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The fate of amino acid and peptide as affected by soil depth and fertilization regime in subtropical paddies

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Abstract

Amino acids and peptides are important regulators of ecosystem functioning due to their potential role as direct nutrient sources for plants and soil microbes. However, the turnover and driving factors of these compounds in agricultural soils remain poorly understood. This study aimed to reveal the short-term fate of ^{14}C-labelled alanine and tri-alanine derived C under flooding conditions of the top (0–20 cm) and sub-horizons (20–40 cm) of subtropical paddy soils taken from four long-term (31 years since treatment) nitrogen (N) fertilization regimes (i.e., without fertilization, NPK, NPK with straw return (NPKS) or with manure (NPKM)). Amino acid mineralization was strongly affected by the N fertilization regime and soil depth, while peptide mineralization was only distinct between soil layers. The average half-life of amino acid and peptide in the topsoil was 8 hours across all treatments, which was higher than previously reported in uplands. The microbial turnover of amino acid and peptide was 7–10 times slower in the subsoil than in the topsoil, with a half-life of about 2–3 days. The half-life of amino acid and peptide for the respired pool was strongly associated with soil physicochemical characteristics, the total biomass, and the structure of soil microbial communities. The N fertilization regime and soil depth affected the substrate uptake rate by microorganisms, with greater uptake observed in the NPKS and NPKM treatments and the topsoil. Microbial amino acid uptake was correlated with the biomass of total and individual microbial groups, whereas microbial peptide uptake was associated with the soil microbial community structure and physicochemical characteristics. This suggests that there are various pathways of amino acid and peptide use by microorganisms under flooding conditions. We conclude that microbial mineralization of amino acid and its peptide in paddy soils under flooding conditions is slower than in upland soils, and that microbial uptake of these substrates is related to soil abiotic factors and the biomass and structure of soil microbial community. These findings have important implications for understanding nutrient cycling and ecosystem functioning in agricultural soils.

Keywords: Nitrogen cycling; Water regime; Oligopeptide-N; Subsoil; Element stoichiometry

Introduction

Nitrogen (N) is a major limiting nutrient in most terrestrial systems (Vitousek & Howarth, 1991; LeBauer & Treseder, 2008). Organic N is an important constituent of soil organic matter and consists of a diverse range of polymeric molecules, including amino acids, peptides, proteins, and nucleic acids (Leinweber *et al.*, 2013). Dissolved organic N (DON) in the soil solution is also equally diverse, containing compounds across a mixture of molecular size and compound types, with high molecular weight (HMW) proteinaceous polymers dominating (>1 kDa; Farrell *et al.*, 2011a; Jones *et al.*, 2012; Warren, 2014, 2017). Given its vital role in soil N cycling and crop production, unraveling the processes that determine turnover rates of organic N in soil is fundamental to improving our understanding of plant-microbial ecosystem functioning.

Increasing evidence suggests that soil microorganisms and many plants can use amino acids and peptides as a source of N without needing further cleavage by extracellular peptidases (Bardgett *et al.*, 2003; Jan *et al.*, 2009; Hill *et al.*, 2011a). This challenges the traditional paradigm of soil N cycling, whereby plants depend entirely on inorganic N to meet their N demands (Schimel & Bennett, 2004; Näsholm *et al.*, 2009; Hill *et al.*, 2011b). The depolymerization of N compounds in soils by extracellular enzyme activity to smaller peptides and amino acids is frequently the rate-limiting step (Schimel & Bennett, 2004; Jan *et al.*, 2009), although plants can use intact protein and even viable microorganisms (Paungfoo-Lonhienne *et al.*, 2008; Hill *et al.*, 2013). As a result, despite the relatively low contents of amino acids and peptides in soil solution, low molecular weight DON (LMW, <1 kDa) is likely to be a critical contributor to microbial metabolism and soil N availability (Farrell *et al.*, 2011a; Jones *et al.*, 2012). Existing evidence suggests that microbial mineralization of LMW DON is universally rapid across various ecosystems. A comparison of amino acids mineralization for 40 soils collected from cropland, grassland, and forest sites around the world Jones *et al.* (2009) found that amino acids added to soil produced the respiration of the same percentage of amino acid-C in all the soils, suggesting that soils possess a similar innate capacity to mineralize amino acids rapidly. The same conclusions were made for the mineralization of peptides in soils across a wide range of

ecosystems (Farrell *et al.*, 2013). Thus, many amino acids and peptides can be taken up directly by both soil microorganisms and plants at rapid rates over a period of minutes to a few hours, depending upon methods and the compounds used and species in question (Farrell *et al.*, 2011b, 2014; Hill *et al.*, 2012; Wilkinson *et al.*, 2014; Prendergast-Miller *et al.*, 2015). However, the mechanisms that regulate the turnover rates of these compounds in the soil remain unclear.

In addition to the rapid turnover of LMW DON, there is evidence that the turnover rates of these LMW compounds are independent of soil environmental factors and microbial diversity or community structure (Jones *et al.*, 2005; Hobbie & Hobbie, 2013). Previous studies have suggested that soil abiotic and biotic factors had no, or minor, effect on the turnover rates of amino acids and peptides in soils from Antarctic tundra (Hill *et al.*, 2011b), Northern Sweden forest (Rousk *et al.*, 2013), and the Hoosfield pH gradient fields at Rothamsted Research (Rousk *et al.*, 2011). Although microbial mineralization of LMW compounds occurred within seconds to a few hours depending upon methods and substrates, microbial uptake rates of these substrates between soils allocated in various climatic zones do not have a consistent trend (Farrell *et al.*, 2013; Wilkinson *et al.*, 2014). In contrast, the variation in the rate of microbial organic N uptake is strongly associated with soil and microbial C parameters (Farrell *et al.*, 2013), which accords with the view that their C requirement primarily drives the microbial utilization of LMW DON because of their starving-survival lifestyle (Hobbie & Hobbie, 2013; Farrell *et al.*, 2014). Therefore, comparing the same soil with various C content and microbial characteristics is required to provide clear evidence for this assumption.

The effect of soil C, nutrient content, and biological characteristics on the fate of LMW DON is not necessarily found between the soils but within the soil provided, i.e., depending on depth. In this aspect, subsoil receives the quality and quantity of organic inputs; entering this horizon differs from the topsoil (Fierer *et al.*, 2003; Spohn *et al.*, 2016). This leads to different resources and environmental gradients through the soil profile. Emerging evidence suggests that microorganisms in the subsoil can actively respond to nutrient inputs and are as sensitive to climate change as in the topsoil (Fontaine *et al.*, 2007; Hicks Pries *et al.*, 2017; Jones *et al.*, 2018). Furthermore, recent

100 evidence reveals the instability of the subsoil organic C (Hobley *et al.*, 2017), which challenges the
101 traditional concept that SOC in the subsoil appears to be more recalcitrant than in the topsoil
102 (Fontaine *et al.*, 2007). Therefore, subsoils should be considered when assessing soil C dynamics and
103 nutrient cycling (Hobley *et al.*, 2018; Jones *et al.*, 2018), especially the dynamics of LMW DON,
104 whose fate can be completely different from the topsoil.

105 In this study, we aimed to examine whether microbial mineralization and uptake of LMW
106 compounds (i.e., alanine and tri-alanine) are affected by soil physicochemical and microbial
107 characteristics. Soil samples possessing distinct differences in physicochemical and microbial
108 parameters were collected from the top (0–20 cm) and subsoil (20–40 cm) horizons of a subtropical
109 paddy field with long-term different N fertilization regimes. Given the intrinsic rapid mineralization
110 of amino acids and peptides as previously reported (Jones *et al.*, 2009; Farrell *et al.*, 2013), we
111 hypothesized that (H1) microbial mineralization of alanine and tri-alanine would occur rapidly in the
112 studied paddy soil and within the previously reported range of upland soils. Owning to higher
113 microbial biomass and activity in the topsoil than in the subsoil (Jones & Shannon, 1999; Jones,
114 1999), our second hypothesis is that (H2) the turnover rates of these substrates would be faster in the
115 topsoil than in the subsoil. Third, we hypothesized that (H3) microbial uptake of these substrates
116 would be strongly related to soil physicochemical characteristics that are indicative of soil C
117 availability but not to the soil microbial community structure (Farrell *et al.*, 2011b, 2013; Wilkinson
118 *et al.*, 2014; Prendergast-Miller *et al.*, 2015).

119 **Materials and methods**

120 *Field experiment and soil sampling*

121 The long-term paddy field experiment was established at Ningxiang County (28°07′ N, 112°18′ E) of
122 Hunan Province, China, in 1986 to monitor the effects of fertilization management on crop production
123 and soil fertility. The soil is classified as Ferallic Cambisol (FAO classification) and has a silt clay
124 loam texture with 13.7% sand and 57.7% silt in the topsoil (0–20 cm). The climate at the site is

125 classified as a humid subtropical climate with a mean annual temperature of 17.2°C, mean annual
126 precipitation of 1331 mm, and a frost-free period of 275 days. The cropping system is dominated by
127 the double rice (*Oryza sativa* L.)-winter crop barley (*Hordeum vulgare* L.) rotation system. Four
128 fertilizer treatments were established in 3.3 m × 6.7 m plots arranged in randomized blocks with three
129 replicates. The four fertilizer treatments included: a control without fertilizer input (Ctrl), chemical
130 fertilizer alone (NPK), rice straw residue plus chemical fertilizer (NPKS), and organic manure plus
131 chemical fertilizer (NPKM). The amount of N fertilizer was the same (530 kg N ha^{-1}), and the PK
132 fertilizers were 218 kg K_2O ha^{-1} and 30 kg P_2O_5 ha^{-1} and slightly different between the fertilized
133 treatments. Late rice straw and manure were applied at a rate of 30% of total N (as in the NPK
134 treatment) for the NPKS and NPKM treatments, respectively, and the remaining N was added as urea.
135 Early rice was transplanted in early May and harvested in the middle of July, and then late rice was
136 transplanted from late July to the end of October. Barley was sown in mid-November and harvested in
137 early May of the following year. In November 2016, topsoil (0–10 and 10–20 cm) and subsoil (20–30
138 and 30–40 cm) samples were collected from four randomly points within each plot, thoroughly mixed,
139 sieved to pass 2 mm, and stored at 4 ºC for further analyses. In total, 48 soil samples were collected
140 for the following analyses. Before the actual incubation, soil samples were pre-incubated under
141 flooding conditions to activate microorganisms.

142 *Soil physicochemical characteristics analyses*

143 The soil samples were analyzed for various physicochemical characteristics along the depth gradient
144 (Fig. S1). Soil pH and electrical conductivity (EC) were determined using standard electrodes in a
145 1:2.5 (*w*/*v*) soil-to-deionized water mixture. Soil available C and N pools were quantified by
146 extracting fresh soil samples after pre-incubation with 0.5 M K_2SO_4 (1:5 *w*/*v*). Concentrations of
147 ammonium (NH_4^+) and nitrate (NO_3^-) were determined colorimetrically using the methods of
148 Mulvaney (1996) and Miranda *et al.* (2001), respectively. Total free amino acid concentrations (FAA)
149 were analyzed fluorometrically by the *o*-phthalaldehyde-β-mercaptoethanol (OPAME) method of
150 (Jones *et al.*, 2002). Soil dissolved organic C (DOC) and total dissolved N (TDN) were quantified

using a Multi N/C 2100 TOC analyzer (AnalytikJena, Jena, Germany). Chemically labile organic C (LOC) was determined colorimetrically by the potassium permanganate oxidizable method (Weil *et al.*, 2003). The particulate organic matter (POM) was assessed by following the procedure of (Gregorich & Beare, 2008). Total C and N content of ground soils and POM samples were determined with a TruSpec® elemental analyzer (Leco Corp., St Joseph, MI, USA). Soil available P (Olsen-P) was extracted in 0.5 M $NaHCO_3$ (1:5 *w*/*v*) and measured colorimetrically via the molybdate blue method (Murphy & Riley, 1962).

Mineralization of ^{14}C-labelled amino acid and peptide

The turnover of amino acid and its peptide were determined as described previously (Jones *et al.*, 2009; Farrell *et al.*, 2011b). Soil from each replicate (5 g fresh weight) was weighed into individual polypropylene tubes (50 cm^3). An anoxic incubation was conducted by adding 5 mL 18.2 MΩ water to each tube to form a thin water layer above the soil throughout the incubation (Devêvre & Horwáth, 2000). After a 24-h pre-incubation, 0.5 mL (10 μM, 1.66 kBq ml^{-1}) of a uniformly radiolabeled ^{14}C-alanine or ^{14}C-tri-alanine in L-enantiomeric forms (American Radiochemicals Inc., St. Louis, MO) solution was added individually to separate tubes. To trap $^{14}CO_2$ evolved, a 6-mL polypropylene vial containing 1 mL 1 M NaOH was placed inside each tube above the soil, and the tube was hermetically sealed and maintained at 22±2°C. This temperature approximates the average temperature during the rice-growing season at this site. To quantify rates of respired $^{14}CO_2$, traps were removed 1, 3, 7, 24, 48, 72, 120, and 168 h after ^{14}C substrates addition. After removal, the amount of $^{14}CO_2$ trapped in the NaOH was determined by liquid scintillation counting after mixing with ScintiSafe 3 scintillation cocktail (Fisher Scientific Ltd.) and a Wallac 1409 scintillation counter (PerkinElmer Life and Analytical Sciences Inc.). After incubating for 7 d, the soil was shaken with 25 mL 0.5 M K_2SO_4 for 30 min at 150 rev min^{-1} to recover any ^{14}C substrate remaining in the solution or the exchangeable phase (Kuzyakov & Jones, 2006). The extracts were determined by liquid scintillation counting, as described above.

The mineralization of LMW compounds followed a biphasic kinetic pattern (Farrell *et al.*, 2011b; Hill *et al.*, 2012). A double exponential first-order kinetic decay model was therefore fitted to the inverse of the mineralization data of the ^{14}C-amino acid and ^{14}C-peptide using a least-squares optimization routine in SigmaPlot v14.0 (Systat Software Inc., San Jose, CA, USA):

$$y = Y_0 + \left(Y_r \times exp^{-k_1 \times t}\right) + \left(Y_b \times exp^{-k_2 \times t}\right) \quad (1)$$

where y represents the amount of ^{14}C remaining in the soil; Y_0 is the asymptote (% of total ^{14}C that was not recovered) and represents ^{14}C that was either synthesized by microbes from ^{14}C substrate and still contained within microbial biomass or was microbially synthesized to a non-extractable pool; Y_r describes the amount of ^{14}C partitioned into the first rapid mineralization pool (*C pool 1*), and k_1 is the exponential decay coefficient, while Y_b describes the second slower mineralization pool (*C pool 2*), and k_2 is the exponential decay coefficient, and t is time (h) after ^{14}C label addition to soil. *C pool 1* was attributed to the rapid use of ^{14}C substrate in catabolic processes leading to the loss of $^{14}CO_2$ in respiration, while *C pool 2* was attributed to the slower turnover of ^{14}C substrate and assumed to be initially immobilized in the microbial biomass via anabolic processes. The assumptions and validation of this modeling approach are provided in (Glanville *et al.*, 2016). The half-life period ($t_{½}$, h) for the first mineralization pool (*C pool 1*) can be calculated using the following equation:

$$t_{1/2} = \ln(2)/k_1 \quad (2)$$

However, the added C substrate to soil may be transformed by several microbial processes and calculating the half-life period for *C pool 2* (k_2) is subject to uncertainty due to the complexity of the connectivity between pool *C pool 1* and *C pool 2* (Boddy et al., 2008; Glanville et al., 2016).

The rate of microbial amino acid and peptide uptake (Φ, μmol N kg^{-1} soil d^{-1}) at a fixed soil solution concentration (10 μM) was adapted from (Farrell *et al.*, 2011b, 2013):

$$\Phi = k_1 \times Q \quad (3)$$

where Q is the soil solution concentration of amino acid or peptide (i.e., 10 μM), values were normalized on a molar N basis (Farrell *et al.*, 2011b, 2013).

Microbial community structure

To investigate the soil microbial community structure, the fresh soil samples were freeze-dried, and the extraction and analysis of phospholipid fatty acids (PLFAs) were carried out according to (Buyer & Sasser, 2012). Two grams of freeze-dried soil was mixed with 4 mL of Bligh-Dyer extractant containing an internal standard added. The samples were then sonicated (10 min, 20 °C), rotated end-over-end (2 h), and centrifuged (10 min). The liquid phase was transferred into clean screw-cap test tubes (13 × 100 mm), and 0.1 mL of chloroform and water were added. The upper phase was discarded, while the lower phase containing the extracted lipids was evaporated at 30 °C. Solid-phase extraction was used to separate lipids using a 96-well SPE plate containing 50 mg of silica per well (Phenomenex Inc., Torrance, CA, USA). Each sample was allowed to evaporate in a glass vial (30 min, 70 °C) with 0.5 mL of 5:5:1 methanol: chloroform: H_2O; the latter process was performed for eluting phospholipids. After evaporation, a transesterification reagent (0.2 mL) was added to each vial, after which the vials were sealed and incubated (37 °C, 15 min). Acetic acid (0.075 m) and chloroform (0.4 mL) were added to each vial; chloroform evaporated to dryness, and the samples were re-dissolved in hexane. Measurements were performed on a 6890 gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with an autosampler, split-splitless inlet, and flame ionization detector. Fatty acid methyl esters (FAMEs) were separated on an Agilent Ultra 2 column, 25 m long × 0.2 mm internal diameter × 0.33 μm film thickness. A total of 21 individual fatty acids were detected across the whole dataset and included for subsequent multivariate statistical analysis. Different taxonomic groups were classified as described in (Frostegård *et al.*, 1993; Sánchez-Rodríguez *et al.*, 2019) with acknowledgment of the caveats raised in (Frostegård *et al.*, 2011).

Statistical analyses

All data were checked for assumptions of normality and log-transformed where necessary. First, we conducted a principal component analysis (PCA) to examine the long-term effects of various N fertilization regimes on soil physicochemical properties. Second, to explore overall differences in soil microbial community structure across all treatments, we used a non-metric multidimensional ordination (NMDS). The nonparametric *adonis* test (PERMANOVA) was used to assess the percentage of variation of soil physicochemical properties and microbial community structure explained by N fertilization, soil depth, and their interaction. Third, we created two independent dissimilarity matrices to determine their overall relationships using the Euclidean distance and Bray-Curtis dissimilarity index for soil physicochemical properties and microbial community structure, respectively. A Mantel test (Pearson, n = 999 permutations) was then used to test for correlation between these two matrices. All these analyses were carried out using the R package *vegan* (Oksanen *et al.*, 2013).

Finally, we performed random forest analysis (Breiman, 2001) to assess which variables (i.e., soil physicochemical properties and the size and structure of soil microbial community) were the most important drivers of the variation found in microbial uptake of amino acid and peptide. The first two components of PCA for soil physicochemical characteristics and microbial community structure, which in total both explained about 80% of the total variance, were used in the random forest analysis. The size of the soil microbial community was reflected by PLFA biomass (nmol g^{-1}) of total and individual taxonomic groups. The ratios of fungal to bacterial and Gram-positive to Gram-negative bacteria were also considered. The random forest analysis with 999 permutations was performed using the R package *randomForest* and *rfPermute* (Breiman, 2001; Archer & Archer, 2019). All analyses were performed in R 3.5.2 (R Development Core Team, 2016).

Results

Soil physicochemical and biological characteristics

As indicated by PCA analysis, soil physicochemical properties varied between different N fertilization regimes and soil layers, where the soil layer alone explained 79.6% of the total variability (PERMANOVA, $P < 0.001$; Fig. 1a). The NPKM treatment was separated from others along the second axis, with 13.1% of the total variability explained by the N fertilization regime ($P < 0.001$). As expected, the selected soil physicochemical characteristics progressively declined with soil depth, except soil pH showed a reverse pattern (Fig. S1).

Total PLFAs content was significantly higher in the NPKS and NPKM treatments than in the Ctrl and NPK treatments ($P < 0.001$; Fig. 1b). Similarly, the content of PLFAs progressively declined with soil depth regardless of the N fertilization regime ($P < 0.001$). The proportional abundances of Gram-negative bacteria generally increased with soil depth, while the proportional abundances of Gram-positive bacteria decreased with soil depth (Fig. S2). The content of fungi and actinomycetes PLFAs was generally higher at the topsoil than at the subsoil. Specific PLFAs indicative of protozoa were detected in the topsoil but not in the subsoil. The ratios of fungal to bacterial and Gram-positive to Gram-negative PLFAs both decreased with depth across all treatments.

A clear separation displayed by NMDS analysis indicated that the soil microbial community structure was significantly affected by N fertilization regime and soil depth (stress = 0.046; Fig. 1c). For the total variability of the soil microbial community structure, soil depth, N fertilization regime, and their interaction explained 58.9, 17.7, and 17.1%, respectively (PERMANOVA, $P < 0.001$). The Euclidean distance matrix for soil physicochemical characteristics was positively and significantly related to the Bray-Curtis matrix of distance for soil microbial community structure (Mantel $r = 0.716$, $P < 0.001$; Fig. 1d).

Mineralization and uptake of amino acid and peptide

In all soils, the mineralization of the ^{14}C-labelled amino acid and peptide occurred in two distinct phases and was best described by a double exponential decay model (Figs. 2 and 3). Regardless of the N fertilization regime and substrate, amino acid and peptide mineralization differed between soil

layers, with the amount of ^{14}C-substrates remaining in soil/microbial biomass in subsoil (~64%) being significantly higher than that in the topsoil (~49%; Fig. 2). In most cases, mineralization kinetics of ^{14}C-amino acid added were strongly affected by fertilization, soil layer, and their interaction, while only soil depth significantly affected mineralization kinetics of ^{14}C-peptide (Table S1, Fig. 3). Specifically, differences between soil depths were evident in the Y_0 of peptide but not amino acid (P = 0.036; Table S1, Fig. 3a, f). A relatively low portion of amino acid and peptide taken up by microorganisms was rapidly respired, with about 16–21% of the substrates respired during the fast-turnover phase across all soils (Y_r; Fig. 3b, g). More ^{14}C-labeled amino acid was allocated to the fast-turnover pool in the NPK than in the Ctrl and NPKM treatments (P = 0.002), but there was no difference in the respired pool of peptide between treatments. The half-life ($t_{1/2}$) of the amino acid (8.1±0.4 h) in the topsoil was comparable to that of peptide (8.2±0.6 h), whereas in the subsoil, the former (2.2±0.2 d) was significantly shorter (3.3±0.3 d) than the latter (Fig. 3e, j). The half-life of amino acid and peptide in the topsoil in our study were substantially higher than those from previous studies (1.4±0.1 h for amino acid and 1.0±0.1 h for peptide; $P < 0.001$, Mann-Whitney Test; Fig. 4).

Assuming an equal soil solution concentration of 10 μM for both substrates, microbial uptake rates were calculated as greater for peptide than amino acid (Fig. 5). Microbial uptake rates of both ^{14}C-substrates were higher in the topsoil than in the subsoil ($P < 0.001$). The microbial uptake rate of ^{14}C-amino acid was higher in the NPKM than in the NPK treatment ($P < 0.01$; Fig. 5a).

Relationship between microbial uptake rate of LMW compound and soil abiotic and biotic factors

To explore the relationship between soil abiotic and biotic factors and the microbial uptake rate of these substrates, we conducted the random forest analysis for both soils and separately for the top and subsoils (Table 1). The important factors controlling microbial amino acid or peptide uptake differed between soil layers. The content of Gram-positive bacterial and actinomycetes PLFAs and the PC1 scores of soil physicochemical characteristics were the important predictors of microbial amino acid uptake (~51% of the variance) in the topsoil. In contrast, the total and bacterial PLFAs significantly affected microbial amino acid uptake in the subsoil (~46% of the variance). By comparison, the PC2

298 scores of soil microbial community structure and fungal PLFAs explained 27.4% of the variance for
299 microbial peptide uptake in the topsoil, while no relationship was detected in the subsoil. Across all
300 soils, 58% of the variance for microbial amino acid uptake was explained by the total, bacterial and
301 actinomycetes PLFAs, while the PC1 scores of microbial community structure and soil
302 physicochemical characteristics, fungal-to-bacterial ratio, and fungal PLFAs accounted for
303 approximately 61% of the variance for microbial peptide uptake.

304 **Discussion**

305 In this study, we assessed microbial mineralization and uptake of amino acid and peptide in paddy
306 soils under flooding conditions. As expected, the turnover rates of amino acid and peptide by
307 microorganisms in the topsoil were fast, with an average half-life of 8 hours, but were slower than
308 those reported previously for uplands (Fig. 4; Farrell et al. 2011b; Farrell et al. 2013; Prendergast-
309 Miller et al. 2015). The microbes under energy and carbon limitation are poised to immediately take
310 up the LMW compounds when they become available (Kuzyakov, 2010; Hobbie & Hobbie, 2013).
311 Thus, this discrepancy can be explained by the decreased metabolic activity of microorganisms in
312 paddy soils under flooding conditions (Devêvre & Horwáth, 2000), which was reported earlier for the
313 uptake and utilization of photosynthetic products in the rice field (Yao *et al.*, 2012; Tian *et al.*, 2013).
314 This suggests that even if LMW compounds are present in soil solution in the paddy field, their
315 uptake by microorganisms is delayed compared to uplands. As a result, we infer that the microbial
316 turnover rate of these LMW DON in paddy soils may be comparable to that in other upland soils if
317 they are under aerobic conditions, but this remains to be examined.
318 The turnover of amino acid but not peptide in the paddy soils was strongly affected by the N
319 fertilization regime; namely, the turnover rate of respired amino acid-C was faster in the NPKS and
320 NPKM treatments compared to the NPK and control treatments, which is likely due to higher
321 microbial activity in the former than in the latter (Wang *et al.*, 2018). This finding contrasts with the
322 results of Jones *et al.* (2005), who showed that microbial use of the amino acid mixture was mostly
323 insensitive to the N fertilizer regime. Indeed, it is supported by the fact that the total microbial

activity, rather than the size or structure of the soil microbial community, is the key determinant governing LMW compounds turnover in soils (Jones *et al.*, 2005; Glanville *et al.*, 2012). Our results showed that the variations in the half-life of respired amino acid and peptide in the topsoil were closely related to soil physicochemical characteristics (PC1; Pearson $r = –0.572$, $P < 0.01$ and –0.467, $P < 0.05$), the structure of the soil microbial community (PC2; Pearson $r = –0.532$ and –0.552, $P < 0.01$), and total PLFAs content (Pearson $r = –0.443$ and –0.438, $P < 0.05$). However, these findings are inconsistent with these studies revealing the lack of any relationship between the half-life of these LMW compounds and any of the soil physicochemical properties with a diverse range of soils along an elevation gradient or at the global scale (Jones *et al.*, 2009; Farrell *et al.*, 2011b, 2013). To verify this, we synthesized the half-life of alanine and tri-alanine from the literature and analyzed their relationships with soil physicochemical characteristics and microbial biomass. Across the global dataset, we find that the amino acid turnover was unrelated to any soil properties and microbial biomass, while soil total N content and C/N ratio were significantly correlated with the peptide turnover ($P < 0.01$; data not shown). Our finding contradicts the claim that the turnover rate of these labeled substrates is controlled by fundamental metabolic pathways common to all heterotrophic microbes and not by the activity of the soil microbial community. We speculate that this may be due to the metabolic patterns of microorganisms in paddy soils that are different from those developed under anaerobic conditions (Kögel-Knabner *et al.*, 2010). In addition, microorganisms have different transport systems for the uptake of amino acids and peptides, which can be a more important factor affecting peptide turnover than the N fertilization effect (Wilkinson *et al.*, 2014). Therefore, we call for future studies that should focus on the effects of different microorganisms on the turnover of different LMW compounds.

The average half-life for amino acid mineralization in the topsoil was similar to that of peptide, consistent with other studies showing a similar half-life of amino acid and peptide mineralization in soils along a grassland productivity gradient (Wilkinson *et al.*, 2014). In contrast, a significantly lower half-life for peptide than amino acid was found for the same soils or 26 soils across six discrete global regions (Farrell *et al.*, 2011b, 2013). Instead, the half-life for the peptide was higher than that of amino acid in soils from native woodland and pasture soils (Prendergast-

352 Miller *et al.*, 2015). Hence, controversy remains as to whether the mineralization of peptide is faster
353 than its monomer. This suggests that comparing mineralization rates of amino acid and its peptides is
354 not evidence of direct peptide uptake by microorganisms. Despite this, the differences in the
355 mineralization kinetics between amino acid and peptide, at least in part, suggest a difference in the use
356 of peptide and its monomer as the substrate for energy mining by soil microorganisms (Hill *et al.*,
357 2011b).

358 The microbial competition for nutrients has the same intensity in the top and subsoil, although
359 nutrient availability and microbial activity progressively decline with depth (Kautz *et al.*, 2013; Jones
360 *et al.*, 2018). Consistent with our second hypothesis, the average half-life of amino acid and peptide in
361 the subsoil was 7–10 fold longer than in the topsoil. This corroborates previous findings that the
362 difference in the average half-life of an amino acid mixture between the top and subsoils is up to 18
363 times (Jones & Shannon, 1999; Jones, 1999) and that mineralization rates of both substances
364 decreased gradually with depth, which is closely related to soil microbial activity and biomass (Jones
365 *et al.*, 2008, 2018).

366 The results presented here partly supported the third hypothesis and showed that factors
367 affecting microbial uptake of amino acid and peptide were different and layer dependent; namely, in
368 most cases, there were no interactions between the N fertilization regime and soil depth with the
369 mineralization patterns of amino acids and peptides, except that in the subsoils, where a clear
370 separation was observed in the NPKS and NPKM treatments (Fig. 2). Moreover, factors affecting
371 microbial uptake rates of studied substrates were distinct along with the soil profile. Previous studies
372 have shown that factors driving microbial uptake of LMW compounds varied considerably. For
373 example, in soils along a grassland productivity gradient, microbial uptake of these substrates was
374 positively correlated with the aboveground net primary productivity (Farrell *et al.*, 2011b). Contrary
375 to this, Wilkinson *et al.* (2014) using the same soils demonstrated that microbial uptake of these
376 substrates declined in less productive sites. Furthermore, various relationships existed between soil
377 physicochemical characteristics and microbial organic N uptake rates, depending on land use and
378 substrate in question (Prendergast-Miller *et al.*, 2015). In a diverse range of soils across various
379 ecosystems, microbial uptake rates of these substrates were closely related to the soil C availability

(Farrell *et al.*, 2013). Through reanalyzing the data from previous studies, we found microbial uptake rates of amino acid and peptide in the topsoil were positively correlated with soil mineral N content but negatively correlated with soil electric conductivity, and microbial uptake rates of peptide were also negatively correlated with microbial biomass (Fig. S3). Our results also clearly showed that factors affecting microbial uptake rates of these substrates across the whole profile are distinct. Since the fate of amino acids and peptides in soil depends on various factors, such as the soil horizon and plant community type, etc., it is difficult to make a generalized conclusion from these individual studies, and the complexity of driving factors should be taken into account.

Conclusions

We investigated the mineralization and uptake of amino acid and its peptide by microorganisms in long-term fertilized paddy soil under flooding conditions. Our findings indicate that the turnover rate of these substrates was about eight times slower compared to previous reports on upland soils. This decrease in turnover rate is probably attributed to the reduced metabolic activity of microorganisms under anaerobic conditions relative to aerobic conditions. The mineralization rate of amino acid and peptide declined with depth, associated with the vertical changes of soil physicochemical characteristics, the content of total PLFAs, and the structure of microbial communities. Variations in soil abiotic and biotic attributes between soil layers significantly impacted microbial mineralization and uptake of amino acid and peptide than the N fertilization regime in this studied paddy soil. Therefore, we conclude that N fertilization does not directly affect the fate of amino acid and peptide but rather modulates soil chemical and biological properties that indirectly influence their mineralization and uptake by microorganisms.

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540

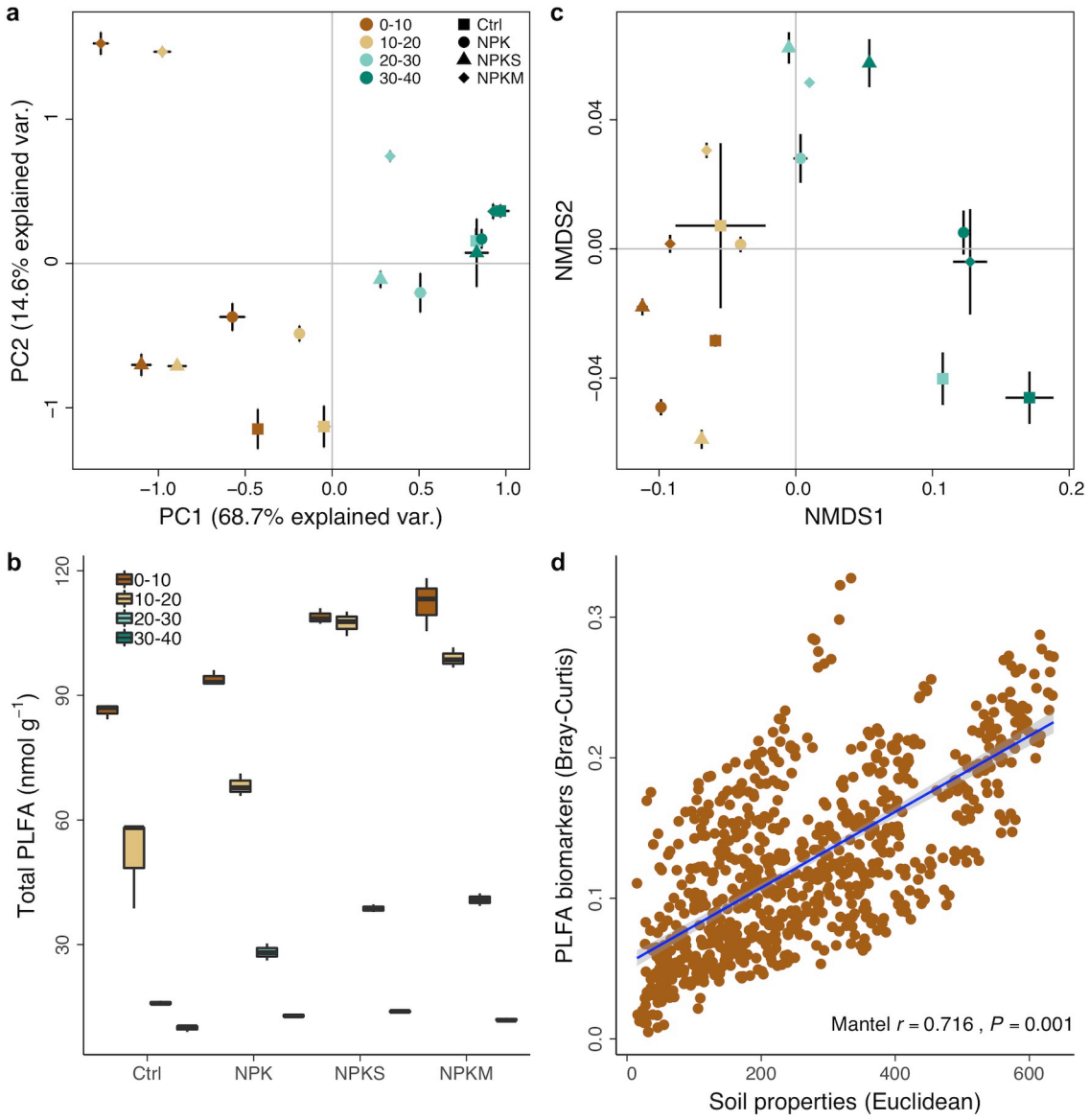
Table 1 Results of random forest analysis of the important predictors for microbial amino acid and peptide uptake in a long-term different N fertilization paddy soil.

	Amino acid			Peptide		
	Variable	Cross-validated R^2	*P*	Variable	Cross-validated R^2	*P*
Topsoil	Gram-positive bacteria, Actinomycetes, Soil PCA_C1	50.9%	< 0.01	PLFA PCA_C2, Fungal PLFA	27.4%	<0.01
Subsoil	Bacterial PLFA, Total PLFA, Gram-positive bacteria, Gram-negative bacteria, Actinomycetes	46.2%	<0.01	NA	NA	NA
All	Actinomycetes, Gram-positive bacteria, Total PLFA, Bacterial PLFA	58.0%	<0.01	PLFA PCA_C1, Fungal-to-bacterial ratio, Soil PCA_C1, Fungal PLFA	60.5%	<0.01

Soil PCA_C1, the first component (C1) of principal component analysis of soil physicochemical characteristics; PLFA PCA_C1 & C2, the first (C1) and second component (C2) of principal component analysis of the soil microbial community using PLFA biomarkers; NA, not applicable.

Figure legends

Figure 1 Soil physicochemical characteristics and microbial community along the soil depths under long-term different N fertilization regimes in paddy soils. (**a**) Biplot of principal component analysis (PCA) of soil physicochemical characteristics in top- and subsoil layers under different N fertilization treatments. (**b**) Boxplot of total PLFAs content. (**c**) Biplot of non-metric multidimensional scaling (NMDS) of the soil microbial community evaluated by PLFA biomarkers (stress = 0.046). (**d**) Scatter plot of the relationship between the matrix of the Euclidean distance from soil physicochemical characteristics and the Bray-Curtis dissimilarity of the soil microbial community. The solid line and gray shading represent the fitted linear regression and 95% confidence interval. In subplot b, the filling color in each treatment corresponds from left to right to the depth of the soil layer from the surface to the bottom. Ctrl, control without fertilization; NPK, chemical N, P, and K fertilizers added; NPKS, NPK with 30% of the total N replaced with the late rice straw return; NPKM, NPK with 30% of the total N replaced with manure application. Values are means ± SEM (n = 3).


a
0-10
10-20
20-30
30-40
Ctrl
NPK
NPKS
NPKM
PC2 (14.6% explained var.)
PC1 (68.7% explained var.)
c
NMDS2
NMDS1
b
Total PLFA (nmol g^{-1})
Ctrl
NPK
NPKS
NPKM
d
PLFA biomarkers (Bray-Curtis)
Soil properties (Euclidean)
Mantel r = 0.716 , P = 0.001

560

561

Figure 2 Mineralization of ^{14}C-labeled amino acid (upper panel) and peptide (lower panel) along the soil depths under long-term different N fertilization regimes in paddy soils. The lines represent fits of a double exponential decay model. Ctrl, control without fertilization; NPK, chemical N, P, and K fertilizers added; NPKS, NPK with 30% of the total N replaced with the late rice straw return; NPKM, NPK with 30% of the total N replaced with manure application. Values are means ± SEM (*n* = 3).

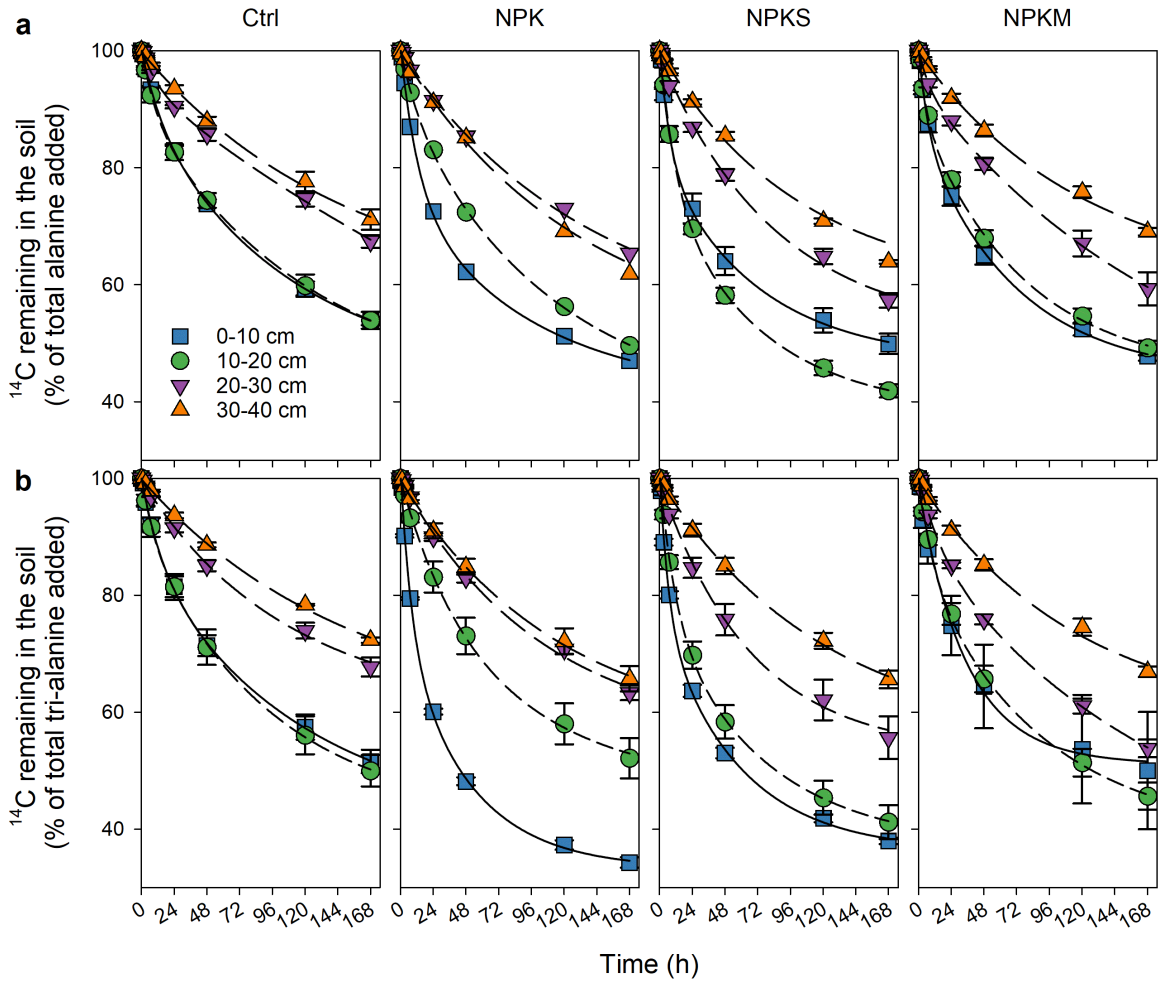


Figure 3 Kinetic model parameters describing the mineralization of ^{14}C-labeled amino acid (**a-e**, upper panel) and peptide (**f-j**, lower panel) along the soil depths under long-term different N fertilization regimes in paddy soils. Y_0 is the asymptote; Y_r and Y_b are the amount of ^{14}C-labeled substrates portioned into microbial respiration and subsequently into biomass production, respectively; k_1 is the rate constant for Y_a during the first rapid mineralization phase; $t_{1/2}$ is the half-life of the labeled substrates for the first rapid mineralization phase. Ctrl, control without fertilization; NPK, chemical N, P, and K fertilizers added; NPKS, NPK with 30% of the total N replaced with the late rice straw return; NPKM, NPK with 30% of the total N replaced with manure application. Values are means ± SEM (n = 3).

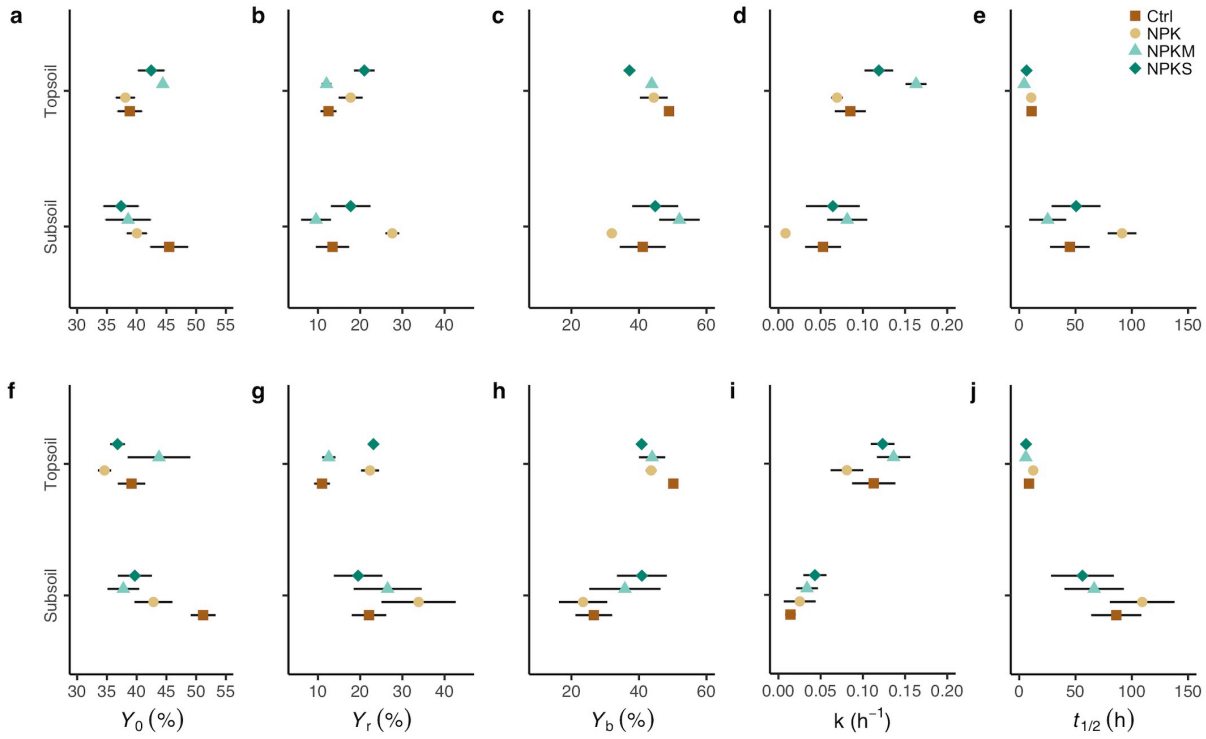


Figure 4 Comparison of the half-life between amino acid and peptide for the first rapid mineralization phase evaluated by measuring $^{14}CO_2$ evolution from this study and previous studies. Values are means ± SEM (n = 3 or 4).

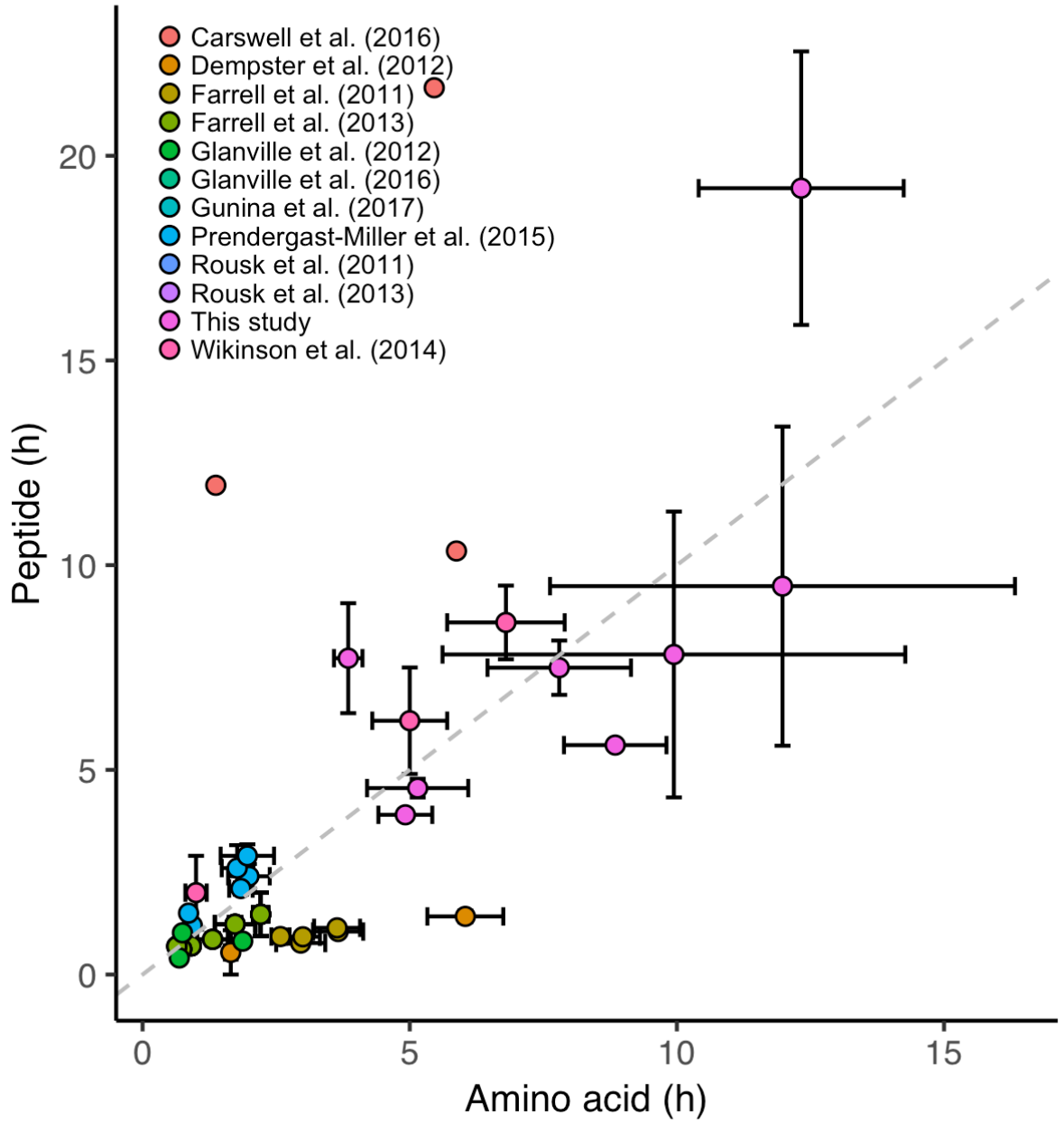


Figure 5 Rates of microbial uptake of amino acid (**a**) and peptide (**b**) from ^{14}C-labeled alanine and tri-alanine along the soil depths under long-term different N fertilization regimes in paddy soils. Ctrl, control without fertilization; NPK, chemical N, P, and K fertilizers added; NPKS, NPK with 30% of the total N replaced with the late rice straw return; NPKM, NPK with 30% of the total N replaced with manure application.