0.1021292	0.186	1
		S	0.2410186	0.0479286	5.029	***
		NPK	0.0026313	0.0324093	0.081	1
		NPKF	0.28682	0.033567	8.545	***
		NPKS	0.172405	0.0422755	4.078	**
		NPF	0.242032	0.0526069	4.601	***
		NPS	0.149309	0.0759556	1.966	1
		NS	0.120865	0.050716	2.383	1
NP	vs	F	0.272445	0.033414	8.154	***
		P	0.0673097	0.0647597	1.039	1
		PK	0.0663458	0.0545715	1.216	1
		PKF	0.608481	0.1100769	5.528	***
		PF	0.184375	0.1008134	1.829	1
		S	0.0376722	0.0450569	0.836	1
		NPKF	0.289451	0.024325	11.899	***
		NPKS	0.175036	0.0353862	4.946	***
		NPF	0.244663	0.0472488	5.178	***
		NPS	0.15194	0.0723479	2.1	1
		NS	0.123497	0.0451339	2.736	0.467175
NPK	vs	F	0.275076	0.0241134	11.408	***
		P	0.0646784	0.0604879	1.069	1
		PK	0.0637146	0.0494268	1.289	1
		PKF	0.611112	0.1076191	5.678	***
		PF	0.187006	0.0981239	1.906	1
		S	0.0350409	0.0386664	0.906	1

NPKF	vs	NPKS	0.1144153	0.0364495	3.139	0.144386
		NPF	0.0447883	0.0480503	0.932	1
		NPS	0.137511	0.0728738	1.887	1
		NS	0.1659547	0.0459723	3.61	*
		F	0.0143752	0.0256484	0.56	1
		P	0.3541297	0.061116	5.794	***
		PK	0.3531659	0.0501936	7.036	***
		PKF	0.321661	0.1079735	2.979	0.239239
		PF	0.1024456	0.0985124	1.04	1
		S	0.3244922	0.0396418	8.186	***
NPKS	vs	NPF	0.069627	0.0544914	1.278	1
		NPS	0.0230958	0.0772727	0.299	1
		NS	0.0515394	0.0526681	0.979	1
		F	0.10004	0.0363086	2.755	0.446965
		P	0.2397145	0.0662996	3.616	*
		PK	0.2387506	0.0563904	4.234	**
		PKF	0.436076	0.1109898	3.929	**
		PF	0.01197	0.1018095	0.118	1
		S	0.210077	0.0472435	4.447	***
NPF	vs	NPS	0.0927228	0.0833747	1.112	1
		NS	0.1211664	0.0612715	1.978	1
		F	0.030413	0.0479435	0.634	1
		P	0.3093415	0.0733206	4.219	**
		PK	0.3083776	0.0644991	4.781	***
		PKF	0.366449	0.1153213	3.178	0.128139
		PF	0.0576573	0.1065149	0.541	1
		S	0.2797039	0.0566764	4.935	***
NPS	vs	NS	0.0284436	0.0821946	0.346	1
		F	0.123136	0.0728035	1.691	1
		P	0.2166187	0.0915296	2.367	1
		PK	0.2156549	0.084628	2.548	0.789023
		PKF	0.459172	0.1276745	3.596	*
		PF	0.035066	0.1197798	0.293	1
		S	0.1869812	0.0788288	2.372	1
NS	vs	F	0.15158	0.0458607	3.305	0.083226
		P	0.1881751	0.0719759	2.614	0.661237
		PK	0.1872112	0.0629663	2.973	0.240823
		PKF	0.487616	0.1144711	4.26	**
		PF	0.063509	0.1055938	0.601	1
		S	0.1585375	0.0549258	2.886	0.305819
F	vs	P	0.3397546	0.0610321	5.567	***
		PK	0.3387907	0.0500914	6.763	***
		PKF	0.336036	0.107926	3.114	0.15541
		PF	0.0880704	0.0984603	0.894	1

		S	0.310117	0.0395123	7.849	***
P	vs	PK	0.000964	0.0747427	0.013	1
		PKF	0.675791	0.121348	5.569	***
		PF	0.251684	0.1130122	2.227	1
		S	0.029638	0.0681069	0.435	1
PK	vs	PKF	0.674827	0.1162307	5.806	***
		PF	0.25072	0.1074988	2.332	1
		S	0.028674	0.0585045	0.49	1
PKF	vs	PF	0.4241065	0.1438571	2.948	0.257825
		S	0.6461532	0.1120788	5.765	***
PF	vs	S	0.2220467	0.1029955	2.156	1

F, farmyard manure; S, straw.

Table. S2 Two-by-two comparison of fertilization levels on SOC in agriculture upland soil

Fertilization	Fertilization levels			Estimate	SE	t	*p*
N	Low Mineral N	vs	Intermediate Mineral N	0.029187	0.024246	1.204	1
			High Mineral N	0.006898	0.032185	0.214	1
			Low Organic N	0.160623	0.040483	3.968	**
			Intermediate Organic N	0.370835	0.046494	7.976	***
			High Organic N	0.194112	0.081376	2.385	0.3641
			Low Organic+Mineral N	0.090685	0.113016	0.802	1
			Intermediate Organic+Mineral N	0.350724	0.02926	11.986	***
			High Organic+Mineral N	0.303056	0.038354	7.901	***
	Intermediate Mineral N	vs	High Mineral N	0.303056	0.038354	7.901	***
			Low Organic N	0.022289	0.027747	0.803	1
			Intermediate Organic N	0.131437	0.037052	3.547	**
			High Organic N	0.341648	0.04354	7.847	***
			Low Organic+Mineral N	0.061498	0.111833	0.55	1
			Intermediate Organic+Mineral N	0.321537	0.024294	13.235	***
			High Organic+Mineral N	0.273869	0.034714	7.889	***
	High Mineral N	vs	Low Organic N	0.153726	0.042672	3.603	**
			Intermediate Organic N	0.363938	0.048412	7.517	***
			High Organic N	0.187214	0.082487	2.27	0.42383
			Low Organic+Mineral N	0.083788	0.113819	0.736	1
			Intermediate Organic+Mineral N	0.343826	0.032221	10.671	***
			High Organic+Mineral N	0.296159	0.040658	7.284	***
	Low Organic N	vs	Intermediate Organic N	0.210212	0.054284	3.872	**
			High Organic N	0.033488	0.086065	0.389	1
			Low Organic+Mineral N	0.069938	0.116438	0.601	1
			Intermediate Organic+Mineral N	0.1901	0.040511	4.693	***
			High Organic+Mineral N	0.142433	0.047498	2.999	0.06185
	Intermediate Organic N	vs	High Organic N	0.176723	0.089051	1.985	0.76164
			Low Organic+Mineral N	0.28015	0.118662	2.361	0.37028
			Intermediate Organic+Mineral N	0.020112	0.046519	0.432	1
			High Organic+Mineral N	0.067779	0.052716	1.286	1
	High Organic N	vs	Low Organic+Mineral N	0.103427	0.136165	0.76	1
			Intermediate Organic+Mineral N	0.156612	0.081391	1.924	0.82127
			High Organic+Mineral N	0.108944	0.085084	1.28	1
	Low Organic+Mineral N	vs	Intermediate Organic+Mineral N	0.260038	0.113027	2.301	0.41257

			High Organic+Mineral N	0.212371	0.115715	1.835	0.93664
	Intermediate Organic+Mineral N	vs	High Organic+Mineral N	0.047668	0.038384	1.242	1
P	Low Mineral P	vs	Intermediate Mineral P	0.02272	0.050855	0.447	1
			High Mineral P	0.023994	0.051154	0.469	1
			Low Organic P	0.137388	0.075033	1.831	0.76353
			Intermediate Organic P	0.222718	0.087474	2.546	0.209371
			High Organic P	0.356513	0.089021	4.005	**
			Low Organic+Mineral P	0.493012	0.125699	3.922	**
			Intermediate Organic+Mineral P	0.191399	0.062588	3.058	0.054373
			High Organic+Mineral P	0.42014	0.053697	7.824	***
	Intermediate Mineral P	vs	High Mineral P	0.001274	0.025454	0.05	1
			Low Organic P	0.114668	0.060507	1.895	0.76353
			Intermediate Organic P	0.199998	0.075385	2.653	0.173621
			High Organic P	0.333793	0.077175	4.325	***
			Low Organic+Mineral P	0.470293	0.117607	3.999	**
			Intermediate Organic+Mineral P	0.168679	0.044141	3.821	**
			High Organic+Mineral P	0.113394	0.060758	1.866	0.76353
	High Mineral P	vs	Low Organic P	0.113394	0.060758	1.866	0.76353
			Intermediate Organic P	0.198723	0.075587	2.629	0.177269
			High Organic P	0.332519	0.077373	4.298	***
			Low Organic+Mineral P	0.469018	0.117737	3.984	**
			Intermediate Organic+Mineral P	0.167405	0.044485	3.763	**
			High Organic+Mineral P	0.396146	0.030742	12.886	***
	Low Organic P	vs	Intermediate Organic P	0.08533	0.093416	0.913	1
			High Organic P	0.219125	0.094866	2.31	0.341792
			Low Organic+Mineral P	0.355625	0.129904	2.738	0.141704
			Intermediate Organic+Mineral P	0.054011	0.070654	0.764	1
			High Organic+Mineral P	0.282752	0.062914	4.494	***
	Intermediate Organic P	vs	High Organic P	0.133795	0.104984	1.274	1
			Low Organic+Mineral P	0.270295	0.137466	1.966	0.748489
			Intermediate Organic+Mineral P	0.031318	0.083749	0.374	1
			High Organic+Mineral P	0.197423	0.077331	2.553	0.209371
	High Organic P	vs	Low Organic+Mineral P	0.1365	0.138456	0.986	1
			Intermediate Organic+Mineral P	0.165114	0.085364	1.934	0.752195
			High Organic+Mineral P	0.063627	0.079077	0.805	1
	Low Organic+Mineral P	vs	Intermediate Organic+Mineral P	0.301613	0.123136	2.449	0.249877
			High Organic+Mineral P	0.072872	0.118864	0.613	1

	Intermediate Organic+Mineral P	vs	High Organic+Mineral P	0.228741	0.047387	4.827	***
K	Low Mineral K	vs	Intermediate Mineral K	0.08735	0.03735	2.338	0.303396
			High Mineral K	0.04852	0.04744	1.023	1
			Low Organic K	0.16311	0.08	2.039	0.548618
			Intermediate Organic K	0.23451	0.06102	3.843	**
			High Organic K	0.56066	0.11749	4.772	***
			Low Organic+Mineral K	0.30281	0.06907	4.384	***
			Intermediate Organic+Mineral K	0.27447	0.06354	4.32	***
			High Organic+Mineral K	0.46041	0.04192	10.983	***
	Intermediate Mineral K	vs	High Mineral K	0.03883	0.03985	0.974	1
			Low Organic K	0.07577	0.07574	1	1
			Intermediate Organic K	0.14716	0.05532	2.66	0.146942
			High Organic K	0.47331	0.11464	4.129	**
			Low Organic+Mineral K	0.21546	0.0641	3.362	*
			Intermediate Organic+Mineral K	0.18712	0.05809	3.221	*
			High Organic+Mineral K	0.37306	0.03308	11.277	***
	High Mineral K	vs	Low Organic K	0.11459	0.0812	1.411	1
			Intermediate Organic K	0.18598	0.06258	2.972	0.063177
			High Organic K	0.51213	0.11831	4.329	***
			Low Organic+Mineral K	0.25429	0.07045	3.609	**
			Intermediate Organic+Mineral K	0.22594	0.06503	3.474	*
			High Organic+Mineral K	0.41189	0.04416	9.328	***
	Low Organic K	vs	Intermediate Organic K	0.07139	0.08981	0.795	1
			High Organic K	0.39754	0.13471	2.951	0.064155
			Low Organic+Mineral K	0.1397	0.09546	1.463	1
			Intermediate Organic+Mineral K	0.11135	0.09154	1.216	1
			High Organic+Mineral K	0.29729	0.0781	3.807	**
	Intermediate Organic K	vs	High Organic K	0.32615	0.12438	2.622	0.154881
			Low Organic+Mineral K	0.0683	0.08023	0.851	1
			Intermediate Organic+Mineral K	0.03996	0.07551	0.529	1
			High Organic+Mineral K	0.2259	0.0585	3.862	**
	High Organic K	vs	Low Organic+Mineral K	0.25785	0.12852	2.006	0.548618
			Intermediate Organic+Mineral K	0.28619	0.12563	2.278	0.326484
			High Organic+Mineral K	0.10025	0.11621	0.863	1
	Low Organic+Mineral K	vs	Intermediate Organic+Mineral K	0.02834	0.08216	0.345	1
			High Organic+Mineral K	0.1576	0.06686	2.357	0.303396

	Intermediate Organic+Mineral K	vs	High Organic+Mineral K	0.18594	0.06112	3.042	0.05302

Table. S3 Two-by-two comparison of moderators MAP, MAT, water condition, tillage types and crop rotation on SOC in agriculture upland soil

Moderators				Estimate	SE	t	*p*
NAP (mm)	< 500	vs	500-1500	0.04227	0.02252	1.877	0.1216
	< 500	vs	> 1500	0.10605	0.041	2.586	*
	500-1500	vs	> 1500	0.06378	0.03689	1.729	0.1216
MTP (℃)	< 5	vs	500-1500	-0.14731	0.03043	-4.841	***
	< 5	vs	> 15	-0.10493	0.03219	-3.26	**
	5-15	vs	> 15	0.04238	0.01855	2.284	*
Water condition	D	vs	W	0.0828	0.03398	2.437	*
Tillage types	CT	vs	DT	-0.0687	0.08896	-0.772	0.88027
			MT	-0.09166	0.05812	-1.577	0.34521
			NT	0.11686	0.04873	2.398	0.08317
			RT	-0.61428	0.09386	-6.545	***
	DT	vs	MT	-0.02296	0.10556	-0.217	0.88027
			NT	0.18556	0.1007	1.843	0.2625
			RT	-0.54558	0.12875	-4.238	***
	MT	vs	NT	0.20852	0.07486	2.786	*
			RT	-0.54558	0.12875	-4.238	***
	NT	vs	RT	0.20852	0.07486	2.786	*
Crop rotation	1	vs	2	-0.044392	0.018264	-2.431	*
			3	0.115963	0.032623	3.555	**
			> 4	-0.005571	0.03897	-0.143	0.88636
	2	vs	3	0.160355	0.031806	5.042	***
			> 4	0.038822	0.038289	1.014	0.62169
	3	vs	> 4	-0.121534	0.046869	-2.593	*

MAP, mean annual precipitation; MAT, mean annual temperature; CT, conventional tillage; DT, deep tillage; RT, reduced tillage; MT: minimum tillage ; NT, no tillage; DW, alternate anaerobic wetting; D, aerobic drying.

3 Study 2

N and P fertilization decrease litter mineralization and priming effect in a semiarid agricultural soil

Contribution: I participated in work, sampling activities, and the experiment incubation, performed most of the analysis in the laboratory, collected and evaluated data, prepared tables and figures, and wrote the manuscript

N and P fertilization decrease litter mineralization and priming effect in a semiarid agricultural soil

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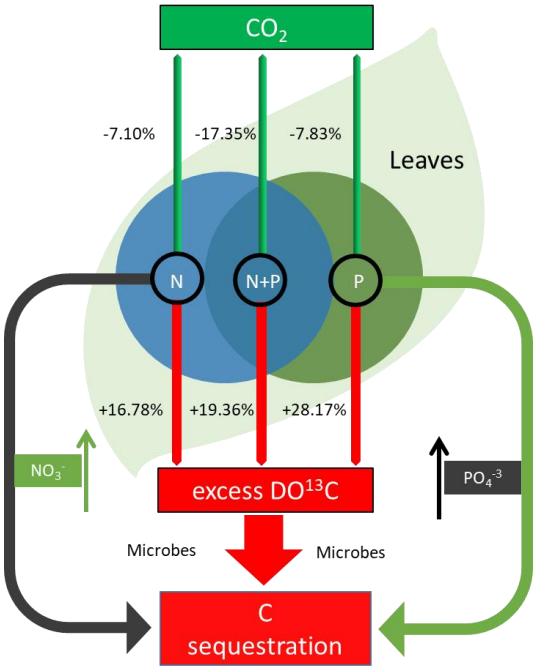
Highlights

- N, P and NP fertilization decreased plant residues mineralization
- N, P and NP fertilization decreased positive priming by alleviating microbial nutrient limitation
- N, P and NP fertilization increased dissolved organic C
- The increasing availability of N and P induces potential C sequestration

Abstract Supply with exogenous nutrients, i.e. fertilized nitrogen (N) and phosphorus (P), may influence the rates of soil organic matter (SOM) formation from plant residues and the microbial decomposition of soil indigenous SOM. However, available N and P limitation is not conducive to SOM sequestration and maintain soil fertility. Our objective was approached by adding mineral N and P, as well as N plus P (NP) with or without litter (*Dactylis glomerata* L.) in a 37-day incubation of a Chernozem under long-term wheat cultivation. Cumulative litter-derived C emissions decreased by 7.10 ± 5.07 %, 17.35 ± 4.09 %, and 7.83 ± 4.92 %, with mineral N, P, and NP, respectively, while litter-derived dissolved organic C (DOC) increased by 16.78 ± 5.25 %, 19.36 ± 4.51% and 28.17 ± 10.28% for the different treatments. The added leaves were mineralized by 24.06 ± 2.76% to 27.59 ± 0.26 % within the incubation time, and increased the soil organic carbon (SOC) mineralization by 75.59 ± 10.81% – 98.50 ± 2.08 % (positive PE) compared to CK. In contrast, mineral N, P, and NP input did not increase SOC mineralization. Compared to litter only, litter + N and litter + NP weakly decreased cumulative SOM-derived C emissions by 8.72 ± 5.62% and 4.42 ± 3.13%, respectively. However, P + litter weakly increased the cumulative SOM-derived C emissions by 3.19 ± 1.08%. The NO_3^- and Olsen-P content were improved along with the maximal velocity of β-1, 4-N-acetyl-glucosaminidase and β-1,4-phosphate for mineral N and P addition, respectively. SOC, NO_3^-, and Olsen-P content was higher in treatments with litter than in treatments without litter across all treatment groups. Thus, we conclude that in N and P depleted semiarid agricultural soils N and P fertilization decrease C emission and induce potential C sequestration for maintaining soil fertility.

Keywords: Fertilization, Priming effect, SOM decomposition, semiarid agriculture

Graphical abstract



1 Introduction

Nowadays, in agricultural ecosystems receive anthropogenic-derived nutrient inputs, which have increased many folds over the last 100 years (Liu et al., 2018; Wei et al., 2019; Zang et al., 2016). Mineral nitrogen (N) and phosphorus (P) are not only essential for plant growth, but also form an integrative part of soil organic matter (SOM) and are released in a plant-available form by the microbial mineralization of SOM (Bobbink et al., 2010; Liu et al., 2018; Zhu et al., 2018; Gan et al., 2020). In this process, the availability of N and P has an influence to decrease (Zang et al., 2016; Ouyuan et al., 2008), increase (Cleveland and Townsend, 2006), or unchange SOM decomposition (Burton et al., 2004). It directly influence the intensity and direction of priming effect (PE) (McGroddy et al., 2004; Ye et al., 2015).

The decomposition of SOM also is regulated by the input of organic substrates (i.e., plant residues) (Zhu et al., 2017; Shahbaz et al., 2017; Soong et al., 2018), leading to PE. Because C limitation is the most important factor for soil microorganism, once high availability of substrates (high C: nutrients ratio) input may alter microorganism activities, especially microbial C and nutrients use efficiency (Shahbaz et al., 2017b). This lead to a stoichiometric imbalance for the microbial needs of C and nutrients (Gan et al., 2020). At high C:nutrients ratio more organic C needs to be mineralized along with a higher investment of extracellular hydrolytic enzymes to acquire nutrients for microorganisms (Wei et al., 2019), leading to a lower C use efficiency (CUE) (Wang et al., 2019) and increase nutrients use efficiency (Mooshammer et al., 2014). Different types of extracellular hydrolytic enzymes being involved in the decomposition of organic C, e.g, β-1, 4-glucosidase (BG), organic N, e.g. β-1, 4-N-acetyl-glucosaminidase (NAG), and organic P, e.g. β-1,4-phosphate (phosphatase (AP))

are considered to be mainly involved in the degradation of plant residues and SOM (Shahbaz et al., 2017b; Zhu et al., 2018; Zhu et al., 2018; Wei et al., 2019). Meanwhile excess C could be also released into soil solution as dissolved organic C (DOC) under nutrients limitation (Hessen & Anderson, 2008).

Kazakhstan is an agrarian country and in semiarid regions with their steppe soils. These areas are largely under agriculture. In northern Kazakhstan native steppes have been largely replaced by arable land in the 1950s and 1960s within the Zelina campaign (Funakawa et al., 2004). Chernozem soil cover approximately 32.1×10^6 ha, accounting for 11.8% of Kazakhstan's area (Funakawa et al., 2004). Land use change and land use went along with soil degradation and loss of agricultural sustainability (Karbozova-Saljnikov et al., 2004). In many northern Kazakhstan soils, one likely reason for the loss in SOC in these soils is the pronounced SOM mining to provide nutrients to crop plants and microorganisms due to lacking fertilization for a few decades. It leads to N and P limitation (http://creativecommons.org/licenses/by-nc-nd/4.0/). Dzhalankuzov and Redkov (1993) reported 28–30% losses of humus in the surface horizon of arable Chernozems of North Kazakhstan from their initial amount before cultivation. Karbozova-Saljnikov et al. (2004) reported 60 kg N ha^{-1} of NH_4NO_3 with wheat straw return increase SOC by 24.2% from 1976 to 1998 and potentially mineralizable C accounts for 5.8% of the SOC in North Kazakhstan. However the research of quantitating SOC degradation of semi-arid Kazakhstan steppe soil in response to mineral fertilization and plant residues return has not been reported. Therefore, we hypothesized that a higher N and P availability due to mineral fertilization (1) decreases plant residue decomposition due to increasing the microbe nutrient supply and enhancing the leaves-C use efficiency; (2) leads to a less pronounced priming of SOM by plant residues

because of alleviating microbial nutrient limitation and preferentially decomposing easily litter-C; and (3) finally the recalcitrant and stabilize litter-C into soil as potential C sequestration. Thus we approached by adding mineral N and P, as well as NP with or without litter using the end-member mixing model distinguish CO_2-C sources, further exploring the mechanism of C turnover in semi-arid Kazakhstan steppe soil.

2 Materials and methods

2.1 Study site and soil sampling

The soils were collected in September 2017 from Ap horizons (0–20 cm) of crop fields near Kokshetau city, at the northeast of Kazakhstan (53°02′N , 69°34′E). The climate of the study area is continental with strong inter-seasonal temperature gradient and large temperature and precipitation fluctuations. The mean annual temperatures and mean annual precipitation in the area are 1.4°C and 336 mm, according long-term weather records at Schuchinsk meteorological station (Yapiyev et al., 2017).

The soil samples were taken from 3 soil profiles (200 cm wide), which were randomly dug within 2000 x 2000 m agricultural field. The crop field was for long time used for wheat cultivation. In addition, soil samples were also collected from six representative “satellites” (0–20 cm depth increments) randomly distributed over the field. All samples were bulked, and fine roots and plant residues were carefully removed manually. After the samples were dried at 40°C, they were stored in closed brown bottles. The physical and chemical properties of soils are: the pH of 7.81 ± 0.07 (1:5 soil/water), soil bulk density of 1.30 ± 0.08 g cm^3, organic C content is 35.20 ± 0.18 g kg^{-1} with an at (%) ^{13}C value of 1.08 ± 0.05; 3.00 ± 0.01 g kg^{-1} total nitrogen, 3.23 ± 0.08 mg kg^{-1} Olsen-P.

2.2 Production and collection of ^{13}C-labelled substrates

^{13}C labeled *Dactylis glomerata* L. leaves (litter) was provided by S. Spielvogel, University of Kiel. As a background, unlabeled litter was used. The plant material was oven-dried during 48 hours under 55°C, cut in small pieces (of about 1 cm) and grinded and stored in dark till being used. The OC content of the ^{13}C labelled and unlabelled leaf litter was 361.70 ± 0.1 g kg^{-1} and 473.4 ± 1.2 g kg^{-1} with an atom % ^{13}C of 31.1 ± 0.01 and 1.08 ± 0.07, respectively.

2.3 Incubation experiment

Prior to the start of the incubation experiment, air-dried soils were re-wetted to 50% of water holding capacity (WHC) and pre-incubated at 22°C for 14 days in dark. Afterwards, the incubation experiment of 37 days duration was conducted. The incubation experiment was set 8 treatments, including: not amended soils (CK) as control (i), C addition with ^{13}C-labeled litter) (ii), mineral N addition in form of NH_4NO_3 (N) (iii), mineral P addition in form of Na_2PHO_4 (P) (iv), concurrent nutrients (NP) (v) and litter and nutrients addition (litter + N, litter + P, litter + NP(vi, vii, viii)). Similarly, the unlabeled plant material, as the exogenous litter-C source solely or in combination with N, P and NP was used as background. The incubation experiment consisted of four replicates per each treatment, the background group has 2 replicates per each treatment. The amount of OC added to the soil with litter, not exceeded 10% of SOC, as it was proposed by Zhu et al. (2014). Mineral N and P were added to the soils in the form of NH_4NO_3 and Na_2PHO_4 at a rate of 100 kg N ha^{-1} and 50 kg P ha^{-1}. After pre-incubation, the soil samples were placed to incubation vessels, consisting a plastic tube (50 mm long with diameter of 36 mm) bounded on one side by nylon mesh using plastic glue and open on the other side. The soils in treatments without plant material addition, were

put to the incubation vessels and compacted close to soil bulk density (1.3 g cm^{-3}). For four treatments with litter addition, the each added soils was divided into six layers of equal weight (every layer about 5.8 g dry soil), then litter (about 68 mg dry mass) was added in between each of the layers (about total 340 mg dry mass for each unit, which is equivalent to 3.52 g C kg dry soil). The soil was compacted close to soil bulk density (1.3 g cm^{-3}) for every layer. In order to keep 60% WHC during the incubation, mineral N and P fertilizers were dissolved in deionized water (10 % WHC) and added to the soils as solutions, and the equivalent of deionized water was added to the control and C treatments (CK and litter). The WHC was kept constant through the chasing period by daily gravimetrical control. All samples were incubated at 24°C for 37 days under dark condition. For CO_2 collecting, every vessel containing soil sample covered with perforated aluminum foil with many holes. Then 32 vessels (Treatments) and 8 vessels (Backgrounds) were placed into the static chambers comprising 800-ml bottle (EU Design NO. 381983, Duran, Germany) covered by lid with two opposing plastic tubes supplied with two-way valves. One of which was connected by a soft plastic tube (7 cm long) above the lid and was connected by a hard plastic tube (7 cm long) with many little holes under the lid. All interfaces were glued together to prevent leaks. The rest samples was incubated in vessels and used to measure dissolved organic C (DOC), microbial biomass C (MBC), $MB^{13}C$, $DO^{13}C$, and extracellular enzymes activities.

2.4 CO_2 efflux, microbial biomass C, available nutrients and extracellular enzymes

Soil CO_2 and $^{13}CO_2$ efflux rates were measured on days 1,2,3,4,6,8,10,12,15,20,27,37 of the incubation. The static chambers were kept opened between the sampling dates. At each sampling date, the bottles was tightly closed with the lid using rubber rings coated with high vacuum grease. Oxygen (200 pa O_2) was used to replacement gas in the bottle before

collecting CO_2. Air samples were collected from the static chambers headspace (the 800-ml bottles) using a 20-ml syringe. At each sampling date, gas samples were collected three times from the chamber headspace at 2, 4 and 6 hours. Every time, after sampling was finished, the lid was opened and soils were incubated aerobically until the next sampling. CO_2 and $\delta^{13}C$ were analyzed by gas chromatography (Agilent 7890, Agilent Technologies, Santa Clara, CA, U.S.A.) and isotope ratio mass spectrometer (Isoprene 100, Elementar, England) coupled with a GasBench (Elementar, Germany), respectively. CO_2 values were evaluated with correction for the CO_2 dissociated in the soil solution in accordance with the method of Sparling & West (1990).

At days 2, 8, 14 and 37, soil samples of about 40 g (fresh weight) were collected to measure dissolved organic C (DOC), microbial biomass C (MBC), $MB^{13}C$, $DO^{13}C$, and the maximal velocity (V_{max}) of BG, NAG and AP. Briefly, microbial biomass C was determined using the chloroform fumigation method (Brookes et al., 1985; Vance et al., 1987). After taking samples, the soil was carefully mixed, and 10 g of fresh soil was sampled for extraction using 40 ml of 0.5 $mol \cdot L^{-1}$ K_2SO_4. Other 10 g of fresh soil were first fumigated with chloroform for 24 h prior to extraction in the same manner. The extracts were analyzed for OC concentration using a Vario TOC CUBE (Elementar, Hanau, Germany). For MBC calculation, converting coefficient (k_c= 0.45) was used (Wu et al., 1990; Ge et al., 2012). The non-fumigated samples were used to measure DOC. The other extracts were frozen (-80 ℃) and dried using a freez-dryer (Lyovapor™ L-300, Germany). Then solid samples were used to measure ^{13}C using an elemental analyzer isotope ratio mass spectrometer (Isotope Cube-Precision, Elementar, Germany). Soil NO_3^- content was analyzed by extraction about 5 g of soils with 0.01 M $CaCl_2$ and measured using a continuous flow analyzer (SEAL

Analytical, Norderstedt, Germany) (Yin et al., 2019). To assess soil Olsen-P content, about 2 g of fresh soil was extracted by 0.5 M NaHCO3 (pH=8.5) and measured using UV/Vis absorbance spectra (Spectro Star Nano; BMG LABTECH GmbH, Ortenberg, Germany) at an absorbance value of 882 nm (Olsen et al., 1954). The potential activities of extracellular enzymes (EEAs) of BG, XYL, CBH were measured based on the method of fluorogenically labeled substrates (Pritsch et al., 2004; Sanaullah et al., 2016; Shahbaz et al., 2017). Three fluorogenic enzyme substrates based on 4-methylumbelliferone (MUF) were used: MUF-β-D-glucopyranoside (MUF-G; EC 3.2.1.21) for β-glucosidase (BG), MUF-N-acetyl-β-D-gluosaminide dehydrate (MUF-N; EC 3.2.1.21) for chitinase (NAG), MUF-phosphate monoester (EC 3.1.3.2) for phosphatase (Nannipieri et al., 2010). Briefly, 1 g of fresh soil was suspended in 50 ml of deionized water for 30 min using an oscillating machine (HS501, IKA®-Werke GmbH & CO. KG, Staufen, Germany). According to preliminary experiments, the saturation concentration of fluorogenic substrates were determined using a range of substrate concentrations: 0, 10, 20, 40, 80, 100, 200, and 400 μmol g^{-1} soil. Then 50 μl of suspension was pipetted into 150-μl specific enzyme substrate solution (containing 50μl of 0.1 M sodium morpholine-4-ethanesulfonate ($C_6H_{13}NO_4Sna_{0.5}$) for MUF substrates) having a final concentration of 200μmol g^{-1} soil. Enzymes activities were measured by the multi-function microplate reader (Infinite ® M Plex, Hamilton Bonaduz AG, Bonaduz, Switzerland) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm and slit width of 25 nm. The activity of enzymes (nmol g h^{-1}) was calculated with the method of Wei et al (2019).

2.5 Calculations and statistical analysis

The CO_2-C values on the days between the measurements points were interpolated by

applying a *cubic spline* function with using R to the measured CO_2-C release (Gentsch et al., 2018). Cumulative SOM mineralization during the incubation period was calculated as the sum of the daily CO_2-C evolution values.

The $\delta^{13}C$ values of *D. glomerata* leaves, SOC, $^{13}CO_2$ were converted to $\delta^{13}C$ (‰) relative to the Pee Dee Belemnite (PDB, 0.0112372) standard and further expressed in atom % as the following:

$$\text{atom \%} = \frac{100 \times 0.0112372 \times (\frac{\delta}{1000}+1)}{1+0.0112372 \times (\frac{\delta}{1000}+1)},$$

where δ is ^{13}C (‰) value from samples.

The ^{13}C in *D. glomerata* leaves, DOC, and SOC (^{13}C excess) was calculated as follows:

$$\text{excess } 13_{C_{sample}} = [(atom\%13_C)_L - (atom\%13_C)_{UL}]/100 \times C_{sample},$$

where (atom% $^{13}C)_L$ and (atom% $^{13}C)_{UL}$ are the atom% ^{13}C in labeled and unlabeled samples, respectively, and C_{sample} is the C contents of each sample.

The ^{13}C incorporated into microbial biomass (excess ^{13}C-MBC) was calculated as the difference in ^{13}C excess in fumigated and un-fumigated soils, divided by a converting factor of 0.45 (Wu et al., 1990; Ge et al., 2012) as follows:

$$\text{excess } 13_{C-MBC} = \frac{[(atom\%13_C)_{f,\ L} - ((atom\%13_C)_{f,UL})] \times C_f - [(atom\%13_C)_{uf,\ L} - ((atom\%13_C)_{uf,UL})] \times C_{uf}}{100 \times 0.45}$$

where f and uf are fumigated and un-fumigated soil extracts, respectively. L and UL indicate extracts from labeled and unlabeled samples, respectively. C_f and C_{uf} represent the total C contents of the fumigated and un-fumigated soils, respectively. The end-member mixing model was used to calculate the fractions of SOM-(C_{SOM}) and litter-derived C (C_{leaves}). The litter-derived $^{13}CO_2$ was calculated by combining mass spectrometric and efflux measurements data (Phillips et al., 2005; Wild et al., 2014; Ye et al., 2015), as follows:

$$C_{litter-derived} = \frac{atom\%CO_{2mix} - atom\%CO_{2ck}}{atom\%C_{litter} - atom\%C_{soil}} \times CO_{2mix}$$

$$C_{SOM-derived} = \frac{atom\%CO_{2mix} - atom\%C_{litter}}{atom\%C_{soil} - atom\%C_{litter}} \times CO_{2mix}$$

where $atom\%CO_{2mix}$ and $atomCO_{2ck}$ are the atom% ^{13}C values of CO_2 derived from the soils amended with ^{13}C-labeled litter and the soil un-amended without ^{13}C-labeled litter, respectively; $atom\%C_{litter}$ and $atom\%C_{soil}$ are the atom% ^{13}C values of litter and soil, respectively. CO_{2mix} is the total CO_2 derived from the soil with ^{13}C-labeled litter.

The PE of SOM was calculated as fellow (Shahbaz et al., 2017b):

$$PE = T_{CO_2-C} - CK_{CO_2-C} - L_{CO_2-C}$$

where T_{CO_2-C} is the total CO_2-C from soils amended with a ^{13}C-enriched litter; T_{CO_2-C} is the CO_2-C derived from the control (CK); and L_{CO_2-C} is the CO_2-C derived from the added ^{13}C-labeled litter.

Microbial C-use efficiency (CUE) of litter-derived C was calculated at each destructive sampling from the following formula (Geyer et al., 2016; Fang et al., 2018),

$$CUE = \frac{13_{C-MBC}}{13_{C-MBC} + R_{cum}}$$

where 13_{C-MBC} and R_{cum} are litter-derived MBC (mg kg^{-1} soil) and the cumulative litter-C mineralization (mg kg^{-1} soil) after incubation for 2, 8, 15 and 37 days, respectively.

The metabolic quotient (qCO_2) was calculated as the ratio of the CO_2 emission rate to microbial biomass (Anderson & Domsch, 1993), as follows:

$$qCO_2 = \frac{CO_2 - C}{MBC}$$

where $CO_2 - C$ and MBC are CO_2 emission rate (mg C kg^{-1} soil d^{-1}) and the content of microbial biomass C (MBC, mg kg^{-1}soil) at 2, 8, 15 and 37 days incubation.

The V_{max} of extracellular enzymes was estimated by using the Michaelis-Menten equation (Tischer et al., 2015),

$$V = \frac{V_{max} \times [S]}{K_m + [S]}$$

where V was reacted rate, V_{max} was the maximal velocity of enzyme, [S] was the substrate concentration, and K_m was the substrate concentration when V was equal to 1/2 V_{max}.

All figures and statistical analyses were made by R software (4.0.0). All data were checked for normality and homogeneity of variance with *qqPlot* and *LeveneTest* function. Two-way analysis of variance was performed to test the effects of fertilizer, leaves returning (Yes and No), and fertilizer-litter interactions using the *aov* function. The mean of each treatment (Control, N, P and NP) were compared using the least significant difference at 5% level (LSD 0.05) in the "agricolae" package. The validity of ANOVA was checked by *outlierTest* Function.

3 Results

3.1 Response of SOC mineralization to litter and N and P addition

The CO_2-C efflux rate was higher for soils amended with exogenous litter as compared to soils without litter addition in all treatments. The CO_2-C emission from soils with litter in all four treatments increased rapidly from the beginning of the chasing period, between days 1and 6, and then, after peaking at day 6, declined sharply from till day 10. Further, from the day 20, remained almost constant until the end of the experiment (Fig.1a). Meanwhile, the shape and magnitude of the CO_2-C emission pattern during the chasing period for was comparable for the treatments without litter addition and control. Consequently, the cumulative emitted SOM-derived CO_2-C was consistently higher ($p < 0.05$) for soils amended with endogenous litter as compared to soils without plant litter addition, while for all not amended with litter soils there were no differences with control (Fig.1b and Table 1).

Herewith, the cumulative amount of SOM-derived CO_2-C was 8.72 ± 5.62% and 4.42 ± 3.13 % smaller, when soils were concurrently amended with litter + N, and litter + NP as compared to solo litter. In contrast, 3.19 ± 1.08 % large in the case when litter + P were added to soils concurrently (Fig.1b and Table 1). The cumulative litter-derived CO_2-C emission was by 7.10 ± 5.07 %, 17.35 ± 4.09 %, and 7.83 ± 4.92 % smaller in response to N, P and NP addition across the entire incubation period as compared to litter only input (Fig.1c). Positive PE was observed for all treatments during the chasing period. Compared to solo litter input, the PE was by 13.28 ± 6.66 % and 9.16 ± 6.48 % lower the case of litter + N and litter + NP addition, respectively, but by 6.59 ± 2.24 % higher in response to litter + P addition (Fig.1d).

3.2 Response of available nutrients to litter and N and P addition

The DOC concentration was higher ($p < 0.05$) in all treatments with plant litter addition (Table 1). The DOC concentration for samples amended with exogenous litter decreased (15-21%) from day 2 to day 37, while the DOC concentration for treatments litter addition peaked at day 6 of the chasing period and then after decreasing up to day 12, remained almost constant until the end of the experiment on day 37 (Fig.2a and Table 1). The excess of ^{13}C in the $DO^{13}C$ pool had maximal values at the day 2 (except litter + P), and changed in the order: litter + P > litter + NP> litter + N > litter. In the end of incubation, compare to solo litter, the excess ^{13}C was 16.78 ± 5.25 %, 19.36 ± 4.51% and 28.17 ± 10.28% for litter + P, litter + N and litter + NP, respectively (Fig.2b). Similarly, the NO_3^- content was consistently higher ($p < 0.05$) in soils amended with exogenous litter-C compared to non-amended with C soils. At the same time, the NO_3^- content was higher ($P < 0.05$) for all treatments with N addition (Fig.2c and Table 1). The Olsen-P content was consistently higher ($P < 0.05$) in all soils with plant residues addition compared to treatments without. Mineral P fertilization increased the

Olsen-P content across the entire incubation period regardless of whether carbon has been added (Fig.2d and Table 1). The SOC concentration was higher ($P < 0.05$) in all treatments with plant litter addition than without plant litter addition in the end of incubation day (Table 1).

3.3 Response of microbial biomass, qCO_2 and CUE to plant residues, N and P application

N, P and NP decreased ($p < 0.05$) MBC in the end of incubation for only four treatments without litter addition (Table 1). MBC and qCO_2 decreased from day 2 to day 37 for four treatments with litter addition (Fig. 3a and b). The tracer incorporation to the microbial biomass was observed already in the beginning of the chasing period. Thereafter, the share of plant litter derived ^{13}C in MBC decreased sharply from day 2 to day 6, and remained almost constant until the end of the incubation. The proportion of carbon, immobilized by microbes from plant litter was largest when litter + N were added to soils concurrently (Fig. 3c). Litter-CUE decreased from day 2 to day 37 for four treatments with litter addition (Fig. 3d).

3.4 Response of soil enzymes on plant residues, N and P application

The V_{max} of BG, NAG and AP were higher ($p < 0.05$) in soils amended with litter across all treatment groups (Table 1). The V_{max} of BG of treatments without litter addition weakly increased across the whole incubation. For the treatments with litter addition (except litter + NP), the V_{max} of BG also increased in the end of day compare to the day 2 (Fig.4a); the V_{max} of NAG increased sharply from day 2 to day 6, then decreased from day 6 to day 14 and was constant until the end of the experiment (Fig.4b). For treatments without litter addition, the V_{max} of NAG decreased from day 2 to day 14 and then did not change significantly till the end of the chasing period (Fig.4b). In the end of incubation day, the V_{max} of NAG was higher (P <

0.05) in treatments with N addition than without N addition treatment (Table 1). The V_{max} of AP for solo litter and litter + N treatments increased from day 2 to day 14 and then has not been changed until the end of the experiment on, while for litter + P and litter + NP treatments a decline of the V_{max} was observed, being much higher ($P < 0.05$) in soils without P addition in the end of incubation period (Fig.4c and Table 1).

4 Discussion

4.1 N, P and NP addition effect on the decomposition of SOM

The soil microbial biomass nutrient pool is considered to be a highly active and heterogeneous pool and controlled by the availability of nutrients, i.e., N, P and other macro- and microelements (Kuzyakov and Xu 2013; Liu et al., 2018). The availability of nutrients also has a direct influence on decomposition of plant residue and SOM (Soong et al., 2018; Wang et al., 2019). However, in our experiment the addition of mineral N, P, and NP only didn't change the C emission rate and accumulative C emission compare to the control. This might be because of microbial C limitation for the built up of microbial biomass (Sinsabaugh et al., 2008; Yayi et al., 2021) and microbial metabolism (Sterner & Elser, 2002; Schimel & Weintraub, 2003). Intermediate degradation products likely led to a peak of the DOC concentration at day 6 of incubation. This is because microbial SOM decomposition affect dissolved organic molecules that are available for microbial use, but whose production is not under immediate microbial control (Schimel & Weintraub, 2003; Allesson et al., 2020).

4.2 The decomposition of plant litter

Compared with the treatments without litter addition, plant litter was added in available nutrients (N and P) limitation Kazakhstan steppe soil, the C efflux from four treatments with

added litter increased sharply at the first day 6 of incubation and then slowly decreased until the end of incubation. It indicated soil microorganisms preferentially utilize freshly and easily decomposable C substrates over native soil organic C (SOC) (Parshotam et al., 2000; Liu et al., 2020; Yuan et al., 2014; Zhu et al., 2016). Microorganism rapidly take it up and metabolize it, producing new microbial biomass (MBC) and increasing respiration (CO_2) to support their growth (Schimel & Weintraub, 2003). Plant residue (e.g., leaves, shoots, roots) include easily decomposable labile C (i.e., starch and glucose), leading its rapid decomposition at initial stage of incubation (Nottingham et al., 2009). Once these easily degradable C was decomposed, CO_2 emission decreased, along with decreasing qCO_2 and CUE was limited, the C emission decreased (Fig. 3b and 3d), as is also reported by Wang et al., 2019. More stable components of plant residues such as lignin are known to be less efficiently utilized by soil microorganisms (Baumann et al., 2009; Waldrop et al., 2012). As parts of the products of litter decomposition are water soluble, particularly those derived from non-complete lignin degradation (Bourbonnais & Paice, 1990; Klotzbücher et al., 2011), litter addition led also to higher DOC concentrations (Chang et al., 2004). Then mineralization would continue to undergoing with increasing DOC concentration (Guggenberger & Zech, 1992; Guggenberger et al.,1994).

4.3 N, P and NP addition effect on the decomposition of plant litter and PE

Besides leaf litter the microbial biomass and activity also responded on mineral N and P fertilization. This exogenous nutrient supply led to a higher availability of N and P and thus more favorable conditions for the microorganisms to align with their C: nutrients stoichiometry. Under alleviating microbial nutrient limitation, the increasing N and P availabilities as related to the C resource after fertilization led to an significantly decreased in

litter-derived C, because litter C is redirected from waste respiration to microbial growth (Schimel & Weintraub, 2003; Wang et al., 2019). Therefore stable C in plant litter was either by absorption or by microbial metabolism and recycling during the incubation (Gunina et al., 2014), resulting in ^{13}C in DOC increased and SOC accumulation (Liebmann et al., 2020; Yu et al., 2020). But increasing N and P availabilities cannot change the fact microbes preferentially utilized the added substrate (Wang et al., 2019). Exogenous easily degraded organic C and other nutrients promoted the mineralization of native SOM though microorganism, resulting in a positive PE. Soil microbes fed on slowly decomposable litter produced hydrolase are able to degrade similar recalcitrant compounds in SOM in a later stage (Chen et al., 2014) . Thus the V_{max} of BG, NAG, and AP increased from day 2 to day 37 with litter combined with N, P, and NP addition. Our results shows that N addition increased NO_3^- and the V_{max} of NAG, this is because NAG activity reflects the microbes (fungal) activity for chitin breakdown (Miller et al., 1998), fungi has a higher N acquisition to maintain its growth (Sinsabaugh et al., 2008; Shi et al., 2018; Yayi et al., 2021). Perhaps, N addition in alkaline soil induces a decrease in soil pH and may produce optimal conditions for NAG activities (Burns et al., 2013). Differently, a better P availability to microorganisms by P fertilization led to a decrease the V_{max} of AP (Fig. 4c) in order to saving energy and N for C sequestration (Zhou, et al., 2017; Wei et al., 2019). Litter + NP also decreased litter-C decomposition, illustrating that the CNP resources may have been diverted towards microbial growth rather than litter decomposition (Hui et al., 2020). However, the V_{max} of BG in all treatments supplied with litter did not significantly change at the end of incubation (Table1). This suggested that the V_{max} of BG is not closely related to C:N:P stoichiometric ratios, and microbial C demand by mineral N and P addition may have been insufficient to induce C

mineralization (Liu et al., 2020;Wei et al., 2019; Zhu et al., 2018), resulting in decreasing plant residues decomposition (Keiblinger et al., 2010; Zhu et al., 2017) and increase protential C sequestration (Williams et al., 2006; Zang et al., 2016).

5 Conclusions

In the present study, increasing the availability of N and P in N and P limitation semi-arid Kazakhstan steppe soil did not change SOM decomposition due to microbial C limitation. Litter addition only largely increased SOM decomposition and induce a positive PE. Compare to litter addition only, N, P, and NP addition led to decreasing by 7.10 ± 5.07 %, 17.35 ± 4.09 % and 7.83 ± 4.92 % in cumulative leaves-derived C emissions respectively, while increasing by 16.78 ± 5.25 %, 19.36 ± 4.51% and 28.17 ± 10.28% leaves-derived dissolved organic C (DOC), respectively. Supply of an easily available C source in form of leave litter to an increase of the SOC mineralization by 89.5–99.1%, i.e. fueling a positive PE due to the fact that microorganisms preferentially utilize degraded organic and other available nutrients for litter and SOM. Application of litter + N force microorganism have a higher N acquisition to increase the V_{max} of NAG. Litter + P limited the V_{max} of AP for saving energy and N, resulting in maintaining recalcitrant and stabilize substrate C. Higher availability of C, N and P stimulated more microbial growth rather than litter decomposition. During the rapidly decomposition stage, the higher CUE led to a decrease of litter-derived CO_2 production. In conclusion, our incubation experiment indicates that N and P fertilization of semiarid soils helps to maintain microbial stoichiometric balance at plant residue return to soil, limiting C emission and promoting potential C sequestration.

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Table. 1

Table 1 Results of two-way ANOVAs showing the effects of fertilizer, litter added (Yes and No), and fertilizer-litter interactions on soil organic matter (SOM)-derived C, soil organic C (SOC), dissolved organic C (DOC), available nutrients (NO_3^-, Olsen-P), microbial biomass C (MBC), C, qCO_2 and the V_{max} of BG, ANG, and AP in the end of incubation.

Fertilizer	Litter	SOC	DOC	NO_3^-	Olsen-P	MBC	qCO_2	BG	NAG	AP
		g kg^{-1}	mg kg^{-1}				μg mg^{-1} h^{-1}	nmol g^{-1} h^{-1}		
Control	Yes	34.88±0.49aA	161.66±6.67bA	335.15±8.28bA	18.39±1.87bA	612.64±39.61aA	0.71±0.13bA	1003.03±99.90abA	526.10±120.19bA	403.94±59.73aA
	No	33.40±0.64αB	147.18±3.90αB	67.21±0.94βB	8.98±0.91βB	418.63±8.26αB	0.60±0.18αA	342.93±32.65αB	48.80±8.78βB	197.28±29.95αB
N	Yes	34.67±0.14aA	166.29±4.45αbA	364.00±2.36aA	17.95±0.33bA	564.23±18.38bA	0.82±0.08abA	823.59±299.73bcA	600.32±93.80abA	438.24±180.14aA
	No	32.66±0.82αB	147.36±3.72αB	103.57±1.66αB	9.00±1.00βB	402.45±10.33αβB	0.62±0.18αA	373.11±147.25αB	74.64±15.45αB	190.91±30.62αB
P	Yes	34.51±0.94aA	164.51±3.10bA	328.47±5.19bA	20.29±1.06bA	550.17±9.68bA	0.83±0.07abA	577.81±168.80cA	690.97±103.58abA	291.44±42.67aA
	No	33.42±0.88αA	146.22±1.88αB	67.59±1.04βB	15.91±1.72αB	398.05±15.36βγB	0.64±0.09αB	307.44±49.27αB	42.13±8.83βB	85.04±7.42βB
NP	Yes	34.45±0.62aA	174.58±9.35aA	362.02±11.86aA	24.66±2.13aA	538.60±34.81bA	0.91±0.08aA	1351.72±395.31aA	734.21±177.05aA	316.16±32.20aA
	No	32.64±0.44αB	149.35±6.03αB	102.67±2.89αB	16.52±3.62αB	382.14±7.43γB	0.75±0.09αB	317.89±96.06αB	55.26±10.03βB	71.28±9.60βB
Factor (Df)		F *P*	F *P*	F *P*	F *P*	F *P*	F *P*	F *P*	F *P*	F *P*
Fertilizer (3)		1.32 0.29	3.14 *	94.22 ***.	28.52 ***	9.6 ***	2.72 0.67	5.33 **	2.04 0.14	7.16 **
Litter (1)		45.47 ***	102.72 **	17258.26 ***	140.29 ***	479.99 ***	15.16 ***	73.121 ***	330.16 ***	80.21 ***
Fertilizer * Litter (3)		0.72 0.55	1.38 0.27	0.96 0.43	3.09 *	1.58 0.22	0.22 0.88	5.40 **	2.269 0.11	0.21 0.89

Note: The fertilizers included no nitrogen and phosphorus fertilization (CK), nitrogen fertilization (N), phosphorus fertilization (P), and combined nitrogen and phosphorus fertilization (NP). Different English and Greek lowercase letters indicate significant differences ($P < 0.05$) between fertilizers in Yes and No, respectively. The English uppercase letters represent significant differences between Yes and No at $P < 0.05$. The symbols *, **, and *** represent significant differences on the effects of fertilizer, leaves returning and fertilizer-litter interactions and No at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. BG, β-1.4-glucosidase; NAG, β-1, 4-N-acetyl-glucosaminidase; AP, phosphomonoesterase (β-1,4-phosphate). All results are means ± standard deviation (n = 4).

Fig.1 CO_2-C efflux (a), cumulative SOM-derived CO_2-C (b), cumulative litter-derived CO_2-C (c) and priming effect (PE, d) over 37 days of soil incubation period under eight different treatments, i.e., CK (only soil); N, soil supplemented with N fertilizers; P, soil supplemented with P fertilizers; NP, soil supplemented with N and P fertilizers; litter, soil supplemented with *Dactylis glomerata* L.(litter); litter + N, soil supplemented with litter and N fertilizers; litter + P, soil supplemented with litter and P fertilizers; litter + NP, soil supplemented with litter, N and P fertilizers. Samples were taken at four times days 2, 6, 14 and 37 days, respectively. All results are means ± standard error (n = 4).

Fig.2 Soil dissolved organic C (DOC) concentration (a), excess dissolved organic ^{13}C ($DO^{13}C$) (b), NO_3^- concentration (c), and Olsen-P concentration (d) over 37 days of soil incubation period under eight different treatments, i.e., CK (only soil); N, soil supplemented with N fertilizers; P, soil supplemented with P fertilizers; NP, soil supplemented with N and P fertilizers; litter, soil supplemented with *Dactylis glomerata* L. (litter); litter + N, soil supplemented with litter and N fertilizers; litter + P, soil supplemented with litter and P fertilizers; litter + NP, soil supplemented with litter, N and P fertilizers. Samples were taken at four times days 2, 6, 14 and 37 days, respectively. All results are means ± standard error (n = 4).

Fig.3 Soil microbial biomass C (MBC) (a), the metabolic quotient (qCO_2) (b), excess microbial biomass ^{13}C ($MB^{13}C$) (c), and C use efficiency (CUE) of added litter, (d) over 37

days of soil incubation period under eight different treatments, i.e., CK (only soil); N, soil supplemented with N fertilizers; P, soil supplemented with P fertilizers; NP, soil supplemented with N and P fertilizers; litter, soil supplemented with *Dactylis glomerata* L. (litter); litter + N, soil supplemented with litter and N fertilizers; litter + P, soil supplemented with litter and P fertilizers; litter + NP, soil supplemented with litter, N and P fertilizers. Samples were taken at four times days 2, 6, 14 and 37 days, respectively. All results are means ± standard error (n = 4).

Fig.4 The maximal velocity (V_{max}) of β-1.4-glucosidase (BG) (a), β-1, 4-N-acetyl-glucosaminidase (NAG) (b) and phosphomonoesterase (β-1,4-phosphate (AP) (c) over 37 days of soil incubation period under eight different treatments, i.e., CK (only soil); N, soil supplemented with N fertilizers; P, soil supplemented with P fertilizers; NP, soil supplemented with N and P fertilizers; litter, soil supplemented with *Dactylis glomerata* L. (litter); litter + N, soil supplemented with litter and N fertilizers; litter + P, soil supplemented with litter and P fertilizers; litter + NP, soil supplemented with litter, N and P fertilizers. Samples were taken at four times days 2, 6, 14 and 37 days, respectively. All results are means ± standard error (n = 4).

Fig1

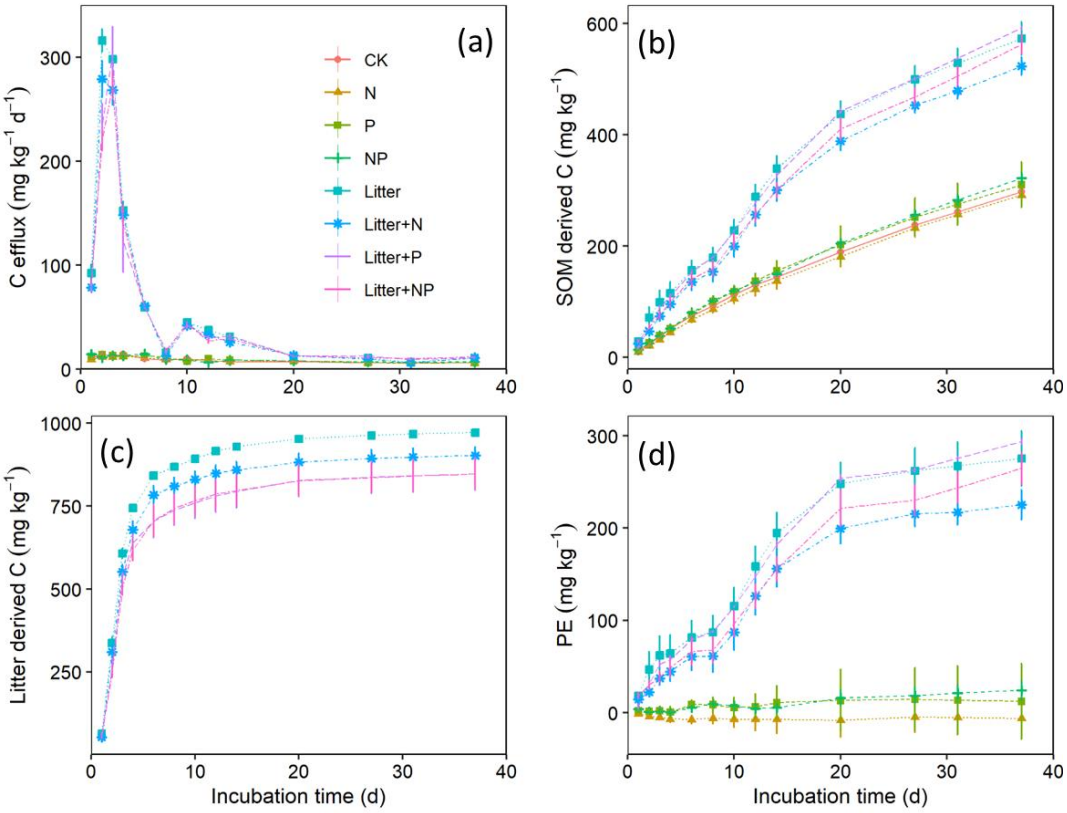

(a)
CK
N
P
NP
Litter
Litter+N
Litter+P
Litter+NP
C efflux (mg kg^{-1} d^{-1})
(b)
SOM derived C (mg kg^{-1})
(c)
Litter derived C (mg kg^{-1})
Incubation time (d)
(d)
PE (mg kg^{-1})
Incubation time (d)

Fig.2

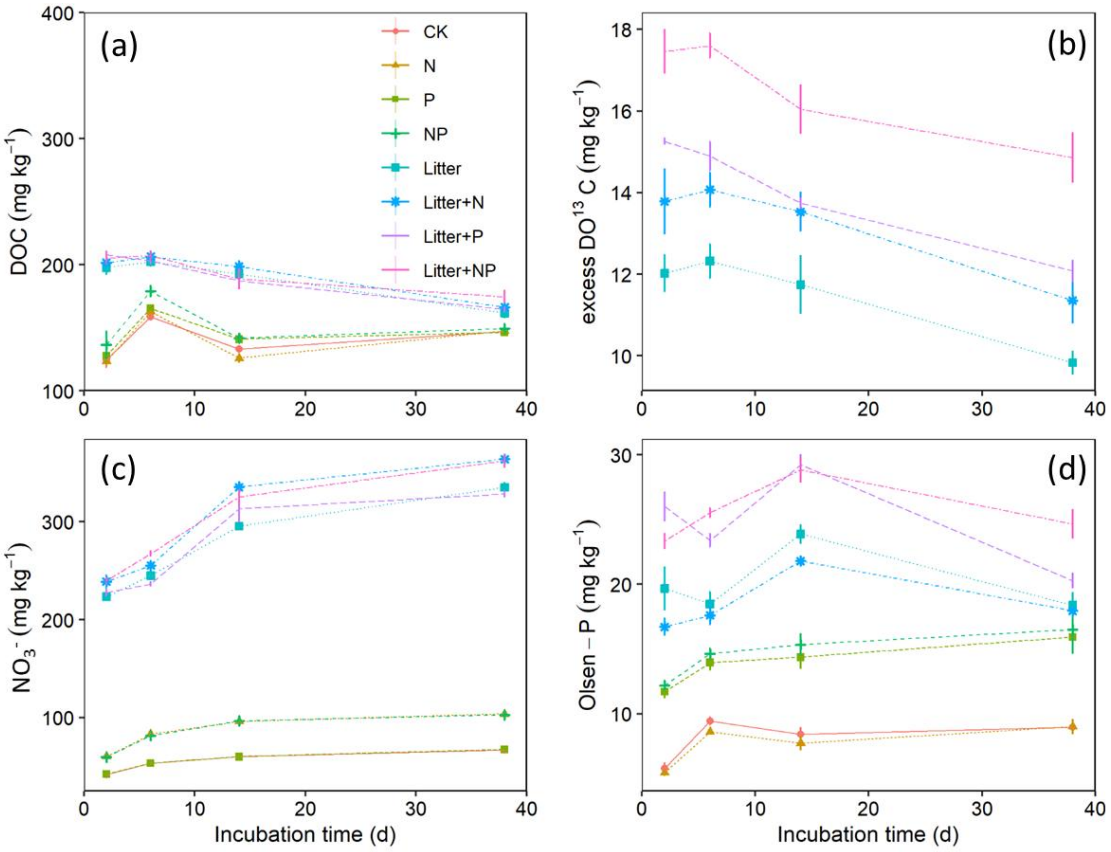

(a)
CK
N
P
NP
Litter
Litter+N
Litter+P
Litter+NP
DOC (mg kg^{-1})
(b)
excess DO^{13}C (mg kg^{-1})
(c)
NO_3^- (mg kg^{-1})
(d)
Olsen – P (mg kg^{-1})
Incubation time (d)
Incubation time (d)

Fig.3

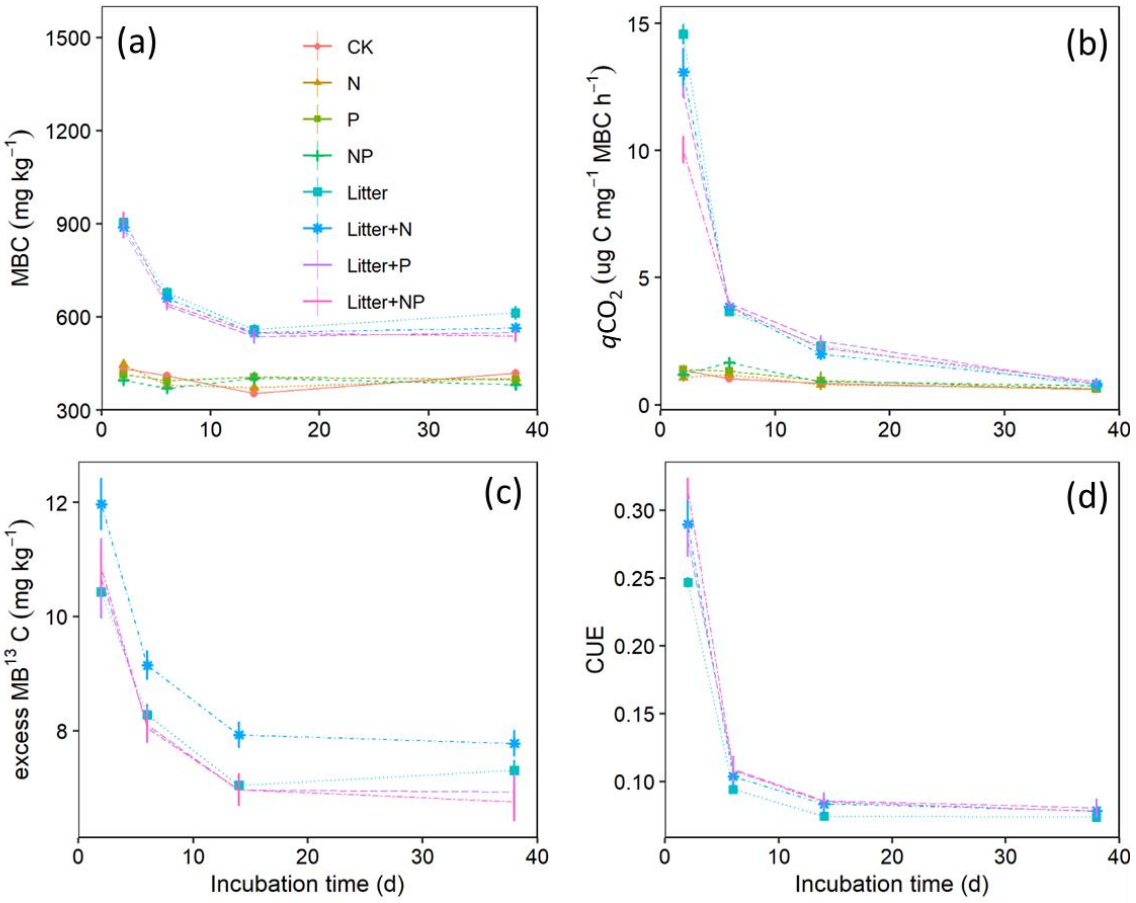

(a)
CK
N
P
NP
Litter
Litter+N
Litter+P
Litter+NP
MBC (mg kg^{-1})
(b)
qCO_2 (ug C mg^{-1} MBC h^{-1})
(c)
excess MB^{13}C (mg kg^{-1})
Incubation time (d)
(d)
CUE
Incubation time (d)

Fig.4

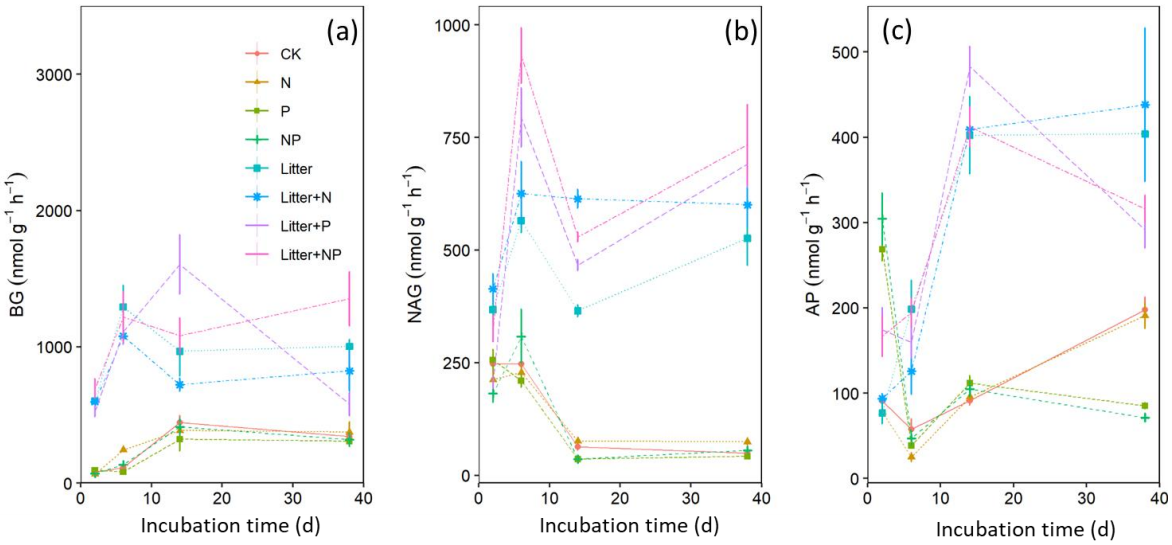
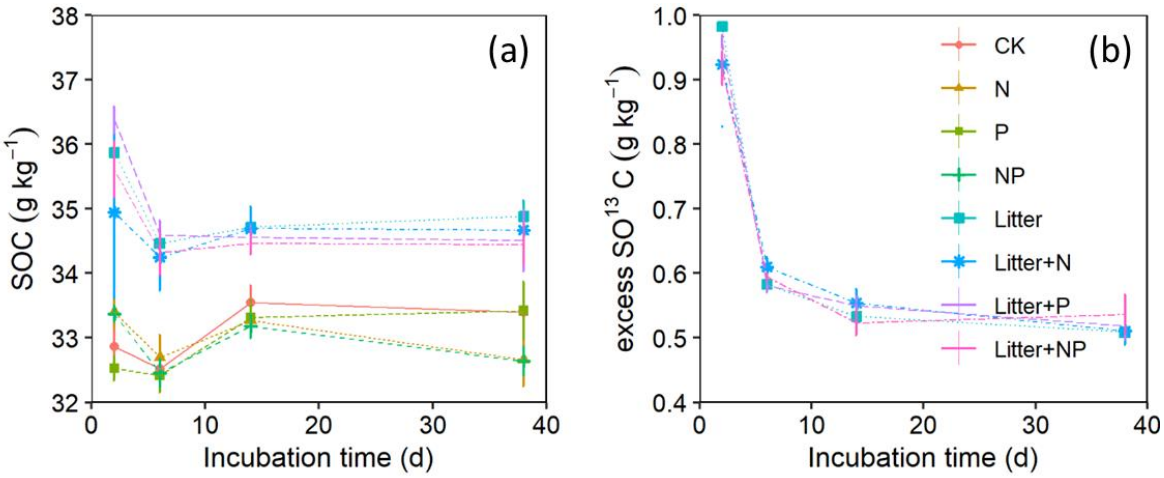

(a)
CK
N
P
NP
Litter
Litter+N
Litter+P
Litter+NP
BG (nmol g^{-1} h^{-1})
Incubation time (d)
(b)
NAG (nmol g^{-1} h^{-1})
Incubation time (d)
(c)
AP (nmol g^{-1} h^{-1})
Incubation time (d)

Fig.S1 Soil organic C (SOC) content (a) and excess organic ^{13}C ($SO^{13}C$) content (b) over day 37 day of the incubation period of the step soil under four different treatments, i.e., CK (only soil) ; N , soil supplemented with N fertilizers; soil supplemented with P fertilizers; soil supplemented with N and P fertilizers; litter, soil supplemented with *Dactylis glomerata* L. (litter); litter + N, soil supplemented with litter and N fertilizers; litter + P, soil supplemented with litter and P fertilizers; litter + NP, soil supplemented with litter, N and P fertilizers at four times (at 2, 6, 14 and 37 days, respectively). All results are means ± standard error (n = 4).



5 Study 3

The stoichiometric ratio of available C and P affects bioavailable P in Kazakhstan steppe soil

Contribution: I participated in work, sampling activities, and the experiment incubation, performed most of the analysis in the laboratory, collected and evaluated data, prepared tables and figures, and wrote the manuscript.

The stoichiometric ratio of available C and P affects bioavailable P in Kazakhstan steppe soil

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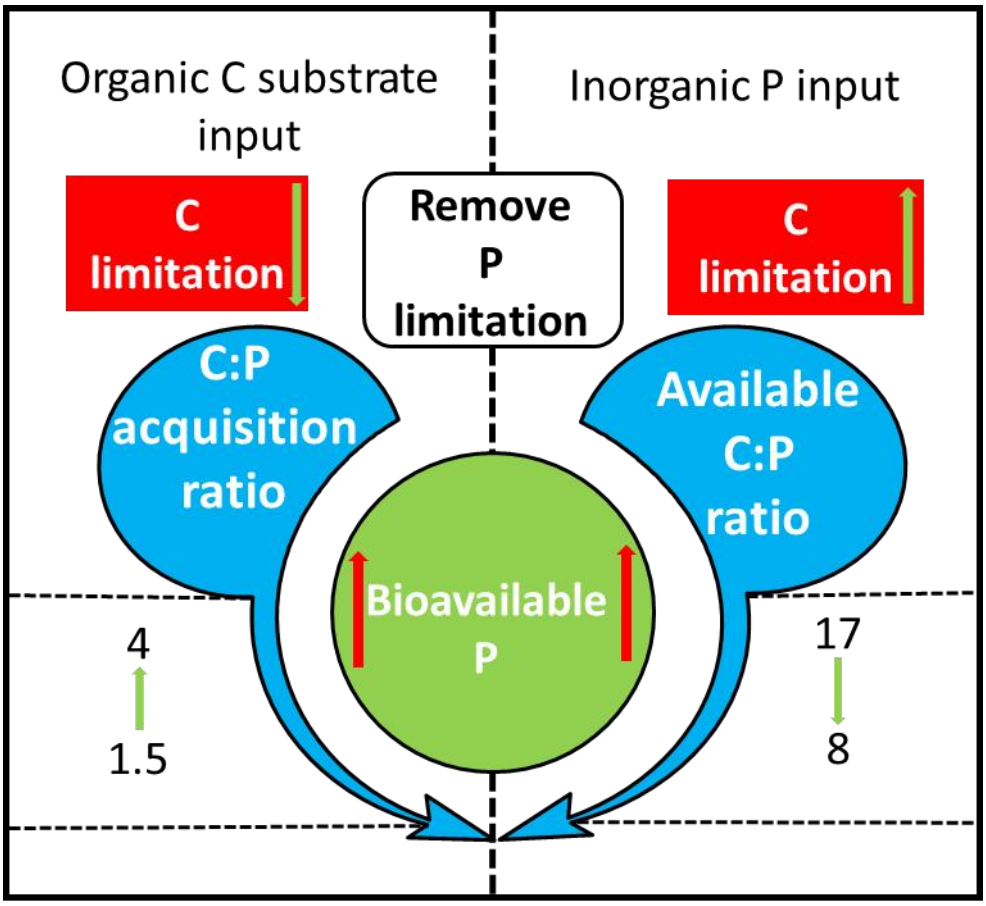
Highlights

- Microbial C limitation decreased with plant residues application, but increased with mineral P addition
- Increased P availability alleviates or eliminates microbial P limitation
- Microbial C:P acquisition ratio was regulated by the ratio of C and P acquisition enzymes
- The C:P acquisition ratio was regulated by the available C:P ratio

Abstract The stoichiometric ratio of carbon (C): phosphorus (P) acquisition is strongly correlated with soil available C:P ratio. However how the stoichiometric relationship between acquiring C and P through microbial metabolism affects bioavailable P is poorly understood in semi-arid agricultural ecosystems. Our objective was to investigate the underlying mechanisms of the P availability in typical P-limited steppe soil from Kazakhstan in response to mineral nutrient (Na_2HPO_4) with and without *Dactylis glomerata* L. leaves addition in a 38-day incubation experiment. Four bioavailable P fractions content ($CaCl_2$-P, Citrate-P, Enzyme-P, and HCl-P) were improved. Sole application of P fertilizer decreased the maximal velocity (V_{max}) of P acquisition enzyme (phosphomonoesterase) but increased microbial C limitation, resulting in increasing the ratio of C to P acquisition but decreasing the ratio of available dissolved organic C: Olsen-P. In contrast, plant residues returning (the application of sole *D. glomerata* leaves and the combined application of *D.glomerata* and mineral P) increased V_{max} of C (β-1, 4-glucosidase, β-1,4-cellobioside, β-1, 4-xylanase) and P acquisition enzymes, however decreasing microbial C and P limitation through improving microbial metabolism. Furthermore, the spearman correlation analysis suggests that microbial C limitation has a negative effect on bioavailable P, illustrating that the decreasing of microbial C limitation can improve soil bioavailable P during the decomposition of organic matter. In conclusion, the decomposition of organic residues eliminated microbial P limitation and increased P availability by allocating C and P acquisition enzymes to balance the stoichiometric ratio of microbial C and P demand.

Keywords: C:P stoichiometric ratio; microbial C and P limitation; bioavailable P

Graphical abstract



1 Introduction

Phosphorus (P) is an essential nutrient and element for all life forms (Correll, 1998; Baligar et al., 2001). The original P source in ecosystems is primary minerals, such as apatite (Nezat et al., 2008), and P occurs in soils as inorganic P (P_i) associated with secondary minerals, or organic P (P_o) (Vitousek et al., 2010), which is also mostly associated with secondary minerals (Andrino et al., 2021). P_i has a low plant availability due to sorption or occlusion within aluminum (Al) and iron (Fe) in acidic soils, or calcium (Ca) and magnesium (Mg) cations in alkaline soils (Sharpley, 1995; Balemi & Negisho, 2012). In agriculture practice exogenous P input can be rapidly immobilized by soil microorganism (Frossard et al., 2000; Bünemann et al., 2012), especially in semi-arid Kazakhstan steppe P-limited soil (Palpurina et al., 2019), influencing the utilized rate of P_i by plants (Johnston et al., 2014; Wei et al., 2017; Yuan et al., 2019). With plant and microbial residues, P_o is returned to the soil, which accounts for about 30–50% and even up to 80 % of the total P pool (Harrison, 1989; Richardson et al., 2009).

Phosphate-solubilizing microorganisms secret phosphomonoesterases to hydrolyze phosphate monoester bonds of P_o, including mononucleotides and sugar phosphates (Nannipieri et al., 2011). Under enzymatic catalysis, the phosphate monoester bond is cut to liberate the phosphate group (Bárta et al., 2014; Tischer et al., 2015), thereby increasing the bioavailability of soil P (Nannipieri et al., 2011). The P_o mineralization is linked to the organic C (OC) mineralization (Harrison, 1982; Cui et al., 2020; Wei et al., 2019b). Higher contents of labile-C in rhizosphere soil limit C and P acquisition to mineralize less organic P,

because plants are a contender for P compare to microorganism (Liu et al., 2021). However changes in the soil P_i content (mineral P fertilizer) would lead to shifts in the C:P stoichiometric ratio along with shifts in extracellular enzyme activities related to the acquisition of C and P (Soong et al., 2018; Wei et al., 2019a). Because P_i rather acids and chelators are needed to mobilize it from Fe- and Al-oxides.

DeLuca et al. (2015) conceptualized four different P fractions based on their bioavailability, including three P_i fractions ($CaCl_2$-P: soluble P_i, Citrate-P: active inorganic P which is chelate-extractable, and HCl-P: more recalcitrant P_i) and one P_o fraction (Enzyme-P: P_o readily attacked by phosphatases) (DeLuca et al., 2015). The size of these four P fractions are considered to be influenced by human activities (Canfield et al. 2010; Zhu et al. 2018), especially such as straw return and mineral fertilization in agricultural ecosystems (Zang et al., 2016; Wei et al., 2019a; Wang et al., 2019). It leads to changes for bioavailable P and also for key stoichiometric ratios of microbes C:P acquisition (Razavi et al. 2016; Wei et al. 2019a,b). Because according to the principle of nutrient stoichiometry, the elemental stoichiometry of microbial biomass determines microbial nutrient demand for environmental nutrient availability and microbial metabolism of one nutrient can also limited by another (Cui et al., 2020). Therefore the synthesis and release of C and P acquisition enzymes are regulated by non-equilibrium flows of energy and nutrients (Ng et al., 2014; Sinsabaugh et al., 2009), maintaining their inner balance. The ratio of C and P acquisition enzymes activity reflects the elemental stoichiometry of soil available C (i.e., dissolved organic carbon (DOC) and soil available P (i.e., Olsen-P) that fulfill the microbial nutrient demand (Sinsabaugh et al., 2009; Zhu et al. 2018; Wei et al. 2019a,b). Therefore the stoichiometry of C and P acquisition

enzymes reflects the demand of microorganisms for C and P (Hofmann et al., 2016; Wei et al., 2019a; Liu et al., 2021) and the characteristics of microbial metabolic limitation represented by C or P (Cui et al., 2020). The optimal growth at the stoichiometric balance of C and P is 42.4-59.5 for soil microorganisms inner homeostasis (Cleveland & Liptzin, 2007; Xu, et al., 2013) and 186 for the average available C:P ratio of SOM as microbial substrate (Cleveland & Liptzin, 2007). To explore the mechanism of microbial metabolism responses to changes in soil available nutrients (Cui et al., 2019) and the characteristics of microbial metabolic limitation (Sinsabaugh et al., 2009), the "lengths" and "angle" of vector in enzymatic activities of C:N vs C:P acquisition have been introduced to quantify the relative investments in C vs nutrient acquisition (vector lengths) or N vs P acquisition (vector angles) (Moorhead et al, 2013, 2016; Cui et al., 2019). Specially, the vector length represents microbial C limitation, the vector angles less than 45° represents microbial N limitation, the vector angles more than 45° represents microbial P limitation.

In agricultural ecosystems, P availability is often is a limiting factor for crop production (Ding et al., 2007) and soil microbial metabolism (Liu et al., 2008; Liu et al., 2021). Compare to mineral P fertilizer, the equilibrium levels of P_o from plant residues in semi-arid arable soils are controlled by a balance between the physical protection offered by the soil matrix and the suitability of the environment for biological productivity (Turner et al., 2003; Palpurina et al., 2019). Therefore the variability in C:P acquisition ratio can explain thought the compound effect of stoichiometric limitation and physical losses (Manzoni et al., 2010; Wei et al., 2019a). However, it is always unclear that the mechanism of the stoichiometry relationship of soil C and P effect soil bioavailable P in semi-arid agricultural ecosystems in

response to mineral P fertilizer and plant residues returning. The goal of our study was to assess the impact of mineral fertilizer P input on P availability of a semi-arid Kazakh steppe soil with and without plant residues application. We hypothesized that (i) solo P_i (mineral P fertilization) decrease soil P_o mineralization due to microbial C limitation; (ii) plant residues returning decreased microbial C limitation to stimulate soil P_o mineralization, causing a decreasing trend in produce higher the V_{max} of C and P acquisition enzymes; (iii) forcing microbes to produce lower dissolved organic C: Olsen-P ratio during the mineralization of plant residues for maintaining the balance of C:P stoichiometric ratio. To test the hypotheses, we used ecological stoichiometry theory to explain the non-equilibrium flows of nutrients (mainly P) and energy (C) (Cui et al., 2020; Manzoni et al., 2010; Ng et al., 2014).

2 Materials and methods

2.1 Study site and soil sampling

The studied soil derives from northeastern Kazakhstan, in vicinity of the city of Kokshetau (53°02′N， 69°34′E). The study area is characterized by a continental climate with cold winters and hot summers, and large inter-seasonal temperature and precipitation fluctuations. According long-term observations at Schuchinsk meteorological station, the mean annual temperature and mean annual precipitation for the area are 1.4°C and 336 mm, respectively (Yapiyev et al., 2017).

A composite soil sample from six small profiles was taken in September 2017 from Ap horizons (0–20 cm). The soil profiles were randomly dug within a 2000 x 2000 m agricultural field under wheat monoculture. Fine roots and other plant residues were carefully removed manually and samples were bulked, dried at 40° C in a ventilated oven, and then stored in a

closed brown bottles. The soil's bulk density was 1.3 ± 0.08 g cm^3, and its main chemical properties were pH (1:5 soil/0.01M $CaCl_2$), 7.59 ± 0.03; soil organic matter (SOC), 35.20 ± 0.18 g kg^{-1}; inorganic C ($CaCO_3$), 21.60 ± 0.16 g kg^{-1}; total nitrogen (TN), 3.00 ± 0.01 g kg^{-1}; Olsen-P, 3.23 ± 0.86 mg kg^{-1}.

2.2 Experimental setup and design

The experiment included four treatments in four replicates each: (i) control (CK – soil only); (ii) soil amended with *Dactylis glomerata* L. leaves (as organic C substrate) (D); (iii) soil amended with inorganic phosphorus (P), and (iv) soil amended with both, *Dactylis glomerata L*. leaves and phosphorus (DP). Prior to the start of the incubation, dried soils were re-wetted to 50% of water holding capacity (WHC) and pre-incubated at 22°C for 14 days in the dark. Approximately 35 g of soil (dry mass equivalent) was placed to an incubation vessel, which was made of a plastic tube (diameter: 3.6 cm; high: 5 cm) sealed at the bottom with nylon mesh using plastic glue. To mimic plant residues input to soil, we used *Dactylis glomerata L*. leaves with a carbon content of 361.6 ± 0.1 g kg^{-1} dw and at an amount of 10% of the soil indigenous OC, i.e. 3.52 g kg^{-1} soil dw. Plant materials were oven-dried at 55°C for 2 days, cut to approximately 1 cm lengths, and crushed to a powder to assure homogeneous mixing of the substrate and the soil. The plant material was stored in the dark until being used. P_i (Na_2HPO_4) was added at a rate of 19.23 mg kg^{-1} soil dw, being equivalent to 50 kg P ha^{-1} distributed over 20 cm soil depth. The rate of P addition was close to values that are recommended for fertilization. Amount of added C was close the annual plant litter input to soil in croplands in Kazakhstan (Takata et al., 2007). For D and DP treatments, the incubated soil samples were divided into six layers of equal weight (every layer about 5.8 g dry soil),

then grass leaf material (about 68 mg dry mass) was added in between each of the layers (about total 340 mg dry mass for each vessel, which is equivalent to 3.52 g C kg dry soil). The Na_2HPO_4 fertilizer was dissolved in deionized water and added into soil. The soil was compacted to soil bulk density (1.3 g cm^{-3}) in the field. The WHC of the soil samples we adjusted to 60% by deionized water. All samples were covered with perforated aluminum foil and incubated at 24°C for 38 days in the dark. The WHC of all samples was monitored and kept constant through the incubation period.

2.3 SOC, TN, dissolved organic C, Olsen-P and microbial biomass C and P

After 38-day of the incubation, soils (of about 40 g of fresh weight) were collected from all treatments to measure SOC, dissolved organic C (DOC), Olsen-P, and microbial biomass C and P (MBC and MBP). To remove $CaCO_3$, the soils were fumigated with 37% HCl for 4 days and solid NaOH for 2 days (Harris et al., 2001). Then the soils were oven-dried (55 ℃) to measure SOC and TN using an elemental analyzer-isotope ratio mass spectrometer (EA-IRMS, Isotope Cube-Precision, Elementar, Germany). MBC were determined using the chloroform fumigation method (Brookes et al. 1985; Vance et al. 1987). In brief, about 10 g of well-mixed soil was used for extraction with 40 ml of 0.5 mol·L^{-1} K_2SO_4. Another aliquot of 10 g soil samples was extracted in the same manner after being fumigated with chloroform for 24 h. The extracts were analyzed for C by a Vario TOC CUBE (Elementar, Hanau, Germany), and MBC was calculated using the conversion factor k_c= 0.45 (Brookes et al. 1985; Vance et al. 1987). The non-fumigated samples were also used for DOC measurements (Liu et al., 2018). MBP was determined using chloroform fumigation–sodium bicarbonate ($NaHCO_3$) extraction, followed by the molybdenum antimony colorimetric method of

Brookes et al. (1982). Briefly, two 2 g of fresh soil (non-fumigated and fumigated) were prepared like above. A third soil sample (2 g of fresh soil) was treated with 0.2 mL of 250 µg P mL^{-1} KH_2PO_4 (to calculate recovery efficiency of P). The three soil samples were then extracted with 80 mL 0.5 M $NaHCO_3$ (pH 8.5). In all samples, P concentration was measured by UV/Vis absorbance spectra (Spectro Star Nano; BMG LABTECH GmbH, Ortenberg, Germany) at an absorbance value of 882 nm. Phosphorus measured in the non-fumigated sample represented Olsen-P (Olsen et al., 1954). The MBP was calculated using a conversion factor *k*p = 0.4 (Wu et al. , 2007), the difference between fumigation-P and un fumigation-P divided by the product of the conversion factor and the recovery efficiency of P.

2.4 Analyses of P fractions of different availability

The four P fractions were extracted with (i) 10 mM $CaCl_2$, (ii) 10 mM citrate, (iii) and 1.0 M HCl, and (iv) phosphatase mixture (0.2 U acid phosphomonoesterase and phytase), respectively, by shaking of 0.5 g fresh soil in each of 50-ml extractant at 200 rpm for 3 h (DeLuca et al., 2015; Wei et al. 2019a). After filtration, then 50 µl suspension for each sample was pipetted into 150-µl Malachite Green solution to color reaction in 96 microwell whiteboard. All samples were measured colorimetrically (630 nm) by UV/Vis absorbance spectroscopy (Spectro Star Nano, BMG LABTECH GmbH, Germany).

2.5 Assay of EEAs

The activities of following extracellular enzymes were measured: (i) C acquisition enzymes, β-1, 4-glucosidase (BG), β-1,4-cellobioside (CBH), and β-1,4-xylosidase (XYL); (ii) N acquisition enzymes, β-1,4-N-acetyl-glucosaminidase (NAG) and L-leucine aminopeptidase (LAP); and (iii) P acquisition enzyme, β-1,4-phosphate (AP). Their maximal

velocity (V_{max}) were measured based on 4-methylumbelliferone (MUF) and 7-amino-4-methylcoumarin (AMC) substrates (Pritsch et al. 2004; Sanaullah et al. 2016; Shahbaz et al. 2017). Five fluorogenic enzyme substrates based on 4-methylumbelliferone (MUF) were used: MUF-β-D-glucopyranoside (MUF-G; EC 3.2.1.21) for BG, MUF-β-D-xylopyranoside (MUF-X; EC 3.2.1) for XYL, MUF-β-D-cellobiohydrolase (MUF-C; EC 3.2.1) for CBH, MUF-N-acetyl-β-D-glucosaminide dehydrate (MUF-N; EC 3.2.1.21) for NAG, MUF-phosphate monoester (EC 3.1.3.2) for AP (Nannipieri et al. 2011). L-Leucine-7-amino-4-methylcoumarin (AMC) substrate was used to estimate L-leucine aminopeptidase (LAP) activity (Shahbaz et al., 2017). Briefly, 1 g of fresh soil was mixed and homogenated in 50 ml deionized water for 30 min using an oscillating device (HS501, IKA®-Werke GmbH & CO. KG, Staufen, Germany). The activities of the enzymes were determined using a reversed range of substrate concentrations: 0, 10, 20, 40, 80, 100, 200, and 400 μmol g^{-1} soil (Wei et al., 2019). Then, 50 μl soil suspension was pipetted into 150-μl specific enzyme substrate solution with 50 μl 0.1 M sodium morpholine-4-ethanesulfonate ($C_6H_{13}NO_4Sna_{0.5}$) and 0.05 M Trizma buffer for MUF or AMC, respectively. Enzyme activities were measured by a multi-function microplate reader (Infinite ® M Plex, Hamilton Bonaduz AG, Bonaduz, Switzerland) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm and slit width of 25 nm (Sinsabaugh et al., 2008; Nottingham et al., 2016; Wei et al., 2019). Enzyme activity (nmol g^{-1} dry soil h^{-1}) was calculated using the linear increase in fluorescence with time during the assay.

2.6 Evaluation of microbial metabolic limitation

The V_{max} of extracellular enzymes was estimated by using the Michaelis-Menten

equation (Tischer et al.2015),

$$V = \frac{V_{max} \times [S]}{K_m + [S]}$$

where V is the reaction rate, V_{max} was the maximal velocity of enzyme, [S] is the substrate concentration, and K_m is the substrate concentration when V was equal to half of V_{max}.

Microbial nutrient limitation was quantified by calculating the vector lengths and angles of the V_{max} of C, N and P acquisition enzymes (Moorhead et al, 2013, 2016; Cui et al., 2019). Vector length which identifies the C limitation, was calculated as:

$$Length = \sqrt{x^2 + y^2}$$

where x is proportional activity of C *vs* P acquisition enzymes ((BG+CBH+XYL)/(BG+CBH+XYL+AP), and y is C *vs* N acquisition enzymes (BG+CBH+XYL)/(BG+CBH+XYL+NAG+LAP).

The angle, representing N or P limitation, was represented by arctangent of the line extending from the plot origin to point (x, y), as

$$Angle(\text{degree}) = \text{DEGREES (ATAN2}(x, y))$$

2.7 Statistical analysis

The significance of differences was examined using a one-way ANOVA (single factor analysis of variance), and multiple comparisons were performed using the Duncan method ($p < 0.05$). Spearman correlation analysis was performed using R with "corrplot" package after the Shapiro-Wilk test. Other figures were made with Origin 8.5. Data are presented as means ± standard deviation (n = 4).

3 Results

3.1 P fractions, DOC and microbial biomass

After 38 days of incubation, all four soil P fractions were significantly higher in P, D and DP treatments as compared to control (CK) (Fig.1). The DOC contents were significantly higher in soils with organic substrate C amendment (D and DP) than at no substrate C addition (CK and P) (Fig.2a). The Olsen-P content was significantly higher in P, D and DP than in the CK treatment (Fig.2b). Consequently, also the DOC:Olsen-P ratio was significantly lower in P only, D only and DP treatment than in the control (Fig.2c). Addition of substrate C increased the MBC content significantly (D and DP) as compared to treatments without substrate C addition (CK and P) (Fig.2d). However, the MBP content didn't change in all treatments (Fig.2e). The MBC:MBP ratio was significantly higher in C treatments (D and DP) than in no substrate C addition treatments (CK and P) (Fig.2f). The SOC contents were significantly higher in soils with substrate C amendment (D) than CK (Fig.S1).

3.2 The V_{max} of extracellular enzymes and their vector characteristics

The V_{max} of C acquisition enzymes (BG + CBH + XYL) was 2.8–3.7 times higher ($p < 0.05$) in C addition treatments (D and DP) as compared to treatments with no substrate C addition (CK and P) (Fig.3a). The V_{max} of P acquisition enzyme (AP) was lower ($p < 0.05$) at P only addition but higher in C addition treatments (P and DP) as compared to CK (Fig.3b). In consequence, the $EEA_{C:P}$ was 1.5–2.1 times higher ($p < 0.05$) in D, P and DP than in CK (Fig.3c). The V_{max} of N acquisition enzymes (NAG + LAP) was 10–12.6 times higher ($p < 0.05$) in C addition treatments (D and DP) than in no C addition treatments (CK and P) (Fig.S2).

Vector lengths and angles ranged from 0.94 to 1.14 and 33.84° to 51.67° for the different variants, respectively. The characteristics of ecoenzymatic stoichiometry varied in response to P and D addition (Fig. 4a). Compared to CK, vector length was significantly higher in P only addition treatment, indicating stronger C limitation (Fig. 4b). In contrast, vector length was significantly lower in substrate C addition treatments (D and DP), illustrating that D addition can relieve microbial C limitation. Vector angles were smaller ($p < 0.05$) in all three treatments (P, D and DP) than in CK. With vector angles <45° this is particularly true for the C addition treatments D and DP (Fig. 4c), indicating that a C source is a necessary factor for decreasing microbial P limitation. In addition, the linear-regression analysis identified significant positive correlations between vector lengths and angles (R^2=0.35, $p = 0.0093$; Fig. 4d).

3.3 Relationships of microbial nutrient limitation with P bioavailability

The spearman correlation analysis showed that vector lengths were negatively correlated with soil $CaCl_2$-P, Citrate-P, Enzyme-P ($p < 0.05$; Fig.5). Vector angles also were negatively correlated with soil $CaCl_2$-P, Citrate-P, Enzyme-P and HCl-P ($p < 0.05$; Fig.5).

4 Discussion

Carbon (C) and P are essential elements for microbial growth (Merchant & Helmann, 2012; Liu et al., 2018; Wei et al., 2019a). To increase the P availability, P solubilizing microorganisms secrete phytase, nuclease, and phosphatase in P-limited soil, hydrolyzing P_o and converting it into P_i (Gyaneshwar et al., 1999; Bashan et al., 2013; Spohn & Kuzyakov, 2013). P_i, e.g. fertilized as soluble orthophosphate in highly recalcitrant forms, can be

immobilized by microorganisms due to low P availability for microorganism (Frossard et al., 2000; Bünemann et al., 2012). It would relieve microbial P limitation to decrease the V_{max} of AP (Allison & Vitousek, 2005; Nannipieri et al., 2011). In our study, the application of mineral P fertilizer decreased V_{max} of AP of dry Kazakhstan steppe soils (P-limited), being in line with the above reports. Because under P-limited conditions soil P cannot satisfy the P demand for microorganism growth, so that microbes would exploit the organic moiety of phosphorylated compounds as a C source for phosphatase synthesis (Wei et al., 2019a; Liu et al., 2021). Once P limitation is alleviated, the V_{max} of AP decrease (Manzoni et al. 2010; Wei et al. 2019a,b; Yuan et al., 2019). Thus, this confirms the first hypothesis that solo P_i (mineral P fertilization) decrease soil P_o mineralization due to microbial C limitation.

Microbial mineralization of P_o is strongly interlinked with C cycling in soil and induces C mineralization (Caruso, 2010; Liu et al., 2021). Our results show that mineral P fertilization increased microbial C limitation (Fig. 4b), also supporting the previous view of simultaneous organic P and C cycling (Harrison, 1982; Wei et al., 2019a; Liu et al., 2021). Microbial C limitation was decreased by the addition of an organic C substrate with *D. glomerata* leaves (D and DP) when compared to the CK and P treatments. The larger DOC concentrations indicate an improved C availability for soil microorganisms, positively affecting the growth and metabolism of microorganisms. Soil microorganisms utilized these available substrate C to build up C storage molecules, increasing MBC (Heuck et al., 2015; Zhu et al., 2016; Liu et al., 2020). The decomposition of these fresh plant residues along with the decomposition of the soil indigenous organic matter provides energy and releases nutrients for microbial acquisition (Kumar et al., 2016; Shahbaz et al., 2017; Wei et al., 2020). Microorganisms

responded on the organic C substrate supply by an increase in the V_{max} of C acquisition enzymes (BG, CBH and XYL) and of AP in the rapidly stage of decomposition (r-strategists of microorganisms) (Burns et al., 2013;Wei et al., 2019a), illustrating more energy and available N and P is necessary for extracellular enzymes synthesis in the microbes rapidly growth (Xu, et al., 2013; Cui et al., 2020; Manzoni et al., 2010; Ng et al., 2014). These increase in C and P acquisition enzymes, indicating that C and P addition caused a shift from oligotrophic to copiotrophic bacteria in fast microbial growth stage after organic C substrate supply (Heuck et al., 2015). This is consistent with the second hypothesis that the V_{max} of C and P acquisition enzymes increased during plant residues decomposition. In our study, it is worth mentioning that the V_{max} of AP were the highest with organic C substrate only addition and forced microbial limitation from P to N. Simultaneous addition of organic C substrate and mineral P (DP) further significantly decreased vector angel (strengthened microbial N limitation) (Fig.4c), inferring that available N source is an important factor for phosphatase synthesis (Wei et al., 2017).

The available C and P needs of microbes reflects the establishment of element homeostasis in the microbial population and their internal balance (Manzoni et al., 2010; Ng et al., 2014). In our study, in the organic C substrate addition treatments (D and DP) the DOC:Olsen-P ratio was lower but the MBC:MBP ratio was higher than at CK. However, the DOC:Olsen-P and MBC:MBP ratio both were less than their average threshold ratios of 186 and 59.5, respectively (Cleveland & Liptzin, 2007; Xu, et al., 2013), illustrating microbial metabolism might be C-limited rather than P-limited (Sinsabaugh & Shah, 2009, 2012; Wei et al., 2019a). In addition, according to the resource allocation model established by Sinsabaugh

& Shah (2012), microorganisms allocate their resources to C and P acquisition enzymes production to hydrolyze soil organic matter and plant residues to gain energy and to take up nutrients (Spohn et al. 2013a; Zhang et al. 2014). Thus enzymatic stoichiometry of C and P acquisition reflects microbial C and P demands (Ng et al., 201; Wei et al., 2020). Our results showed that the C:P acquisition ratio is in the range of 3 to 4.5, illustrating a strongly C demand in plant residues decomposition. These both indicate organic C substrate dominates the behavior of microbial plant residues mineralization (Wei et al., 2019), especially non-homeostatic behavior in C and P (Vance et al., 2003; Cleveland and Liptzin, 2007; Scott et al., 2012; Mooshammer et al., 2014; Wei et al., 2020). This supports our third hypothesis that C limitation decreased the DOC:Olsen-P ratio though C:P stoichiometric balance (Sinsabaugh & Shah, 2009, 2012).

With the organic matter mineralization, soil microorganisms are releasing orthophosphate (predominantly as HPO_4^{2-} and $H_2PO_4^{1-}$) immobilizing parts of the P as MBP (Hinsinger 2001; Barea et al. 2005; Heuck et al., 2015). Additional C and P input could help copiotrophic organisms to outcomplete oligotrophic organisms (Fierer et al., 2007; Heuck et al., 2015), which are likely dominating in these nutrient limited soils (Tada et al., 1995; Heuck et al., 2015). The spearman correlation analysis further identified that P bioavailability ($CaCl_2$-P, Citrate-P, Enzyme-P) was negatively affected by microbial C limitation. This result suggests that decreasing C limitation could improve soil bioavailable P (Elser et al., 2000a,b; Sinsabaugh et al., 2009; Wei et al., 2019a; Cui et al., 2020). This is also the reason why P limitation of heterotrophic microorganisms can be mitigated and/or overcame in the presence of exogenous labile organic C from plant residues (*D. glomerata* leaves) to provide energy for

P_o mineralization by increasing the V_{max} of C and P acquisition enzymes (Liu et al., 2018; Zhu et al., 2018; Wei et al., 2019a, 2020), maintaining the balance of C and P stoichiometric ratio.

5 Conclusions

P fertilization directly increased bioavailable P and microbial C limitation. Plant residues (*D. glomerata* leaves) application decreased microbial C limitation and provided more available P with the decomposition of plant residues. This caused a decreasing ratio of available carbon (energy) to available phosphorus (DOC:Olsen-P ratio). However available C:P ratio decreased from 17 to 8, resulting in limiting the optimal growth of soil microorganisms. Thus it forced microbes to produce higher the V_{max} of C, N and P acquisition enzymes during the mineralization of plant residues. Therefore a positive feedback occurred, leading to a further increase in bioavailable P. In general, the combined application of plant residues and mineral P is important for improving soil fertility by maintaining C and P stoichiometric ratio.

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Figure captions

Fig.1 Contents of soil phosphorus fractions based on the biological availability $CaCl_2$-P (a), Citric-P (b), Enzyme-P (c) and HCl-P (d) in soil after 38 days of incubation under four different treatments, i.e., no substrate addition (CK), phosphorus addition (P), *Dactylis glomerata* L. addition (D), *Dactylis glomerata* L. and phosphorus addition (DP). All results are means ± standard error (n = 4).

Fig.2 Soil DOC (a) and Olsen-P (b) contents, the ratio of DOC to Olsen-P (c), MBC (d) and MBP (e) contents, and the ratio of MBC to MBN (f) in soil after 38 days of incubation under four different treatments, i.e., no substrate addition (CK), phosphorus addition (P), *Dactylis glomerata* L. addition (D), *Dactylis glomerata* L. and phosphorus addition (DP). All results are means ± standard error (n = 4).

Fig.3 The maximal velocity (V_{max}) of C acquisition enzymes (the sum of the maximal velocity of β-1, 4-glucosidase (BG), β-1,4-cellobioside (CBH), andβ-1,4-xylosidase (XYL)) (a), the maximal velocity of P acquisition enzyme (β-1,4-phosphate (AP)) (b), and the ratio of C- to P acquisition enzyme ($EEA_{C:P}$, c) in soil after 38 days of incubation under four different treatments, i.e., no substrate addition (CK), phosphorus addition (P), *Dactylis glomerata* L. addition (D), *Dactylis glomerata* L. and phosphorus addition (DP). All results are means ± standard error (n = 4).

Fig.4 Extracellular enzyme stoichiometry of the relative proportions of C to N acquisition versus C to P acquisition (a), the variation of vector length (b) and angle (c) and their relationships (d) in soil after 38 days of incubation under four different treatments, i.e., no substrate addition (CK), phosphorus addition (P), *Dactylis glomerata* L. addition (D), *Dactylis glomerata* L. and phosphorus addition (DP). All results are means ± standard error (n = 4).

Fig.5 Spearman correlation heat map of between microbial nutrient (N or P) limitation and soil physicochemical properties.

Fig.1

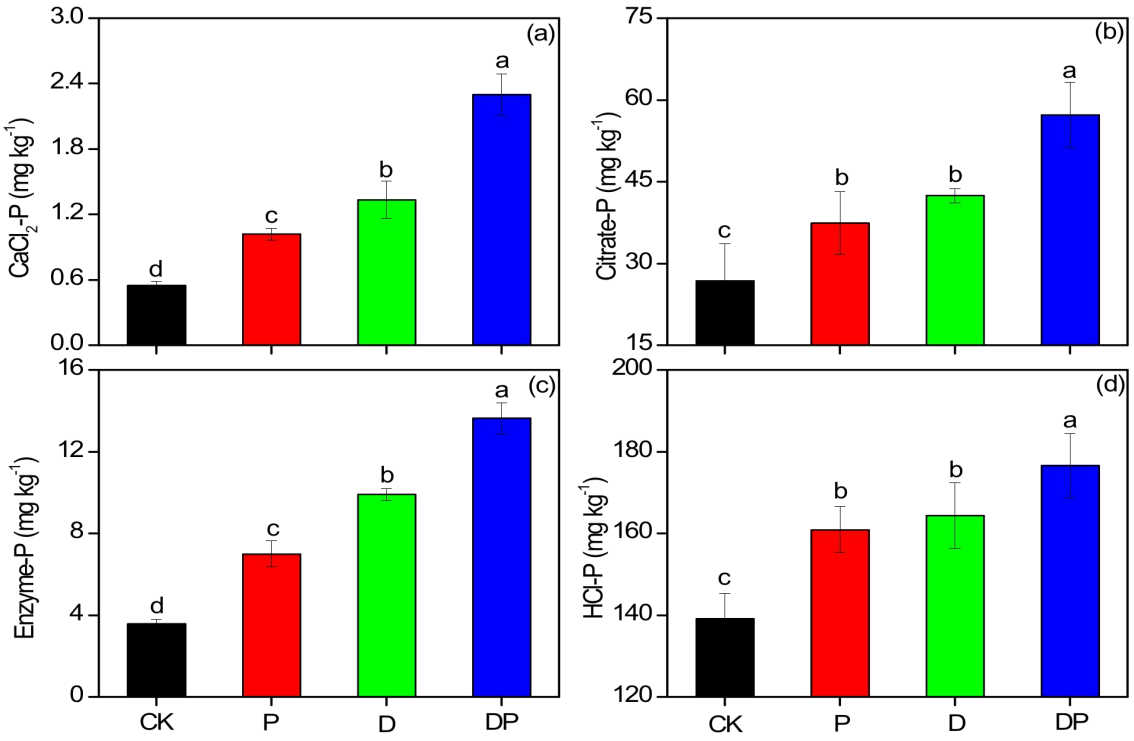

(a)
$CaCl_2$-P (mg kg^{-1})
3.0
2.4
1.8
1.2
0.6
0.0
a
b
c
d
(b)
Citrate-P (mg kg^{-1})
75
60
45
30
15
a
b
b
c
(c)
Enzyme-P (mg kg^{-1})
16
12
8
4
0
a
b
c
d
(d)
HCl-P (mg kg^{-1})
200
180
160
140
120
a
b
b
c
CK
P
D
DP

Fig.2

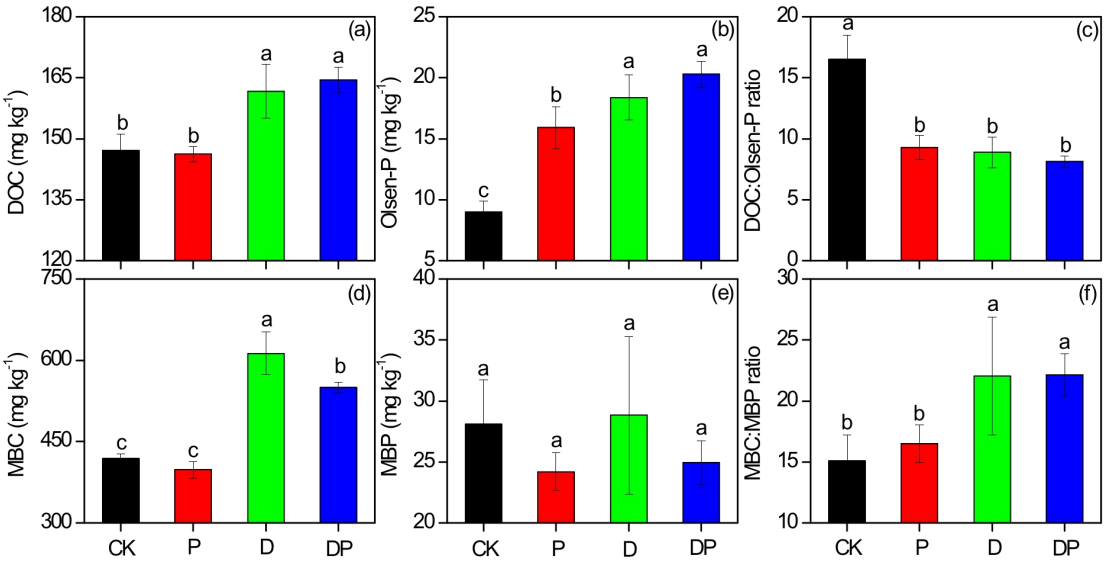

(a)
DOC (mg kg-1)
180
165
150
135
120
b
b
a
a
(b)
Olsen-P (mg kg-1)
25
20
15
10
5
c
b
a
a
(c)
DOC:Olsen-P ratio
20
15
10
5
0
a
b
b
b
(d)
MBC (mg kg-1)
750
600
450
300
c
c
a
b
(e)
MBP (mg kg-1)
40
35
30
25
20
a
a
a
a
(f)
MBC:MBP ratio
30
25
20
15
10
b
b
a
a
CK
P
D
DP

Fig.3

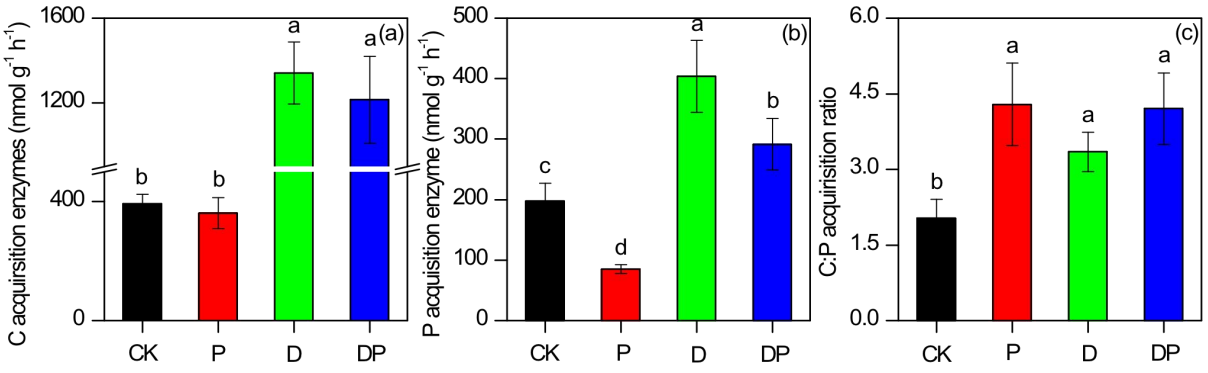

C acquisition enzymes (nmol g-1 h-1)
1600
1200
400
0
b
b
a
a
(a)
CK
P
D
DP
P acquisition enzyme (nmol g-1 h-1)
500
400
300
200
100
0
c
d
a
b
(b)
CK
P
D
DP
C:P acquisition ratio
6.0
4.5
3.0
1.5
0.0
b
a
a
a
(c)
CK
P
D
DP

Fig.4

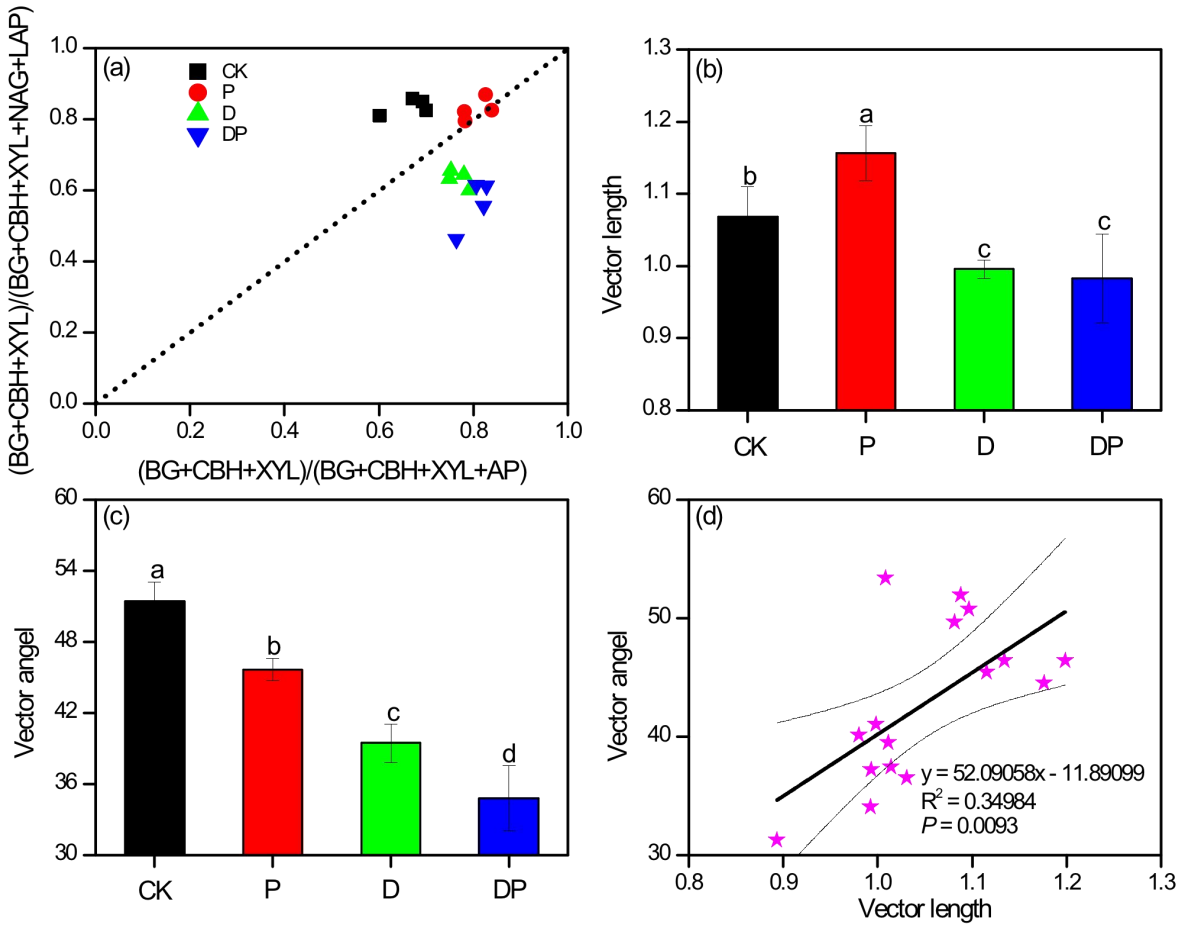

(a)
CK
P
D
DP
(BG+CBH+XYL)/(BG+CBH+XYL+NAG+LAP)
(BG+CBH+XYL)/(BG+CBH+XYL+AP)
(b)
Vector length
a
b
c
c
CK
P
D
DP
(c)
Vector angel
a
b
c
d
CK
P
D
DP
(d)
Vector angel
y = 52.09058x - 11.89099
R2 = 0.34984
P = 0.0093
Vector length

Fig.5

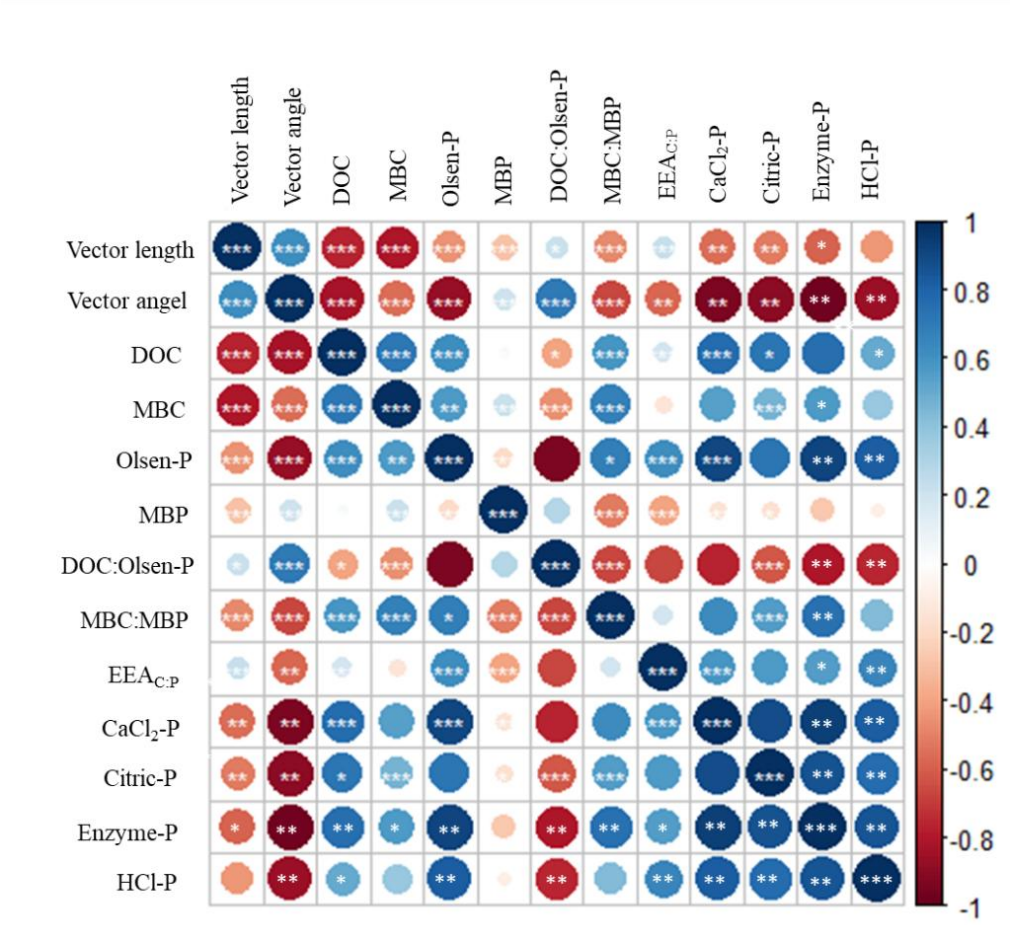

Vector length
Vector angle
DOC
MBC
Olsen-P
MBP
DOC:Olsen-P
MBC:MBP
EEA$_{C:P}$
$CaCl_2$-P
Citric-P
Enzyme-P
HCl-P
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Table S1 The maximal velocity (V_{max}) of C acquisition enzmyes (β-1, 4-glucosidase (BG), β-1,4-cellobioside (CBH) and β-1,4-xylosidase (XYL)), the N acquisition enzymes (β-1,4-N-acetyl-glucosaminidase (NAG) and L-leucine aminopeptidase (LAP)), and the P acquisition enzyme (β-1,4-phosphate (AP)).

	C acquisition enzymes						N acquisition enzymes				P acquisition enzyme	
	V_{max} (nmol MUF or AMC g^{-1} h^{-1})											
Treatment	BG	R^2	CBH	R^2	XYL	R^2	NAG	R^2	LAP	R^2	AP	R^2
CK	342.93±32.65c	0.91	31.76±6.14c	0.87	19.43±4.54c	0.83	48.80±8.78c	0.88	29.01±6.42b	0.90	197.38±29.95c	0.95
P	307.44±49.27c	0.91	27.78±12.12c	0.85	26.45±7.34c	0.91	42.13±8.83c	0.91	32.98±32.98b	0.91	85.04±7.24d	0.96
D	1003.03±99.90a	0.92	205.21±45.66b	0.82	133.40±14.85b	0.87	526.10±120.19b	0.97	259.69±30.25a	0.95	403.94±59.73a	0.96
DP	577.82±168.80b	0.94	476.18±185.21a	0.90	161.76±11.85a	0.92	690.97±103.58a	0.96	249.93±36.69a	0.95	291.44±42.67b	0.97

Fig. S1 Soil organic carbon (SOC) contents in soil after 38 days of incubation under four different treatments, i.e., no substrate addition (CK), phosphorus addition (P), *Dactylis glomerata* L. addition (D), *Dactylis glomerata* L. and phosphorus addition (DP).

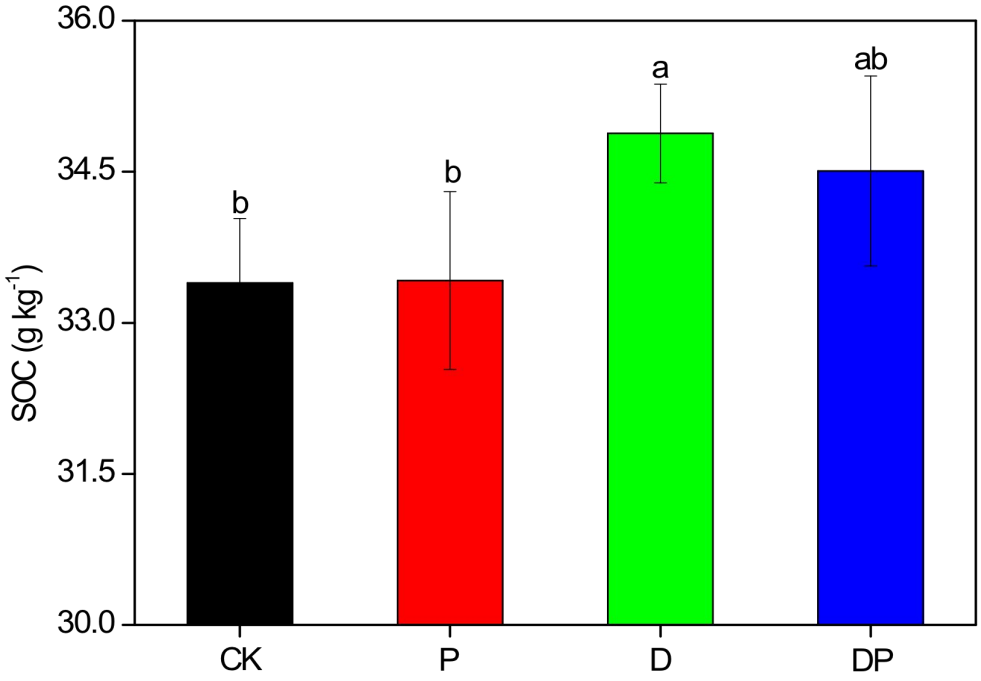
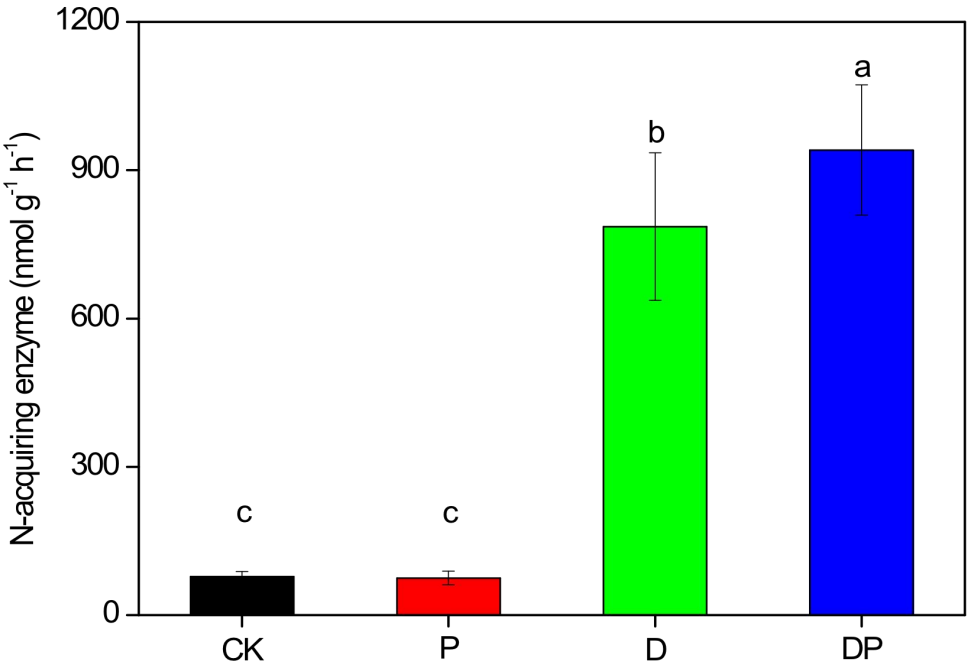


Fig. S2 The maximal velocity (V_{max}) of N acquisition enzymes (the sum of the potential maximum activity of N-acetyl-β-D-gluosaminide (NAG) and L-leucine aminopeptidase (LAP)) in soil after 38 days of incubation under four different treatments, i.e., no substrate addition (CK), phosphorus addition (P), *Dactylis glomerata* L. addition (D), *Dactylis glomerata* L. and phosphorus addition (DP).



6 Study 4

Stoichiometric theory shapes enzyme kinetics in paddy bulk soil but not in model rhizosphere soil

Contribution: I participated in fieldwork, sampling activities, and the experiment incubation, performed most of the analysis in the laboratory, collected and evaluated data, prepared tables and figures, and wrote the manuscript.

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Stoichiometric theory shapes enzyme kinetics in paddy bulk soil but not in model rhizosphere soil

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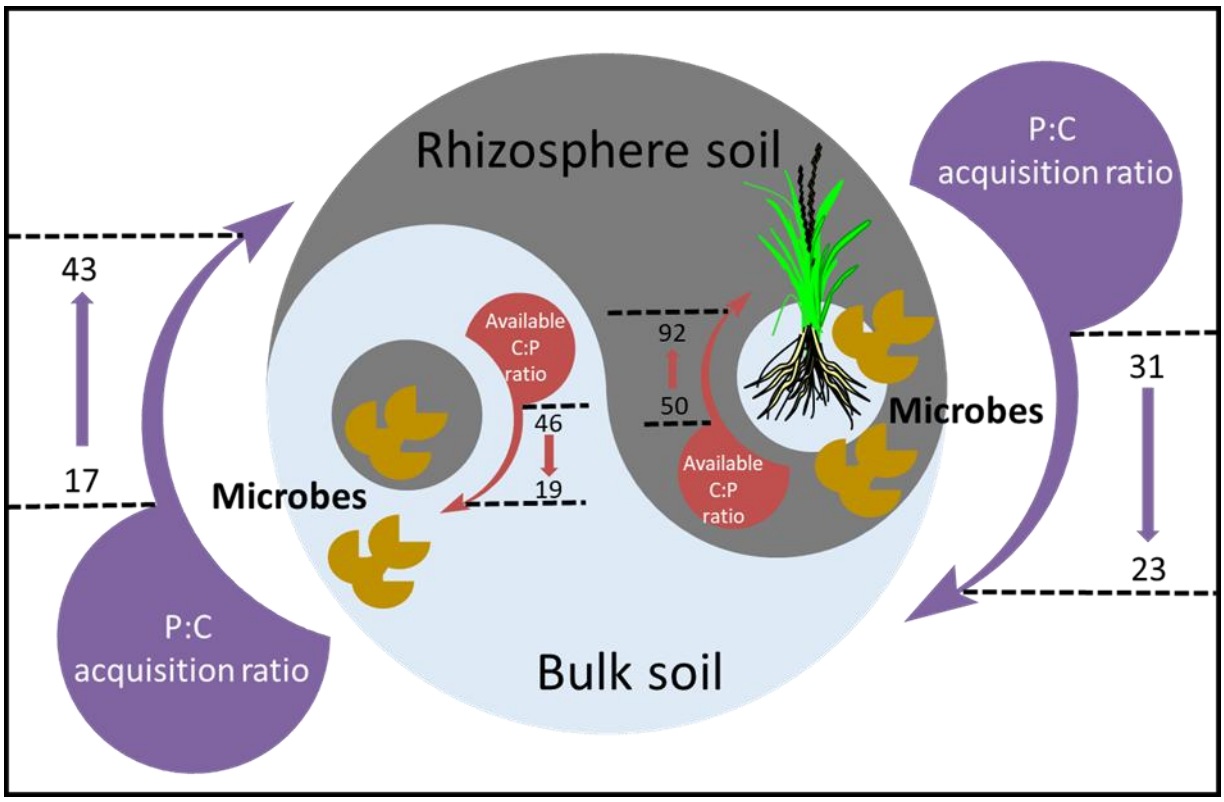
Highlights

- C and P acquisition stoichiometry were studied in paddy bulk and model rhizosphere soils
- Enzyme activity for C and P acquisition was lower in a model rhizosphere than in bulk soils
- P:C acquisition ratio was regulated by C and P acquisition enzymes and rhizosphere-C
- Nutrient turnover was regulated by the P:C acquisition ratio

Abstract The available carbon (C) to phosphorus (P) ratio in soil is regulated by extracellular hydrolases for C and P acquisition by microbes and plants. However, the stoichiometric relationship between acquiring C and P in paddy rhizosphere and bulk soils remains unclear. The objective was to explore the underlying mechanisms of C and P acquisition stoichiometry in model rhizosphere and bulk soils in response to P fertilization and cellulose addition. Amendment with either cellulose or P separately caused a significant increase in the maximal velocity (V_{max}) of C acquisition enzymes (β-1,4-glucosidase and β-cellobiohydrolase) but decreased that of P acquisition enzymes (acid and alkaline phosphomonoesterases) in bulk soil. In contrast, lower V_{max} values of C and P acquisition enzymes were observed in model rhizosphere soil than in bulk soil. The co-application of cellulose and P increased the V_{max} of P acquisition enzymes in model rhizosphere soil but decreased that of only alkaline phosphomonoesterase in bulk soil. Results show that P availability and labile-C content co-regulated the P:C acquisition ratio, and two inverse linear relationships were observed. Specifically, the P:C acquisition ratio was negatively related to both the dissolved organic C:Olsen-P ratio and the microbial biomass C:P ratio in model rhizosphere soil. However, the P:C acquisition ratio was positively related to both the dissolved organic C:Olsen-P ratio and the microbial biomass C:P ratio in bulk soil. Overall, microbes mineralized less organic P to acquire P in model rhizosphere (i.e. containing higher labile-C) than in bulk soil (i.e. having lower labile-C contents).

Keywords: Enzyme; Paddy soil; P:C acquisition ratio; Phosphorus fertilization; Microbes

Graphical abstract



1 Introduction

Phosphorus (P) is a major nutrient that is indispensable for plant growth (Schnitzer, 1991; Correll, 1998; Baligar et al., 2001). In acidic soils, up to 80% of fertilizer P (presented as soluble orthophosphate ions [$H_2PO_4^-$]) reacts with iron (Fe) and aluminum (Al) ions in the soil to form less soluble $FePO_4$ and $AlPO_4$ or is sorbed to Fe and Al oxides (Gyaneshwar et al., 2002; Wei et al., 2019a), which significantly reduces P availability to plants. A large pool of P also occurs in an organic form, representing an important indicator of soil fertility (Schnitzer, 1991; Correll, 1998). Because both plants and microbes have large P requirements, competition exists (Zhang et al., 2014), leading to direct and indirect microbial P nutrient mining of the fertilizer P fixed by Fe and Al, and of soil organic matter (SOM). To acquire P from SOM, phosphate-solubilizing microorganisms secrete hydrolytic enzymes (phosphatases) to hydrolyze monophosphoesters and release orthophosphate ions (Sinsabaugh et al., 2009; Nannipieri et al., 2011; Hofmann et al., 2016). Phosphatases are categorized as acid phosphomonoesterase (ACP) and alkaline phosphomonoesterase (ALP) depending on their pH optima (Hofmann et al., 2016; Wei et al., 2019b), contributing to P acquisition (Spohn et al., 2013b; Wei et al., 2019a). ACP is produced by both plants and microorganisms (such as ectomycorrhizae), whereas ALP is produced by microorganisms only (Nannipieri et al., 2011; Hofmann et al., 2016).

Microbes mineralize organic phosphorylated compounds extracellularly to utilize the organic moiety of the compounds as a C source (Spohn & Kuzyakov, 2013; Hofmann et al., 2016). That is, microbial mineralization of organic P is strongly interlinked with C cycling in

soil (Caruso, 2010; Wei et al., 2019b) and induces C mineralization (Peng et al., 2016; Cui et al., 2020). Such microbial processes are associated with changes in the activity of C and P acquisition enzymes, resulting in a further stoichiometric imbalance between C and P (Moro et al., 2015; Wei et al., 2019a,b). The P:C acquisition ratio, a useful index for assessing microbial P acquisition in response to the changing soil environment (Godin et al., 2015; Ge et al., 2020), mainly depends on the demand and competition for P uptake between crop roots and soil microbes (Spohn et al., 2013a; Wei et al., 2019b; Liu et al., 2022). Optimizing P application to maintain the stoichiometric balance between C and P is required for maximizing crop productivity (Wei et al., 2019a; Yuan et al., 2019).

Rhizosphere and bulk soils are two different ecosystems in which P acquisition occurs (Hofmann et al., 2016). The rhizosphere is the most active area for microbe–soil–plant interactions (Kuzyakov & Xu, 2013) and is also a hotspot for P mineralization (Spohn et al., 2013b). Plant–microbe interactions can be mutualistic as well as competitive (Hofmann et al., 2016). Application of P to P-limited soil can not only increase root exudates (Jones et al., 2004; Liu et al., 2020) for enzymatic reactions and growth of microbes (Bais et al., 2006; Zhu et al., 2018) but also increase the preservation of rhizodeposit-C by reducing the microbial energy required for nutrient mining (He & Dijkstra, 2015; Yuan et al., 2019; Wei et al., 2021a). Root exudates (i.e., organic acids and siderophores) directly solubilize $FePO_4$ and $AlPO_4$ and further induce the production of organic and inorganic acids (such as acetic acid and nitric acid, respectively) by phosphate-solubilizing microorganisms (Hinsinger, 2001; Spohn et al., 2013b). However, microbial C limitation in bulk soil is enhanced by P fertilizer addition (Hofmann et al., 2016). Therefore, mineralization of organic P tends to occur at a higher rate in bulk soil

than in rhizosphere soil (Spohn et al., 2013b; Gianfreda, 2015; Razavi et al., 2017). Furthermore, the metabolism of soil microorganisms is strongly influenced by labile-C content in the roots of rhizosphere soil (Marschner et al., 2012; Liu et al., 2020; Wei et al., 2021a), which leads to different organic P mining strategies of microbial phosphatase between rhizosphere soil and bulk soil. However, the enzyme profiles of a model rhizosphere and bulk soils have rarely been distinguished, particularly in paddy soils. Additionally, the effect of P fertilization and root exudates (rhizosphere soil) on microbial acquisition of P from SOM in paddy soils is yet to be established (Ge et al., 2017; Zhu et al., 2018).

Here, we aimed to improve the current understanding of the characteristics of microbial C and P acquisition to optimize P fertilizer application in P-limited paddy soil. Specifically, we examined the underlying mechanisms of C and P acquisition stoichiometry in rhizosphere and bulk soils in response to P fertilization and C substrate addition. We examined the substrate-dependency of extracellular hydrolytic enzymes under a changing soil environment using kinetic parameters. These parameters included maximal velocity (V_{max}) and saturation affinity constant (K_m), derived from the Michaelis–Menten equation (Ma et al., 2017; Wei et al., 2019). This approach helped to establish links between the abiotic soil environment and microbial C and P acquisition in paddy ecosystems (Tischer et al., 2015). We hypothesized that (1) cellulose addition would increase the V_{max} of C acquisition enzymes involved in SOM decomposition and nutrient release, and (2) P addition would reduce the V_{max} of P acquisition enzymes to alleviate microbial and plant demand for P and (3) the microbial P:C acquisition ratio would be reduced in a model rhizosphere soil but not in bulk soil, owing to low levels of labile-C in bulk soil and different microbial nutrient mining strategies in a model rhizosphere

and bulk soils.

2 Materials and methods

2.1 Study site and soil sampling

The plough layer (0–20 cm) of paddy soils were sampled from the Taoyuan Agro-ecological Experimental Station, Hunan Province (111° 27′ E, 28° 55′ N), which is a typical double-rice cropping area in southern China. This region features a subtropical humid climate and has an annual average temperature of 16.5 °C and precipitation of 1448 mm. Paddy soils were classified as Stagnic Anthrosols derived from quaternary red clay.

Soils were collected using a stainless-steel drill (diameter: 5 cm) in November 2016. Fine roots and other plant residues were manually removed from moist soil (water content: 27.9%) and sieved through a 4-mm screen. Soils were then pre-incubated in a 50 L plastic bucket, flooded to a depth of 2–3 cm at 25 °C, and stored in the dark for 14 days. The upper layer of water was poured out and the soils were then used in the incubation experiment. Soil samples (approximately 20 g) were air-dried for physical and chemical characterization (<0.149 mm). The basic soil properties were as follows: pH, 5.1; organic C, 12.2 g kg^{-1}; total N, 1.6 g kg^{-1}; total P, 0.8 g kg^{-1}; and available P (Olsen-P), 4.5 mg kg^{-1}. The soil was composed of 82.1% clay, 11.9% silt, and 6% sand.

2.2 Soil nutrient addition and rice planting

The soils were fertilized at a rate of 100 mg K_2O-K kg^{-1} soil (oven-dried basis), 40–80 mg P_2O_5-P kg^{-1} soil, and 120–150 mg urea-N kg^{-1} soil in the field, according to the 2014 subtropical rice application recommendations of the Chinese Ministry of Agriculture Fertilizer

(http://www.moa.gov.cn/ztzl/2014nsxsc/sxsc_jszd/201404/t20140410_3846602.htm). The amount of fertilizer applied in the pot experiment was 1.5–3 times that of the field experiment. Before the pot experiment, potassium chloride (KCl) and ammonium nitrate (NH_4NO_3) were added to the pre-incubated soil as base fertilizers at 160 mg K kg^{-1} soil and 250 mg N kg^{-1} soil, respectively. Then, the soils were uniformly mixed and divided into quarters, for four different treatments. The four treatments (with three replicates) were (i) no cellulose (substrate) or phosphorous addition (CK), (ii) cellulose addition (E), (iii) phosphorous addition (P), and (iv) cellulose and phosphorous addition (EP). Cellulose and P (as NaH_2PO_4) were added at rates of 1 g C kg^{-1} soil and 80 mg P kg^{-1} soil, respectively. Wet soil (approximately 1.2 kg dry soil) was then evenly packed into a rhizobox (20 cm × 2 cm × 32 cm) with one removable side, at a bulk density of 1.3 g cm^{-3}. Rice seedlings were transplanted to the center of each rhizobox, and distilled water was added up to approximately 3 cm above the soil surface. The boxes were incubated in a greenhouse under a day/night temperature regime of 28 ± 1/16 ± 1 °C, relative humidity of 50%, and photosynthetically active radiation of 500 mmol m^{-2} s^{-1} for 12 h per day. The rhizoboxes were kept inclined at 60°, so that the root system adhered to the glass wall, facilitating the distinction between rhizosphere soil and bulk soil. The rhizoboxes were wrapped with aluminum foil to avoid algae growing on the glass surface during the experiment period.

2.3 Sampling and soil analyses

A model rhizosphere soil is defined as soil that is 2–3 mm from the root center, while the remaining soil is defined as bulk soil (York et al., 2016; Ma et al., 2017; Razavi et al., 2017). When the rice root system had matured 45 days after transplanting the rice seedlings, the

rhizobox was opened and the upper layer of water was removed. First, shoot samples were collected. Then, the rhizosphere soil was collected. In brief, sterilized blades and tweezers were used to delineate the rough area of the rhizosphere according to the growth area of the root. The roots were taken out as carefully as possible to avoid cross-contamination with the bulk soil area. Next, the rhizosphere and bulk soils were put in separate plastic zip lock bags and stored at 4 °C. All indexes were measured within 5 days. Approximately 50 g of soil was sampled from the rhizosphere soil and bulk soil to determine dissolved organic C (DOC), NH_4^+, NO_3^-, Olsen-P, and microbial biomass C, N, and P (MBC, MBN, and MBP, respectively) content. Another 5 g of wet model rhizosphere soil or bulk soil was used to analyze extracellular enzymes.

Soil MBC and MBN were determined using chloroform fumigation (Vance et al., 1987; Wu et al., 1990). In brief, a 20 g soil sample was fumigated with chloroform for 24 h, followed by extraction using 80 mL of 0.5 M K_2SO_4. Another 20 g soil sample was extracted directly using 80 mL 0.5 M K_2SO_4 without chloroform fumigation. The extracted soil C concentration was analyzed using a Shimadzu TOC-VCPH analyzer (Vwp, SHIMADZU, Japan). The dissolved N concentration was analyzed using an auto analyzer (AA3, SEAL, Germany). MBC and MBN were calculated using k_c = 0.45 and k_n = 0.45, respectively (Jenkinson & Ladd, 1981). The K_2SO_4 soil extracts from non-fumigated samples were also used to determine DOC, NH_4^+ and NO_3^- concentrations in rhizosphere and bulk soil (Liu et al., 2018). Soil MBP was determined using chloroform fumigation–sodium bicarbonate ($NaHCO_3$) extraction, followed by the molybdenum antimony colorimetric method (Brookes et al., 1982; Wu et al., 2007) using a UV-Vis spectrophotometer (UV-2450, Japan). In brief, two 4 g soil samples

(non-fumigated and fumigated) were prepared. The third 4 g fresh soil sample was treated with 0.4 mL of 250 µg P mL^{-1} KH_2PO_4 (to calculate recovery efficiency of P). Three soil samples were then extracted with 80 mL 0.5 M $NaHCO_3$ (pH 8.5). MBP was calculated with k_p = 0.4 (Brookes et al., 1982; Wei et al., 2019b). Olsen-P was determined from the soil $NaHCO_3$ extracts (1:20 w/v) (Olsen et al., 1954; Wei et al., 2019b).

The V_{max} of BG, CBH, ACP, and ALP was determined from their enzyme kinetics (Michaelis & Menten, 1913; Tischer et al., 2015). In brief, 1 g of fresh wet model rhizosphere or bulk soil was mixed in 50 mL deionized water and oscillated for 30 min using an oscillating machine. Enzyme activity was determined using substrate concentrations of 0, 20, 40, 60, 100, 200, 600, and 800 µmol g^{-1} soil, respectively (Wei et al., 2021b). Then, 50 µL suspension was pipetted into 150 µL specific enzyme-substrate solution containing 50 µL sodium morpholine-4-ethanesulfonate ($C_6H_{13}NO_4SNa_{0.5}$) buffer for 4-methylumbelliferone (MUF) (except for ACP and ALP). The buffer was adjusted to pH 6.5 and 9 to determine ACP and ALP activities, respectively, using 1 M NaOH solution and a Multimode Reader (Scientific Fluoroskan Ascent FL, Thermo) at an excitation wavelength of 365 nm, an emission wavelength of 460 nm, and a slit width of 25 nm (Marx et al., 2005; German et al., 2011). The activity of each enzyme was determined at indoor temperatures (24 °C) at 0 min, 30 min, 1 h, 2 h, and 3 h. Enzyme activity (nmol g^{-1} h^{-1}) was calculated using the linear increase in fluorescence with time during the assay (Wei et al., 2021b).

2.4 Calculations and statistics

V_{max} and K_m were estimated using the Michaelis–Menten equation (Michaelis & Menten, 1913; Li et al., 2020):

$$V = \frac{V_{max} \times [S]}{K_m + [S]},$$

where V is the reaction rate, V_{max} is the maximal velocity of enzyme, [S] is the concentration of the substrate, and K_m (substrate affinity) is the substrate concentration when V is equal to half of V_{max}. V_{max} and K_m were fitted using the non-linear regression routine of Origin 8.5.

Statistical analyses were performed in R software (4.0.0). Two-way analysis of variance was performed to test the effects of treatment, sampling location (rhizosphere and bulk soils), and treatment–location interactions using the *aov* function (Wei et al., 2019b; Cui et al., 2020). After *Levene* Test for Homogeneity of Variance, the means of each treatment (CK, E, P or EP) were compared using the least significant difference at the 5% level ($LSD_{0.05}$) with the "agricolae" package (Kabacoff, 2011; Wei et al., 2019b). Pearson's correlation analysis was performed using the "corrplot" package after confirming normal distributions with the Shapiro-Wilk test and log-transforming the data if required. The linear regressions were determined after the potential outliers were tested with *outlierTest* Functions according to Cook's distance and the relationship of residuals vs fitted (Kabacoff, 2011).

3 Results

3.1 Effects of cellulose and P addition on available nutrients and microbial biomass

Soil DOC content was consistently higher ($P < 0.001$) in a model rhizosphere soil than in bulk soil across all treatments. In a model rhizosphere soil, DOC was higher ($P < 0.05$) in P-addition treatments (P, EP) than in no-P-addition treatments (CK, E). In bulk soil, compared

to CK, DOC was not affected by the single or combined addition of cellulose and P (Table. 1). In both a model rhizosphere and bulk soil, Olsen-P content was 2–4 times higher ($P < 0.05$) in P-addition treatments (P, EP) than in no-P-addition treatments (CK and E). Interestingly, the P treatment resulted in higher Olsen-P ($P < 0.05$) in bulk soil than in a model rhizosphere soil (Table. 1). Therefore, the DOC:Olsen-P ratio was 1.4–1.9 times higher ($P < 0.05$) in a model rhizosphere soil than in bulk soil. The DOC:Olsen-P ratios of P-addition treatments (P and EP) were 1.5–2 times lower ($P < 0.05$) than those of no-P-addition treatments (CK and E).

Similar to DOC, soil MBC was consistently higher ($P < 0.05$) in a model rhizosphere soil than bulk soil across all treatments. In a model rhizosphere soil, MBC was higher ($P < 0.05$) in P-addition treatments (P, EP) than in no-P-addition treatments (CK, E). In bulk soil, compared to CK, MBC was significantly higher in the E, P, and EP treatments (Table. 1). In both a model rhizosphere and bulk soils, MBP was 2–5 times higher ($P < 0.05$) in P-addition treatments than in no-P-addition treatments. Therefore, the MBC:MBP ratios of P-addition treatments were lower ($P < 0.05$) than those of no-P-addition treatments in both a model rhizosphere and bulk soils (Table. 1). Overall, P addition resulted in a lower DOC:Olsen-P and MBC:MBP. DOC, MBC, and the DOC:Olsen-P ratio were significantly higher in a model rhizosphere soil than in bulk soil.

3.2 Effects of cellulose and P addition on enzyme kinetics

The E and P treatments resulted in approximately 2 times higher V_{max} of BG ($P < 0.05$) and approximately 4–5 times higher V_{max} of CBH in bulk soil than in a model rhizosphere soil ($P < 0.05$). In bulk soil, compared to CK, the E and P treatments had a higher V_{max} of CBH ($P < 0.05$), even though these treatments had no effect on the V_{max} of BG. In a model rhizosphere

soil, the P treatment had a lower V_{max} of BG (2-fold decline; $P < 0.05$), whereas the EP treatment had a higher V_{max} of BG in a model rhizosphere soil ($P < 0.05$); however, these treatments did not affect the V_{max} of CBH (Table. 2).

The V_{max} of ACP and ALP was 1.5–3 times higher ($P < 0.05$) in bulk soil than in a model rhizosphere soil (except for in the EP treatment). In bulk soil, compared to CK, the E and P treatments had a lower V_{max} of ACP ($P < 0.05$), while the E, P, and EP treatments had a lower V_{max} of ALP ($P < 0.05$). In a model rhizosphere soil, compared to CK, the E treatment had a lower V_{max} of ACP, whereas the P treatment had a lower V_{max} of ALP ($P < 0.05$). In contrast, the EP treatment had higher V_{max} values of both ACP and ALP ($P < 0.05$) in a model rhizosphere soil than in bulk soil (Table. 2).

The K_m of BG did not significantly differ between soil sampling zones or among treatments; however, in the E and P treatments, the K_m of CBH was approximately 10-fold higher in bulk soil than in a model rhizosphere soil ($P < 0.05$). Compared to CK, the E and P treatments had a higher K_m of BG and K_m of CBH in bulk soil but not in a model rhizosphere soil ($P < 0.05$; Table. 2). The K_m of ACP in a model rhizosphere soil was approximately 1.4–2 times higher ($P < 0.05$) than that in bulk soil for all treatments except the EP treatment. Compared to CK, the E and EP treatments had a lower K_m of ACP in bulk soil and a model rhizosphere soil ($P < 0.05$); however, the P treatment had a higher K_m of ACP in a model rhizosphere soil ($P < 0.5$; Table 2). The K_m of ALP was higher in a model rhizosphere soil than in bulk soil in the EP treatment ($P < 0.05$). Compared to CK, the EP treatment had an approximately 1.3–2 times lower K_m of ALP in bulk soil ($P < 0.05$), but there was no difference in a model rhizosphere soil (Table. 2).

Overall, the addition of cellulose or P alone significantly increased the V_{max} of C acquisition enzymes (CBH) but decreased that of P acquisition enzymes (ACP and ALP) in bulk soil. The co-application of cellulose and P increased the V_{max} of ACP and ALP in a model rhizosphere soil but decreased that of ALP in bulk soil compared to that in CK.

3.3 Relationship between the C:P ratio and enzyme kinetics

Pearson correlation analysis showed that, in both a model rhizosphere and bulk soils, the DOC:Olsen-P ratio was significantly positively correlated with the MBC:MBP ratio. In a model rhizosphere soil, the V_{max} of BG was positively correlated with the V_{max} of ALP, and the V_{max} of BG and V_{max} of CBH were negatively correlated with the K_m of ACP and K_m of ALP, respectively. However, in bulk soil, the V_{max} of BG and V_{max} of ACP were negatively correlated with the V_{max} of ALP and K_m of CBH, respectively (Fig. 1).

Linear regression analysis showed that the DOC:Olsen-P ratio was negatively ($R^2 = 0.358$, $P = 0.04$) correlated with the V_{max} of C acquisition enzymes in bulk soil (Fig. 2a). The DOC/Olsen-P ratio was negatively correlated with the V_{max} of P acquisition enzymes in a model rhizosphere soil ($R^2 = 0.88$, $P < 0.001$) and was positively correlated with the V_{max} of P acquisition enzymes in bulk soil ($R^2 = 0.732$, $P = 0.003$) (Fig. 2b). The DOC:Olsen-P ratio was negatively correlated with the P:C acquisition ratio in a model rhizosphere soil ($R^2 = 0.37$, $P = 0.036$) and was positively correlated with the P:C acquisition ratio in bulk soil ($R^2 = 0.536$, $P = 0.006$) (Fig. 2c). The MBC:MBP ratio was not significantly correlated with the V_{max} of C acquisition enzymes in a model rhizosphere or bulk soil (Fig. 2d); however, it was negatively correlated with the V_{max} of P acquisition enzymes in a model rhizosphere soil ($R^2 = 0.47$, $P = 0.057$) and was positively correlated with the V_{max} of P acquisition enzymes in bulk soil ($R^2 =$

0.635, P = 0.011) (Fig. 2e). The MBC:MBP ratio was negatively correlated with the P:C acquisition ratio in a model rhizosphere soil (R^2 = 0.467, P = 0.014) and was positively correlated with the P:C acquisition ratio in bulk soil (R^2 = 0.378, P = 0.033) (Fig. 2f).

4. Discussion

Rhizosphere and bulk soils had significantly different enzyme activities (Table 2). Cellulose addition alone (compared to CK) increased the V_{max} of C acquisition enzymes (BG and CBH), with their V_{max} being much higher in bulk soil than in a model rhizosphere soil (Table 2) due to enzyme specificity for the hydrolysis of cellobiose (Caruso, 2010). Labile-C (glucose and mucopolysaccharides) in root exudates can alleviate microbial C limitation and is directly used for growth by heterotrophic bacteria (Godin et al., 2015), decreasing the V_{max} of C acquisition enzymes in rhizosphere soil (Table 2). Therefore, large amounts of labile-C from rice root exudates in a model rhizosphere soil impedes the production of C acquisition enzymes (Schliemann, 1984; Elser et al., 2003; Tischeret al., 2015). Microorganisms that are r-strategists might be more dominant in a model rhizosphere soil than bulk soil because of the constant input of labile-C in root exudates (Hofmann et al., 2016; Wei et al., 2019a; Yuan et al., 2019). Less amounts of labile-C in bulk soil can increase the V_{max} of C acquisition enzymes (Table 2), which would enhance SOM breakdown (Caruso, 2010; Peng et al., 2016; Liu et al., 2018; Wei et al., 2019a; Li et al., 2020). P addition can not only stimulate rice root growth to increase the release of root exudates but also reduce dephosphorylation of organic compounds (Hofmann et al., 2016; Wei et al., 2019a; Yuan et al., 2019), which would facilitate microbial metabolism and growth in rhizosphere soil (Caruso, 2010; Zhu et al., 2018; Zhu et al., 2020), resulting in

more C released for microbial use in a model rhizosphere soil than in bulk soil (Spohn & Kuzyakov, 2013; Hofmann et al., 2016). Consequently, P addition would have increased the C limitation already present in bulk soil, increasing the V_{max} of both BG and CBH (Table 2). Accordingly, the K_m of CBH was also significantly higher in bulk soil than in a model rhizosphere soil after the addition of cellulose or P (Table 2). This might be because cellulose-degrading microorganisms are highly sensitive to available nutrients in bulk soil (Tischer et al., 2015). However, combined addition of cellulose and P could have increased available C for microbial growth while maintaining C balance without affecting V_{max} of C acquisition enzymes (Table1 and 2; Fisk et al., 2015; Liu et al., 2020; Liu et al., 2022).

In our study, cellulose addition reduced the V_{max} and K_m of ACP in both rhizosphere and bulk soils (Table 2). This is because SOM mineralization driven by microbial C and/or nutrient demands can increase mineral P availability, facilitating the growth of plants and microbes (Spohn & Kuzyakov, 2013; Peng et al., 2016; Wei et al., 2019a; Yuan et al., 2019; Wei et al., 2020). In comparison, cellulose addition alone increased the V_{max} and K_m of ALP, but only in bulk soil (Table 2). This difference indicates that, under P-limited conditions, soil P cannot satisfy the P demand for rice growth, so that microbes would exploit the organic moiety of phosphorylated compounds as a C source for phosphatase synthesis under such conditions (Schliemann, 1984; Hofmann et al., 2016; Peng et al., 2016). If P limitation is alleviated, a negative feedback in phosphatase activity can emerge (Su et al., 2007). This could explain why the P addition reduced the V_{max} of ACP in bulk soil and the V_{max} of ALP in a model rhizosphere soil compared to those in CK (Table 2). On the other hand, the V_{max} of both ACP and ALP was higher in bulk soil than in a model rhizosphere soil treated with either cellulose or P (Table 2).

This suggests that root exudates might help phosphate-solubilizing microorganisms to mobilize P (Wei et al., 2019a; Wei et al., 2019b; Wei et al., 2019c). Furthermore, root exudates and microorganisms can reduce soil pH (Table S1), which directly increases the dissolution of P in the soil matrix (Jones, 1998) and indirectly decreases the adsorption of orthophosphate ions to soil particles (Jones, 1998; Aoki et al., 2012). This can increase P availability and limit phosphatase activity (Zhang et al., 2014; Wei et al., 2019a). The addition of both cellulose and P together might have changed microbial community composition and increased microbial activity, which could increase rice P acquisition by diffusion and mass flow (Watt et al., 2006; Zhang et al., 2014). However, in a model rhizosphere soil, rice roots produce ACP, which can explain the lower ALP activity (Table 2), as suggested by previous studies (Sinsabaugh et al., 2009; Nannipieri et al., 2011; Zhang et al., 2014; Hofmann et al., 2016; Razaviet al., 2016; Ge et al., 2017). Interestingly, the V_{max} of ALP in P treatments was approximately two times lower in rhizosphere soil than in bulk soil (Table 2), indicating that less organic P was mineralized in a model rhizosphere soil than in bulk soil under P-unlimited conditions (Hofmann et al., 2016). Application of both cellulose and P together increased the V_{max} of ALP but did not affect the K_m of ALP in a model rhizosphere soil (Table 2); conversely, this treatment decreased both the V_{max} and K_m of ALP in bulk soil (Table 2). The results suggest that cellulose (i.e., stimulated by straw return) can facilitate the mineralization of organic P, and in turn, P acquisition by microbes and rice. That is, a high labile-C concentration in the rice rhizosphere might increase rice demand for available P (Wei et al., 2019a). This also explains why the V_{max} of BG was positively related to that of ALP (Fig.1).

The P:C acquisition ratio reflects the C and P needs of microbes (Tischer et al., 2015; Ge

et al., 2017; Wei et al., 2019c; Wei et al., 2020), along with the establishment of element homeostasis in the microbial population and the potential activity of C and P acquisition enzymes (Ng et al., 2014; Wei et al., 2019a; Ge et al., 2020; Liu et al., 2020). Our results showed that the DOC:Olsen-P ratio ranged between 50 and 92 in a model rhizosphere soil and between 19 and 46 in bulk soil (Table 1). These values were much lower than the average threshold value of 186 recorded by Sinsabaugh et al. (2009); thus, microbial metabolism might be C-limited, rather than P-limited (Sinsabaugh et al., 2009; Wei et al., 2019b; Liu et al., 2022). P addition significantly reduced the DOC:Olsen-P ratio and increased the V_{max} of C acquisition enzymes (CBH and BG in P and EP treatments) (Tables 1 and 2). This could be attributed to an increase in organic acid exudation resulting from the promotion of plant growth by P addition. Additionally, orthophosphate ions released from SOM mineralization are thought to have a positive priming effect (Vance et al., 2003; Spohn & Kuzyakov, 2013). This would create different nutrient requirements between model rhizosphere soil and bulk soil, which explains the negative correlation of DOC:Olsen-P ratio and the P:C acquisition ratio in rhizosphere soil (Fig.2c). Considering available C and P can be used to satisfy the growth demands of microbial populations and rice (Elser et al., 2003; Sinsabaugh et al., 2009; Spohn & Kuzyakov, 2013; Soong et al., 2018; Wei et al., 2019c; Liu et al., 2020; Liu et al., 2022), large amounts of P in the a model rhizosphere soil might have altered the P mining strategies of microbes, consequently decreasing SOM decomposition and the capacity to acquire P (Zhang et al., 2014; Hu et al., 2018; Yuan et al., 2019; Wei et al., 2021a). This may have altered microbial access to C and P resources (Ho et al., 2005), as reflected by the changes in the V_{max} of C and P acquisition enzymes (Table 2). Large amounts of labile-C in a model rhizosphere soil impede

the production of C acquisition enzymes (Elser et al., 2003; Tischeret al., 2015); however, microbes in bulk soil need to allocate their resources to the production of C acquisition enzymes in response to P availability to achieve their optimal growth (Ng et al., 2014; Soong et al., 2018; Wei et al., 2019a; Liu et al., 2020). Consequently, the P:C acquisition ratio decreased in a model rhizosphere soil but increased in bulk soil (Fig. 3).

5 Conclusions

P addition to P-limited paddy soil during the early stages of rice growth increased the K_m of P acquisition enzymes but reduced their V_{max} in a model rhizosphere soil. In comparison, cellulose addition reduced both the K_m and V_{max} of P acquisition enzymes. The V_{max} of ALP was positively associated with the V_{max} of BG, demonstrating that microorganisms can utilize the organic moiety of compounds as C sources for phosphatase synthesis. Results also demonstrated that the available C:P ratio and MBC:MBP ratio are two important indicators for the P:C acquisition ratio. Microbes mineralized less organic P to acquire P in a model rhizosphere soil (high labile-C content) than in bulk soil (low labile-C content). This causes the P:C acquisition ratio to decline in a model rhizosphere soil but to increase in bulk soil. Therefore, the P:C acquisition ratio is regulated by both P availability and rhizosphere-C. In conclusion, appropriate C and P fertilization regimes could be exploited to facilitate sustainable development of subtropical agriculture, based on the growth demands of crops such as rice.

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Tables

Table. 1 Results of two-way ANOVAs showing the effects of treatment, sampling location (rhizosphere soil (RS) and bulk soil (BS)), and treatment–location interactions on soil available nutrients, microbial biomass C and P, available C/P ratio, and microbial biomass C:P 45 days after rice transplantation.

Treatment	Location	DOC (mg kg^{-1})	Olsen-P (mg kg^{-1})	MBC (mg kg^{-1})	MBP (mg kg^{-1})	DOC/Olsen-P	MBC/MBP
CK	RS	384.16 ± 25.95bA	4.87 ± 0.19bA	711.85 ± 86.97bA	6.44 ± 0.10dA	78.83 ± 2.58aA	110.63 ± 15.13aA
	BS	217.22 ± 7.94αB	5.20 ± 0.74γA	406.51 ± 31.79γB	7.65 ± 1.62βA	42.31 ± 6.02αB	54.18 ± 7.41αB
E	RS	369.22 ± 6.39bA	4.10 ± 0.56bA	869.43 ± 2.34bA	10.72 ± 2.02cA	91.38 ± 13.94aA	83.07 ± 15.71bA
	BS	218.25 ± 18.44αB	4.85 ± 0.75γA	482.62 ± 8.63βB	7.74 ± 1.29βA	45.32 ± 3.51αB	63.51 ± 10.37αA
P	RS	432.29 ± 18.30aA	7.79 ± 0.04aB	1260.62 ± 111.14aA	22.04 ± 2.75bA	55.52 ± 2.46bA	57.61 ± 6.89cA
	BS	191.98 ± 29.75αB	10.10 ± 0.06αA	540.59 ± 44.89αβB	27.88 ± 9.65αA	19.00 ± 2.85βB	20.61 ± 5.37βB
EP	RS	426.13 ± 27.82aA	8.61 ± 1.14aA	1107.35 ± 189.55aA	30.18 ± 0.39aA	50.28 ± 9.45bA	36.75 ± 6.76cA
	BS	226.60 ± 22.05αB	9.00 ± 0.22βA	597.13 ± 40.23αB	22.48 ± 5.52αA	25.17 ± 2.51βB	28.00 ± 8.87βA

Factor (df)	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
Treatment (3)	2.719	0.08	98.01	***	19.81	***	35.48	***	33.74	***	35.05	***
Location (1)	478.88	***	15.41	**	183.05	***	0.28	0.60	173.60	***	53.03	***
Treatment × Location (3)	5.20	*	3.70	*	6.45	**	2.87	0.07	2.45	0.10	6.24	**

Note: The fertilizer treatments were: no cellulose or phosphorus addition (CK), cellulose addition (E), phosphorus addition (P), and combined cellulose and phosphorus addition (EP). DOC, dissolved organic C; Olsen-P, available P; MBC, microbial biomass C; MBP, microbial biomass C. Different English and Greek lowercase letters indicate significant differences ($P < 0.05$) between fertilizers in RS and BS, respectively. The English uppercase letters represent significant differences between RS and BS at $P < 0.05$. The symbols *, **, and *** represent significant differences in the effects of treatment, sampling location, and treatment–location interactions at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. All results are means ± standard deviation (n = 3).

Table. 2 Results of two-way ANOVAs showing the effects of treatment, sampling location (rhizosphere soil (RS) and bulk soil (BS)), and treatment–location interactions on soil kinetic parameters of C- and P-acquiring enzymes (maximal velocity (V_{max}) and saturation affinity constant (K_m)) 45 days after rice transplantation.

Treatment	Location	C-acquiring enzymes				P-acquiring enzymes			
		V_{max} of BG (nmol g^{-1} h^{-1})	K_m of BG (μmol g^{-1})	V_{max} of CBH (nmol g^{-1} h^{-1})	K_m of CBH (μmol g^{-1})	V_{max} of ACP (nmol g^{-1} h^{-1})	K_m of ACP (μmol g^{-1})	V_{max} of ALP (nmol g^{-1} h^{-1})	K_m of ALP (μmol g^{-1})
CK	RS	43.33 ± 2.03bA	39.80 ± 14.89aA	6.60 ± 2.11aA	63.11 ± 24.40aA	411.44 ± 6.22bB	67.12 ± 8.14bA	763.65 ± 57.03bB	67.62 ± 21.98aB
	BS	44.05 ± 0.65αA	27.72 ± 10.66βA	7.98 ± 2.40γA	67.85 ± 13.89βA	774.99 ± 5.12αA	47.33 ± 7.74αB	1450.18 ± 42.63αA	120.40 ± 13.57αA
E	RS	39.83 ± 1.10bB	49.93 ± 7.35aA	5.60 ± 6.47aB	33.89 ± 19.29aB	276.38 ± 59.26cB	57.68 ± 5.64bcA	833.49 ± 47.06bB	85.03 ± 21.14aA
	BS	46.17 ± 2.17αA	52.94 ± 9.07αA	16.51 ± 1.73βA	370.75 ± 18.25αA	552.66 ± 121.13βA	31.21 ± 0.63βB	1242.77 ± 42.05βA	92.40 ± 7.15βA
P	RS	28.84 ± 0.27cB	38.72 ± 7.58aA	3.03 ± 0.15aB	23.50 ± 14.87aB	425.83 ± 62.18bB	83.56 ± 4.03aA	464.49 ± 4.56cB	102.52 ± 30.50aA
	BS	49.03 ± 6.13αA	47.97 ± 15.78αA	29.00 ± 3.19αA	258.26 ± 119.02αA	656.65 ± 30.94βA	58.56 ± 9.81αB	702.54 ± 86.06γA	91.38 ± 10.15βA
EP	RS	56.56 ± 4.91aA	57.21 ± 13.22aA	7.17 ± 4.72aA	51.87 ± 53.03aA	800.47 ± 33.14aA	51.20 ± 4.79cA	1125.33 ± 49.91aA	81.07 ± 1.23aA
	BS	49.58 ± 4.78αA	46.08 ± 2.50αβA	9.18 ± 0.29γA	64.87 ± 24.24βA	822.30 ± 8.98αA	56.73 ± 8.43αA	564.58 ± 148.94γB	37.66 ± 0.57γB
Factor (df)		F *P*	F *P*	F *P*	F *P*	F *P*	F *P*	F *P*	F *P*
Treatment (3)		18.03 ***	3.58 *	8.46 **	10.45 ***	54.23 ***	15.96 ***	64.11 ***	6.51 **
Location (1)		12.95 **	0.37 0.55	55.48 ***	48.84 ***	98.53 ***	35.51 ***	43.57 ***	0.04 0.84
Treatment × Location (3)		16.59 ***	1.40 0.28	17.96 ***	15.36 ***	10.41 ***	7.32 **	83.64 ***	8.80 **

Note: The fertilizer treatments were: no cellulose or phosphorus addition (CK), cellulose addition (E), phosphorus addition (P), and combined cellulose and phosphorus addition (EP). BG, β-1,4-glucosidase; CBH, β-cellobiohydrolase (β-1,4-cellobioside); ACP, acid phosphomonoesterase; ALP, alkaline phosphomonoesterase. Different English and Greek lowercase letters indicate significant differences ($P < 0.05$) between fertilizers in RS and BS, respectively. The English uppercase letters represent significant differences between RS and BS at $P < 0.05$. The symbols *, **, and *** represent significant differences in the effects of treatment, sampling location and treatment–location interactions at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. All results are means ± standard deviation (n = 3).

Figures

Fig. 1 Pearson correlation between physicochemical properties and enzyme kinetic parameters (V_{max} and K_m) of rhizosphere soil (RS) and bulk soil (BS) 45 days after rice transplantation. The symbols *, **, and *** represent significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. DOC, dissolved organic C; Olsen-P, available P; NH_4^+, ammonium nitrogen; NO_3^-, nitrate; MBC, microbial biomass C; MBP, microbial biomass P; V_{max}, maximal velocity; K_m, half-saturation constant; BG, β-1,4-glucosidase; CBH, β-cellobiohydrolase (β-1,4-cellobioside); ACP, acid phosphomonoesterase; ALP, alkaline phosphomonoesterase.

Fig. 2 Linear relationship of C (V_{max} of [BG + CBH]) and P (V_{max} of [ACP + ALP]) acquisition and their ratio (P:C) in relation to soil available nutrients (DOC:Olsen-P) (a–c) and microbial biomass (MBC:MBP) (d–f) in rhizosphere soil (RS) and bulk soil (BS) 45 days after rice transplantation. DOC, dissolved organic C; Olsen-P, available P; MBC, microbial biomass C; MBP, microbial biomass P; V_{max}, maximal velocity; BG, β-1,4-glucosidase; CBH, β-cellobiohydrolase (β-1,4-cellobioside); ACP, acid phosphomonoesterase; ALP, alkaline phosphomonoesterase.

Fig. 3 Schematic of stoichiometric theory that shapes enzyme kinetics in the bulk soil (but not the rhizosphere soil) of rice after cellulose and phosphorus addition.

Fig.1

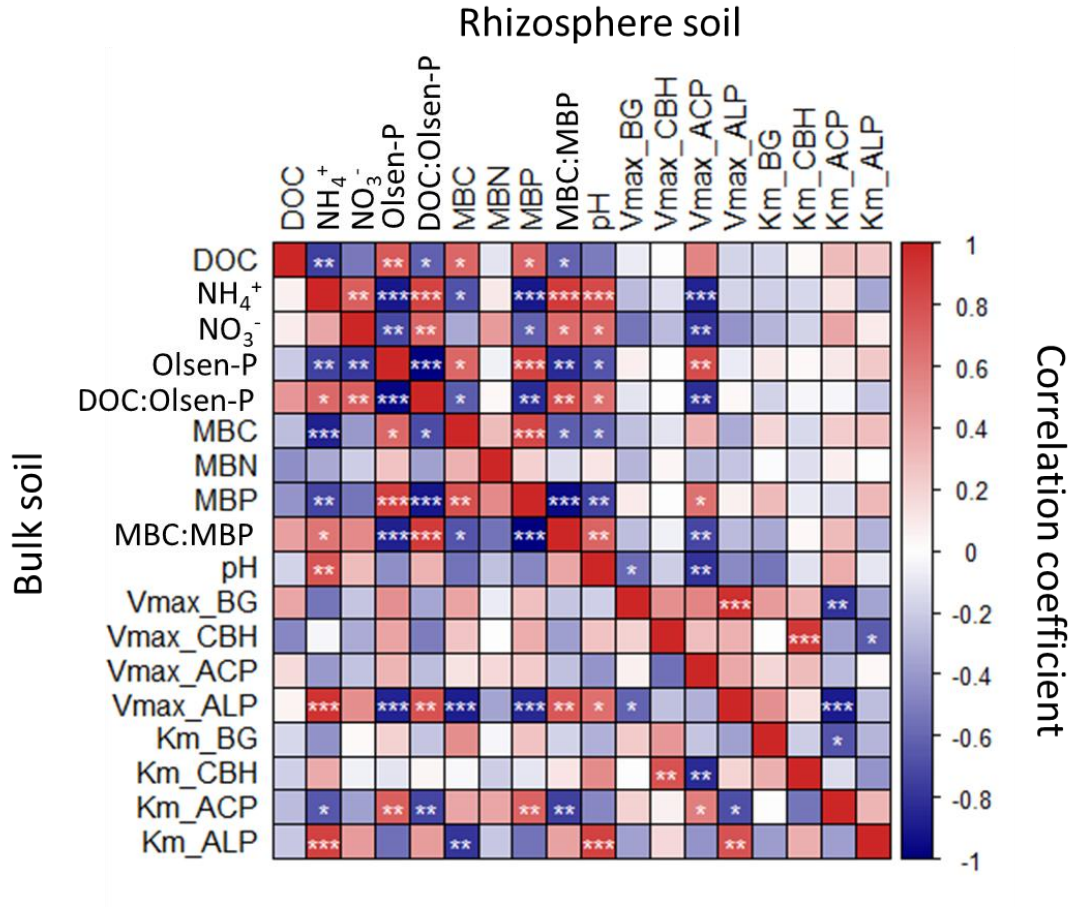

Rhizosphere soil
Bulk soil
Correlation coefficient
DOC
NH4+
NO3-
Olsen-P
DOC:Olsen-P
MBC
MBN
MBP
MBC:MBP
pH
Vmax_BG
Vmax_CBH
Vmax_ACP
Vmax_ALP
Km_BG
Km_CBH
Km_ACP
Km_ALP
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Fig.2

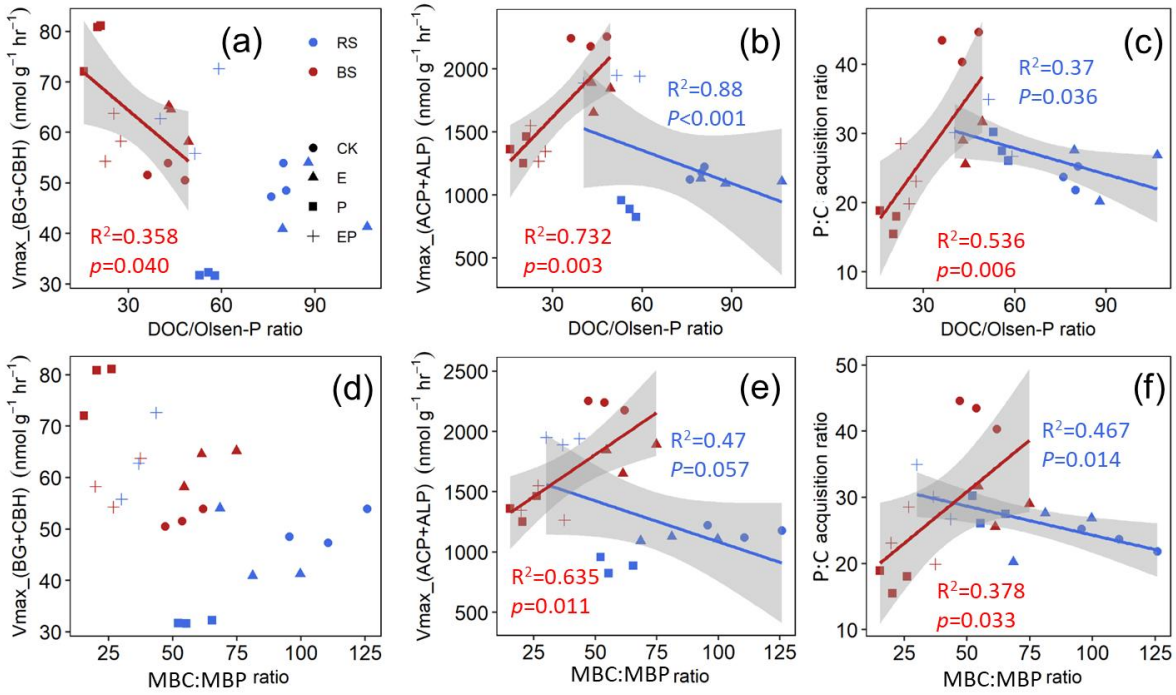
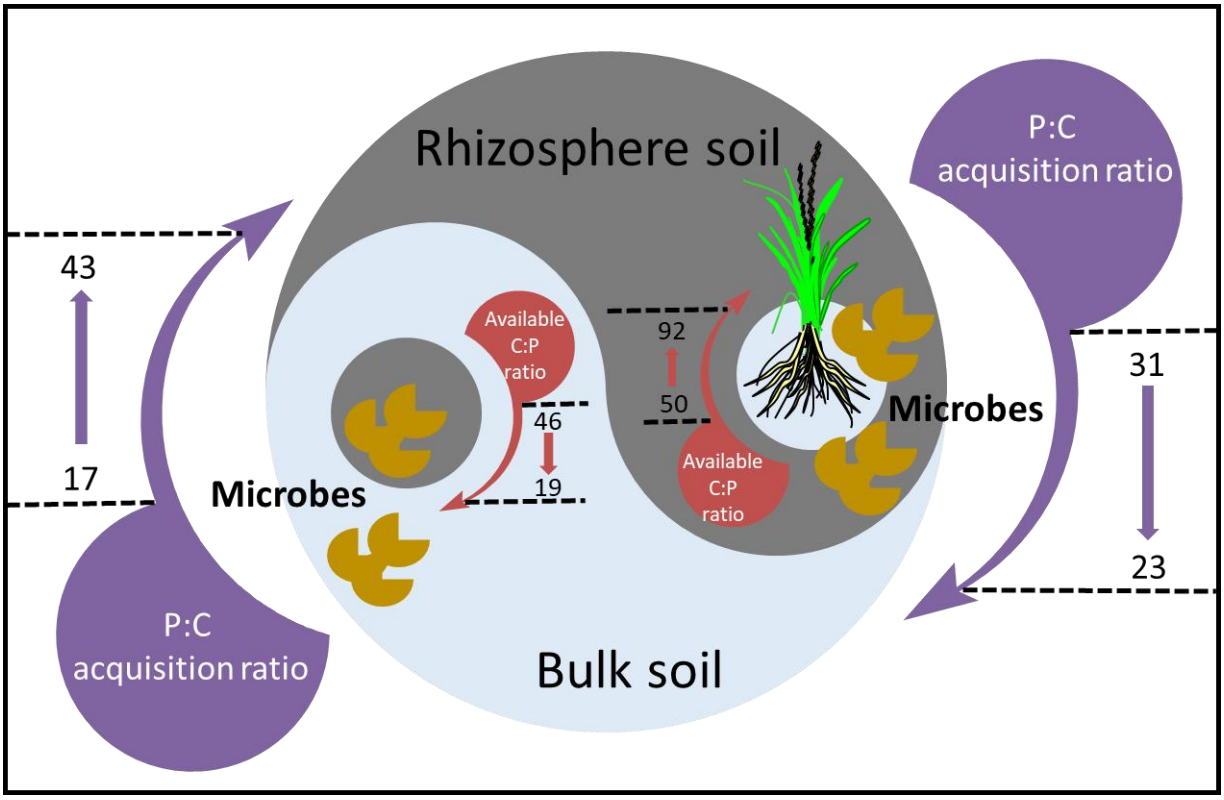

(a)
RS
BS
CK
E
P
EP
R²=0.358
p=0.040
Vmax_(BG+CBH) (nmol g⁻¹ hr⁻¹)
DOC/Olsen-P ratio
(b)
R²=0.88
P<0.001
R²=0.732
p=0.003
Vmax_(ACP+ALP) (nmol g⁻¹ hr⁻¹)
DOC/Olsen-P ratio
(c)
R²=0.37
P=0.036
R²=0.536
p=0.006
P:C acquisition ratio
DOC/Olsen-P ratio
(d)
Vmax_(BG+CBH) (nmol g⁻¹ hr⁻¹)
MBC:MBP ratio
(e)
R²=0.47
P=0.057
R²=0.635
p=0.011
Vmax_(ACP+ALP) (nmol g⁻¹ hr⁻¹)
MBC:MBP ratio
(f)
R²=0.467
P=0.014
R²=0.378
p=0.033
P:C acquisition ratio
MBC:MBP ratio

Fig. 3


Rhizosphere soil
P:C acquisition ratio
43
17
Microbes
Available C:P ratio
46
19
92
50
Available C:P ratio
Microbes
31
23
P:C acquisition ratio
Bulk soil

7 General Discussion

5.1 The effects of fertilization on SOC stocks

Soil fertility is dominated by SOC, which is related to the types, levels, and amounts of fertilization. In my meta-analysis, we investigated 217 published studies to identify consequences of different types, levels, and relative proportion of N, P, and K fertilization on SOC across global agricultural upland soils. The results show that fertilization has a positive effect on SOC in agricultural upland system. Fertilization not only increases crops yield, but also decreases C losses in agricultural soils due to the increase in the amount of crops residues input. Furthermore, fertilization type has an important impact on SOC stocks, with decreasing SOC stocks in the order of mineral + organic fertilization > organic fertilization > mineral fertilization. First, organic fertilization is an important source of OM per se (Ding et al., 2017). Besides, mineral nutrient addition to organic residues produces a lower available C: nutrients ratio (i.e., N, P, K and other macronutrients), leading to favor the anabolic pathway of organic carbon utilization over the catabolic pathway (Wang et al., 2019). The addition of nutrients in mineral form to crop residues (with a wide C: nutrients) allows microorganisms to use them directly and do not need to acquire the nutrients by decomposition of organic residues. Vice versa, in the combined organic + mineral fertilization, the application of crop residues with wide C: nutrient ratios such as straw leads to a decrease of mineral N, P and K fertilizer losses by causing N, P and K immobilization in the short term. This enhanced microbial immobilization also increases C sequestration in soil and enhances soil quality (Plante et al.,

2006; You et al., 2014; Zhao et al., 2014).

Considering the level of fertilization, intermediate N (100–300 kg ha^{-1} $year^{-1}$) and K (50–150 kg ha^{-1} $year^{-1}$), but high P ($\geqslant$60 kg ha^{-1} $year^{-1}$) fertilization produce the biggest effect on SOC stocks. Optimum mineral N, P, and K fertilization contribute not only to the maximum crop yield but also the amount of plant residues returned to the soil is maximum (Geisseler & Scow, 2014). Compared with intermediate N, excess N fertilization (> 300 kg ha^{-1} $year^{-1}$) combined with a low N use efficiency led to N loss, causing a negative effect on C sequestration (Zhu et al., 2016). At low N fertilization rates (<100 ha^{-1} $year^{-1}$), roots exudate less organic substances into the soil to gain nutrients through SOM decomposition for the growth of crops, thus causing a reduction in SOC content (Zhao et al., 2019). Bansal et al. (2020) found that SOC increased by about 21% in response to a high P rate (88 kg ha^{-1} $year^{-1}$) in Springfield, averaged over 5 years at a depth of 0–15 cm, compared with that in control soil. In addition, SOC also is influenced by human activities (i.e., tillage types). This is consistent with the study of Haddaway et al. (2017), who reported that SOC in the upper soil layer (0–15 cm) was significantly higher under NT than that under tillage in boreo-temperate agricultural regions. Because compare to tillage, NT decrease physical disturbance and destruction of macro-aggregates, the surface soil with high OM contents is not incorporated to deeper soil dept. It is more conductive to increase the SOC stock in surface soil (Mazzoncini et al., 2011).

Of course, environmental variables also affect SOC stocks in agricultural upland soils. Increasing water supply with precipitation and irrigation also increases SOC stocks by higher biomass production and crop residue return to soil (Márton, 2008; Liu et al., 2016; Wei et al., 2021).

5.2 The effects of N and P fertilizer on litter mineralization and priming effect

Exogenous N and P input may influence the microbial decomposition of soil indigenous SOM and litter. In my first incubation experiment, the microbial decomposition of soil indigenous SOM depending on nutrient availability were studied by adding mineral N and P with or without plant residues (*Dactylis glomerata* L. leaves) for a 37-day in a Chernozem soil of long-term planting wheat. It is surprising that N, P and NP addition didn't significantly change CO_2 emission, illustrating increasing N and P availability under C limitation can't change microbial respiration in semi-arid agricultural soil. This is match well with Tian et al. (2016) and Qian et al. (2016), who reported that there was no response of soil microbial CO_2 emission to N and P addition in tropical forest soil, respectively. In order to demonstrate that microbial C is limited, Mori et al. (2016) found that CO_2 emission rates in tropical forest soils were stimulated by P addition at high C concentrations (2000 μg C g^{-1} soil was added as glucose) but not at low C concentrations (100 μg C g^{-1} soil was added as glucose). It illustrates that C emission is related to microbial C limitation. This is consistent with Chen et al. (2018) N addition increased two indicators of C-limitation significantly (i.e., activity of BG and vector length), both of which indicated that N addition aggravated microbial C-limitation due to reduced recalcitrant C decomposition.

CO_2 emission was significantly higher with plant residues addition, because plant residues with easily decomposable biomolecules C (i.e., starch and cellulose) can be rapidly decomposed by microbes (Brant et al., 2006; Nottingham et al., 2009). Increasing C emissions with plant residues addition also indicates the growth of the microbial population,

decomposition of the added labile substrate, and/or increasing microbial activity through priming (Blagodatskaya & Kuzyakov, 2008; Hui et al., 2020). My results also show that compared to the control, input of leaves increased SOC mineralization by 90-99%, indicating a positive PE. Shahbaz et al. (2017) also reported an increase of up to 20% root, 44% stem, and 51% leaf mineralization can stimulate native SOC mineralization, resulting in a positive PE. Mineral N, P and NP addition decreased litter-derived CO_2 losses by 7.10 ± 5.07 %, 17.35 ± 4.09 %, and 7.83 ± 4.92 %, but increased litter-derived dissolved organic C by 16.78 ± 5.25 %, 19.36 ± 4.51% and 28.17 ± 10.28%, respectively. This is because litter C is redirected from waste respiration to microbial growth under alleviating microbial C and nutrient limitation (Schimel & Weintraub, 2003; Wang et al., 2019), increasing the litter-C use effectivity (Wang et al., 2019). Therefore this also is a consequence of increased condensation reactions and decreases in the degradability of recalcitrant C (Craine et al., 2007). Combined application of an organic substrate and nutrients in mineral form reduces C losses either by adsorption of DOC to minerals and by microbial metabolism and recycling (Gunina et al., 2014), finally leading to SOC accumulation (Liebmann et al., 2020; Yu et al., 2020). It match well with my meta-analysis that lower organic C: inorganic nutrient ratios (i.e., straw + mineral nutrients) lead to higher C stocks. Because the decomposition and storage of SOC is regulated and controlled by the critical C: N and C: P ratios. Furthermore, my results also show that N addition increased NO_3^- and the V_{max} of NAG. Since NAG activity reflects the fungal activity for chitin breakdown (Miller et al., 1998), fungi have a higher N acquisition to maintain its growth (Sinsabaugh et al., 2008; Shi et al., 2018; Yayi et al., 2021). However the availability of P decrease the V_{max} of AP in order to saving energy and N for C sequestration

(Zhou, et al., 2017; Wei et al., 2019a).

5.3 Stoichiometric theory shapes enzyme kinetics in bulk soil

Microbial mineralization of organic P is strongly interlinked with C mineralization (de Neergaard & Magid, 2015; Peng et al., 2016; Wei et al., 2019; Cui et al., 2020). Microbes mineralize organic phosphorylated compounds extracellularly to utilize the organic moiety of the compounds as a C source (Spohn & Kuzyakov, 2013; Hofmann et al., 2016). In my second experiment, the objective was to investigate the underlying mechanisms of the P availability in response to P-fertilization and *Dactylis glomerata* L. litter application to a typical P-limited steppe soil from Kazakhstan after 38 days of incubation. According to "lengths" and "angle" of vector in enzymatic activities of C:N vs C:P acquisition (Moorhead et al, 2013, 2016; Cui et al., 2019), I found that mineral P fertilization relieved microbial P limitation (decreased the V_{max} of P acquisition enzyme) but increased microbial C limitation. However the addition of plant residues increased the content of dissolved organic C (measured as DOC) and bioavailable P (measured as $CaCl_2$-P, Citric-P, Enzyme-P and HCl-P) to decrease microbial C limitation and remove microbial P limitation (Study 3, Fig.3). This led to DOC: Olsen-P ratio but increase the C:P acquisition of stoichiometric ratio. This is consistent with a previous study, showing that the decomposition of root-detritus with a low C:P ratio has the potential to improve soil P availability (de Neergaard & Magid, 2015; Wei et al., 2019a). However the DOC:Olsen-P and MBC:MBP ratio both were less than their average threshold ratio of 186 and 42-60 recorded by Sinsabaugh et al. (2009) and Cleveland & Liptzin (2007), respectively, indicating microbial C limitation rather than P limitation. Our

results from steppe (Study 3) and paddy soils (Study 4; Liu et al., 2021) show that once P limitation is alleviated, the V_{max} of P acquisition decreased and microbial C limitation increased.

Plants, as a competitor for microorganisms with respect to P uptake, are growing in agriculture soil. Rhizosphere and bulk soils are two different ecosystems in which P acquisition occurs (Hofmann et al., 2016). Lower V_{max} values of C and P acquisition enzymes are usually observed in the model rhizosphere soil than in bulk soil, illustrating that large amounts of labile-C from rice root exudates in a model rhizosphere soil impedes the production of C acquisition enzymes (Schliemann, 1984; Elser et al., 2003; Tischer et al., 2015). Further, V_{max} of P acquisition was higher in the bulk soil than in the model rhizosphere soil treated with either cellulose or P. This suggests that root exudates might help P-solubilizing microorganisms to mobilize P (Wei et al., 2019a; Wei et al., 2019b; Wei et al., 2019c). An increase in organic acid exudation resulting from the promotion of plant growth by P addition. Orthophosphate ions released from SOM mineralization are thought to have a positive PE (Vance et al., 2003; Spohn & Kuzyakov, 2013). Large amounts of P in the model rhizosphere soil thus might have altered the P mining strategies of microorganisms due to plant growth, consequently decreasing SOM decomposition (Zhang et al., 2014; Yuan et al., 2019; Wei et al., 2021). This may have altered microbial access to C and P resources (Ho et al., 2005) and created different nutrients requirements between model rhizosphere soil and bulk soil. Large amounts of labile-C in the model rhizosphere soil impede the production of C acquisition enzymes (Elser et al., 2003; Tischer et al., 2015); however, microbes in bulk soil need to allocate their resources to the production of C acquisition enzymes in response to P

availability to achieve their optimal growth (Ng et al., 2014; Soong et al., 2018; Wei et al., 2019; Liu et al., 2020). Consequently, microorganism in model rhizosphere mineralize less organic P than in bulk soil (Liu et al., 2021). Thus optimizing P application to maintain the stoichiometric balance between C and P is required in agricultural soil (Wei et al., 2019; Yuan et al., 2019).

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8 Conclusion and Outlook

In this thesis, the role of organic substrate in input and mineral fertilization for SOM decomposition and accumulation was studied in a meta-analysis and in laboratory incubation experiments.

According to the meta-analysis, fertilization significantly increased SOC, whereas in short-term incubation experiments mineral N and P addition didn't change microbial reparation (CO_2 emission). Plant residues (or plant litter + mineral nutrients) addition significantly increased CO_2 emission from indigenous SOM, i.e positive PE. However, CO_2 emission from both, plant residues and SOM, significantly decreased in response to N, P, and NP addition compare to only plant litter addition due to increasing litter-C use effectivity. This is mirrored by the results of the meta-analysis, showing that a balanced addition of organic substrates and nutrients has the best potential to increase SOC stocks in agricultural soils. Furthermore, bioavailable P also is improved due to a decrease in the microbial C limitation. However, microorganisms to mineralize less organic P in a model rhizosphere than in bulk soil due to the rhizosphere effect. In conclusion, microbial C and nutrient limitation impede SOC accumulation and P availability in agricultural soil. Mineral nutrients fertilization with organic residues is very important to maintain the stabilization of SOM. In the future, different microbial taxa residues in the soil reuse and stability as well as contribution to SOM still need to be deeply studied.

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